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Comparative Analysis of Argonaute-Dependent Small RNA Pathways in *Drosophila*

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Abstract of the Dissertation

Comparative Analysis of Argonaute-Dependent Small RNA Pathways in *Drosophila*

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The specificity of RNAi pathways is determined by several classes of small RNAs, which include siRNAs, piRNAs, endo-siRNAs, and microRNAs (miRNAs). These small RNAs are invariably incorporated into large Argonaute (Ago)-containing effector complexes known as RNA-induced silencing complexes (RISCs), which they guide to silencing targets. Both genetic and biochemical strategies have yielded conserved molecular components of small RNA biogenesis and effector machineries. However, given the complexity of these pathways, there are likely to be additional components and regulators that remain to be uncovered. I have undertaken a comparative and comprehensive RNAi screen to identify genes that impact three major Ago-dependent small RNA pathways that operate in *Drosophila* S2 cells. I identify subsets of candidates that act positively or negatively in siRNA, endo-siRNA, and miRNA pathways. These studies indicate that many components are shared among all three Argonaute-dependent silencing pathways, though each is also impacted by discrete sets of genes.

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Chapter 1

Introduction

Small RNA-mediated silencing

Small RNA-mediated silencing is a gene regulatory mechanism that is conserved throughout animal, plants, and fungi. In this pathway, small RNAs are incorporated into Argonaute (Ago)-containing effector complexes, known as RNA-induced silencing complexes or RISCs. The RISCs then use these small RNAs as a guide to find their targets, leading to sequence-specific gene silencing.

There are several core components in the small RNA-mediated silencing pathway; small RNAs that serve as the guide to the target mRNAs, catalytic enzymes that generate and load these small RNAs, and the effector complexes that suppress the target mRNAs. The small RNA components are comprised of single-stranded (ssRNA) or double-stranded RNA (dsRNA) molecules ranging from 20-30 nucleotides in length, and are classified into three species based on their biogenesis and mechanism of activity: microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNA (piRNAs).

Another key component of the small RNA-mediated silencing pathway is the myriad of proteins that are involved in the biogenesis of the small RNAs. These include Drosha and Dicer (Dcr-1/Dcr-2), important catalytic enzymes shown to be involved in the generation of small RNAs from dsRNA precursors. They are accompanied by a group of accessory proteins, such as Pasha, Loquacious (Loqs) and R2D2, which provide specificity or stability to the small RNAs being generated.

The last key component of the small RNA-mediated silencing pathway comprises the various effector complexes, each with a member of the Argonaute family at its core. In *Drosophila*, the Argonaute family is subdivided into two clades, the Ago subfamily and the Piwi subfamily, and associates with different small RNA components. Their mechanisms

of action vary from translational inhibition or sequestration of mRNAs to cleavage of target transcripts.

In *Drosophila*, RNA-mediated silencing can be subdivided into three separate pathways based on the small RNAs that act in each mechanism: the miRNA pathway, the siRNA (or classical RNAi) pathway, and the piRNA pathway. Each has different small RNA species and protein complexes that function almost exclusively within their respectively pathway (though there are exceptions). Although this thesis will concentrate on the miRNA and siRNA pathways, a better understanding of all of these silencing pathways would allow researchers to reconstruct the complex small RNA-based regulatory network, and perhaps manipulate the system to their advantage.

The Ago/Piwi protein family

Aside from the small RNAs, the key effector component of small RNA-mediated silencing is the Argonaute family. In *Drosophila* and mammals, the Argonaute family can be divided into two subfamilies based upon their sequence similarity; the PIWI subfamily and the eIF2C/Ago subfamily [1]. PIWI subfamily genes have 28.3–50.2% identity in their amino acid sequences and are expressed mainly in gonads, whereas the eIF2C/Ago subfamily genes have 77.6 % 77.6–83.6% identity and are expressed ubiquitously.

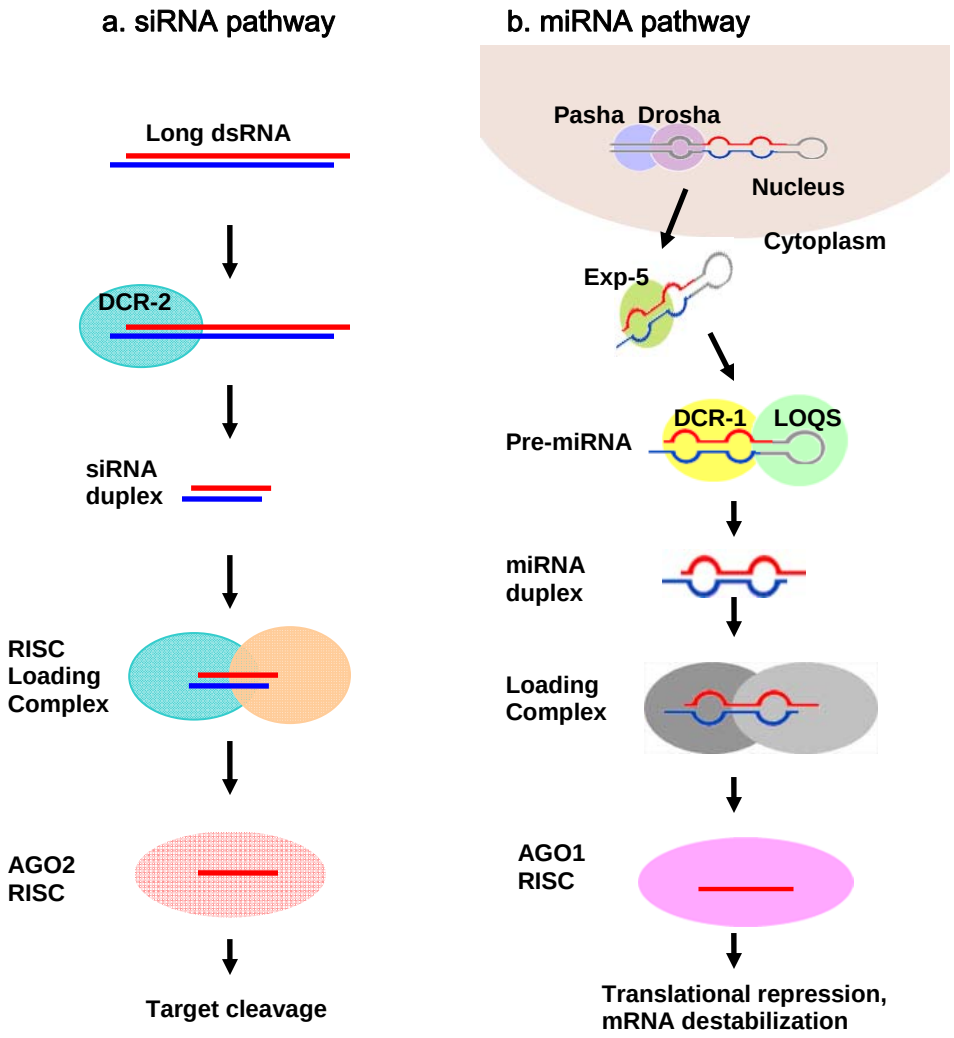


Figure 1.1. Argonaute-dependent small RNA silencing pathways in *Drosophila*
In *Drosophila*, there are two Argonaute-dependent small RNA silencing pathways; the small interfering RNA (siRNA) and microRNA (miRNA) pathways. **a** | Long dsRNA precursors are processed by DCR-2 to generate siRNA duplexes. DCR-2 and the dsRNA-binding protein R2D2 load the duplex into Argonaute2 (AGO2). The siRNA directs AGO2 to the target RNA and triggers target cleavage. **b** | miRNAs are transcribed from the genome as primary transcripts (pri-miRNAs). The transcribed pri-miRNAs have long stem-loop structures (hairpins). These precursors are processed in the nucleus into ~65-70 bp hairpins (pre-miRNAs) by the Drosha/ Pasha complex. pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin-5(Exp-5), and are further processed by DCR-1 to generate mature miRNAs. miRNAs are loaded into AGO1. The miRNA guides translational repression and mRNA destabilization of target gene by the AGO1 complex.

In *Drosophila*, five Argonaute proteins (Piwi, Aubergine, dAgo1 and dAgo2, dAgo3) have been identified [2]. dAgo1 and dAgo2 are assigned to Ago family whereas Piwi, Aubergine, dAgo3 belong to Piwi family. (Table 1) *Drosophila* Ago1 is most similar to mammalian Ago2 amongst the *Drosophila* Argonaute members, and they are considered as homologues [1].

Table 1. Type of small RNA and Argonaute protein in Drosophila

Subfamily	Protein	Small RNA	Length of small RNA	Function
Ago	AGO1	miRNA	21-23nt	Translation repression, mRNA degradation
	AGO2	siRNA	~21nt	RNA cleavage
Piwi	PIWI	piRNA	24-27nt	Repression of transposable element
	AGO3	piRNA	24-25nt	
	AUB	piRNA	23-26nt	

Drosophila Piwi family proteins associate with piRNAs, and are required for germ line development. Piwi contributes to the self-renewal of germ line stem cells and underlies transposon mobility control [3,4], and Aubergene (Aub) is important for pole-cell formation [5]. Unlike siRNAs and miRNAs, piRNAs do not require Dicer for processing. Piwi proteins have demonstrated slicing activity, cleaving the target complementary to position 10 and 11

of the piRNA, and might be involved in piRNA generation. A proposed hypothesis on their function is that Aub and Piwi-associated piRNAs guide the complexes to cleave targets. The products of these reactions are incorporated into Ago3 and are used to guide and cleave of Aub-Piwi-associated piRNA precursors [6,7]. The Argonaute clade is -100kD in size and part of a highly conserved protein subfamily.

The Argonaute subfamily is characterized by two common domains, known as PAZ domains and PIWI domains [8]. The PAZ domain is named after the proteins Piwi, Argonaut and Zwille, and has an OB fold structure that is involved in single-stranded DNA or RNA binding [9]. The PAZ domain from Ago2 can bind to two-nucleotide overhangs at the 3' end of small RNAs, and is thought to distinguish siRNA from degraded RNAs through this mechanism.

Drosophila Ago2 was isolated as a component of RISC complex [10], and was shown to co-fractionate with the *Drosophila* homolog of the fragile X mental retardation protein (FMRP), Drosophila Fragile X related protein (dFXR), and VIG (Vasa intronic gene). In size exclusion chromatography, fractions around ~500-kD and show RISC activity [11]. In contrast, the majority of miRNAs appeared around the ~250-kD fraction, and co-fractionate with Ago1 protein. Co-fractionation was observed in both size fractionation and ion exchange. Knock down of Ago2 causes decreased target cleavage activity for siRNAs, but Ago1 depletion has no effect. This result implies that Ago1 and Ago2 have different roles for specified part of RNAi, as shown by their association with the miRNA and siRNA pathways respectively.

The miRNA pathway

MicroRNAs (miRNAs) are double-stranded RNAs approximately 22 nucleotide in length. They are generated from ~70nt RNA precursors which form stem loop structures [12]. These stem-loop structures are themselves the product of a long primary transcript. The active strand (guide strand) of miRNAs functions to guide the effector complex to target mRNA. The miRNA recognizes the target through imperfect complementarity to sites in 3' untranslated regions, UTRs, This binding is required for the negative regulation of the target gene, through mechanisms such as translational repression and mRNA destabilization. Although the majority of miRNAs targets are found at 3'UTR sites, the 5' UTR and protein-coding sequences can also be the targets of miRNAs, e.g. as is observed for Nanog, Oct4 and Sox2 in mouse [13,14].

More than 1000 miRNAs have been identified in organisms as diverse as *C. elegans*, *Drosophila*, *Arabidopsis thaliana* and mammals [15]. Many miRNAs are highly conserved between species, with ~55 % of *C. elegans* miRNAs having homologues in humans [16]. Like protein-coding genes, each miRNA gene has a different expression pattern that is dependent on the cell type and developmental timepoint. miRNAs have been shown to have important roles in the control of developmental timing, cell proliferation, differentiation and apoptosis, and organ development [17].

miRNAs are transcribed from the genome as primary transcripts (pri-miRNAs), with ~40% of miRNA genes being located in intergenic regions and 10% being found in exonic regions of non-coding transcription units [18]. While the majority of pri-miRNAs are

generated by RNA polymerase II (Pol II) [19,20], a minor group of miRNAs associated with Alu repeats can be transcribed by RNA Pol III [21].

The transcribed pri-miRNAs are usually several kilobases in size, and have long stem-loop structures (hairpins). These long precursors are processed in the nucleus into ~65-70 bp hairpins (pre-miRNAs) by a 130-160kD RNase III enzyme, Drosha. The enzyme interacts with a dsRNA-binding protein partner called Pasha (DGCR8 in human) and is found in the nucleoplasm and the nucleolus [6,22]. The Drosha/Pasha complex is known as the microprocessor, and is involved in the generation of most pre-miRNAs.

A small group of pre-miRNAs has been shown to be generated without the aid of Drosha. These pre-miRNAs (found in flies, worms and mammals) are embedded in short introns and are called mirtrons. Their precursors are produced by the splicing machinery [23-26]. When these introns are spliced out, a lariat-debranching enzyme opens the lariat structure, to generate mirtron pre-miRNAs. miRNAs can also be derived from other non-coding RNAs, such as tRNAs or small nucleolar RNAs (sno RNAs) [27].

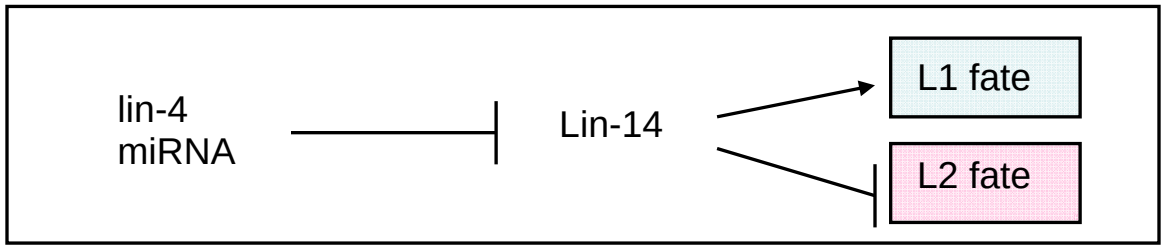
Processed pre-miRNAs are exported to the cytoplasm, a process mediated by the nuclear transport receptor, exportin5 (EXP5) [28-30]. The pre-miRNAs are then cleaved by Dicer 1 (Dcr-1), an Rnase III enzyme, into mature miRNAs. After Dicer cleavage, small dsRNAs are loaded into the Ago protein complex. The guide strand (miRNA) remains in this complex, whereas the passenger strand (miRNA*) is degraded [31].

The widely accepted functions of miRNAs are translational inhibition and exonucleolytic mRNA decay. However, translational activation [32] and heterochromatin formation [33] have also been reported.

miRNA targets

Bioinformatic predictions of miRNA targets suggest that at least 20-30% of animal transcripts have one or more conserved miRNA binding sites in their 3'UTR [34-36]. Although some mRNAs in animals have sequences with high complementarity to miRNAs (and these might lead to mRNA cleavage), the majority of miRNA target sites have imperfect complimentary sequences. There is an important sequence, known as the seed region, spanning nucleotide positions 2-8 at 5' end of miRNAs. This region shows high complementarity with target mRNAs, and pairing of the seed region with the target is crucial for miRNA-mediated regulation [17]. At nucleotide position 9-12, miRNAs and mRNA targets generally have imperfect complimentary sequences, resulting in bulged structures. This prevents endo-nucleolytic decay, and leads to translational inhibition and exo-nucleolytic mRNA decay [37].

Figure 1.2. microRNA regulation in *c.elegans* developmental pathway



miRNAs can function as regulatory switches to control the timing of developmental transitions. *Let-7* and *lin-4* were the first miRNAs to be discovered. They were uncovered in *C. elegans* through genetic screens [38]. Mutants in these miRNAs lack the ability to control developmental timing. It was later shown that *lin-4* negatively regulates the level of

LIN-14 protein (coding for a transcription factor) promoting the termination of the first larval stage (L1) and progression into the second larval stage (L2) [39]. (Figure 1.2) Multiple sequences complementary to *lin-4* were found in the 3' untranslated region of *lin-14* mRNA.

Translation inhibition by microRNA

The major miRNA-mediated effector function is translational inhibition of target mRNAs. SILAC (Stable isotope labeling by amino acids in cultured cells) analysis showed that transfection of mature miRNA into Hela cells lead to strong changes in proteins levels, frequently accompanied by slight decreases in transcript levels [40]. For example, for mammalian Dicer (a target of let-7b), translation rates increased by more than 4-fold when let-7b was blocked, while its mRNA level increased by only 30%. This implies that translational inhibition is a major mechanism of miRNA mediated silencing, although the exact route through which this occurs is still being debated.

Accumulating evidence suggests that miRNAs might block translation initiation. Several groups have shown that 5' cap structure (m7G) and 3' polyA tail, but not an IRES or non-functional ApppN cap, are necessary for the miRNA-mediated repression [41,42]. In addition, tethered eIF-4E and eIF-4G (which promotes cap-independent translation), alleviates miRNA-mediated repression of a reporter [41,42]. This suggests that miRNA mediated repression might interfere upstream of eIF-4E's recruitment of eIF-4G.

One mechanism of translation initiation control is polysome loading on the mRNA. Using polysome gradient experiments, one group reported that upon let-7 miRNA binding or hAgo2 tethering to a target site, mRNA was shifted to lighter fractions in a polysome

gradient [41]. This suggests that polysome recruitment to mRNA is blocked, and mRNA is released from the ribosome. This result supports the idea that miRNA mediated repression occurs during translation initiation.

However, there are conflicting observations that raise the possibility of post-initiation translational repression. Extracts from *C. elegans* L1 and L2 larvae were analyzed by sucrose gradient, with polyribosomes from both L1 and L2 stages remaining associated with the *lin-14* mRNA [43,44]. However, LIN-14 protein was down regulated approximately12 to 15-fold. Another group also showed similar results with KRAS mRNA, a target of let-7 miRNA. Three endogenous miRNAs and KRAS were co-fractionated with polysomes [45]. These results implied that translation regulation occurs after translational initiation.

Since binding of a single miRNP (microRNA ribonucleoprotein) to mRNA frequently has no significant repressive effect, co-fractionation of mRNA, miRNP and polysomes does not necessarily imply repression. Therefore, it has been suggested that those conflicting studies do not disprove the initiation-repression hypothesis [41,46]. However, the possibility of post-initiation repression is still possible, since reduction in the translational elongation and termination rates mediated by miRNPs could lead to changes in protein levels.

Another possibility is a rapid destruction of the nascent protein. However, pulse-chase experiments, were unable to show either the labeled full-length peptide or labeled nascent polypeptide chains from a miRNA-targeted transcript [47]. In addition, when reporter proteins were targeted to endoplasmic reticulum (ER), which sequestered the nascent protein from proteolysis, reporter activity remained repressed by miRNAs. Suppression

levels were also unchanged by the proteasome inhibitors, MG132 and Z-Leu-Leu-Leu-al [41]. These results strongly argue against the possibility of posttranslational degradation of the completed polypeptide chain.

The mechanism of miRNA-mediated repression remains unclear, and heavily debated. There are contradictory data, but it should be noted that initiation and post initiation repression may not be mutually exclusive. It is possible that the target mRNA might have different status according to the cell environment and cell status, leading to different modes of translational repression.

RNA degradation by miRNA

Although miRNAs generally lead to decreased levels of target proteins through translational inhibition, they are also associated with mRNA destabilization [48]. In most cases, miRNAs do not cause endo-nucleolytic cleavage but target mRNA can be degraded by the general mRNA-decay machinery.

In eukaryotes, mRNA degradation occurs in cytoplasmic foci, called P-bodies (mRNA-processing body). Degradation begins with the shortening of poly(A) tail followed by removal of 5' cap structure by a decapping enzyme (Dcp1p and Dcp2p). Then, mRNAs are degraded from 5' to 3' by an exonuclease, Xrn1p [49]. One protein that accumulates in the P-body is GW182, which is essential for p-body integrity. Argonaute proteins have been shown to co-localize and interact with GW182, and that this is required for miRNA mediated mRNA repression. Mutations in PAZ domain of Argonaute lead to a failure to localize in P-body and to suppress the target [39,50] .

A proposed model suggests that the recruitment of Argonaute proteins to the P-body leads to mRNA degradation, as GW182 promotes mRNA deadenylation by recruiting the CCR4-NOT deadenylation complex [51]. In addition, Dcp1p and Dcp2p are also found in the P-body, and might be involved in decapping of target mRNA. Depletion of CCR4-NOT complex or decapping proteins, DCP1 and DCP2 prevents miRNA mediated mRNA decay.

Regulation of miRNAs

Since miRNAs can be important regulators of numerous cellular processes, it is important to be able to control their levels and activities. In addition to transcriptional regulation of primary transcripts, miRNA activity can be regulated by: 1) controlling miRNA biogenesis, 2) competitive binding to 3' UTR, or 3) target mimicry.

As components of the microprocessor, Drosha and DGCR8/Pasha can be regulated to control miRNA biogenesis. In mammals, a negative feedback loop occurs where DGCR8 up-regulates Drosha by stabilizing the protein via protein-protein interaction. When Drosha and DGCR8 levels are high, the Drosha/DGCR8 complex down-regulates DGCR8 by cleaving a hairpin structure that exists in the DGCR8 mRNA. This would destabilize and reduce Drosha transcripts, lowering microprocessor activity [52]. Genetic observations suggest that there is an intricate homeostasis of DGCR8 levels in the cell, with *Dgcr8* heterozygous cells (with one functional copy of the gene) maintaining DGCR8 levels comparable to those of wild-type cells. Most of miRNAs are unaffected in *Dgcr8* heterozygous mice [53]. This regulatory mechanism of the microprocessor might control miRNA production in the cells, and help in maintaining a homeostatic level of miRNA-mediated repression.

Dicer, a key component of multiple small RNA pathways, has been shown to be negatively regulated by let-7. Mammalian Dicer has let-7 target site in 3'UTR, and transfection of *let-7* precursor molecules can reduce Dicer protein levels in cultured 293T cells [54]. Since let-7 itself is a product of dicer, depletion of Dicer protein reduces let-7 levels and the associated suppression. This dicer/let-7 negative feedback loop might contribute to regulation of mature miRNA expression levels.

In plants, Ago1 is controlled by a miR168 negative feed back loop through three miR168 target sites. Mutants with decreased Ago1/miR-168 complementarity at the target site exhibited increases in Ago1 mRNA, leading to developmental defects. The mutants also show decreased miRNA levels, and a corresponding accumulation of miRNA targets. miRNAs that are complementary to the mutant Ago1 can rescue the developmental defects caused by the mutant [55].

Direct interference with miRNA biogenesis can also exert control over miRNA activity. In humans, Lin28, a RNA-binding protein, suppresses let-7 production by blocking let-7 maturation [56-58]. This is achieved by the uridylation of the precursor let-7 at its 3′ end. The uridylated pre-let-7 is unable to be cleaved by Dicer and undergoes degradation [59]. Interestingly, let-7 can target Lin-28 and reduce its protein level, thus potentially up-regulating let-7 activity.

Examples of positive regulators of miRNAs are transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP). Both can enhance the Drosha-mediated processing of pri-miR-21 into pre-miR-21, leading to an increase in mature miR-21 [60]. TGF-β and BMP signal through SMAD proteins, which interact with RNA helicase p68 (also known as DDX5), a component of the DROSHA microprocessor subunit. TGF-beta-

and BMP-specific SMAD signal transducers are recruited to microprocessor by p68, and facilitate pre-miR-21 accumulation through enhancement of its processing rate.

Competition for binding at the 3'UTR can serve as a useful regulatory mechanism for miRNA activity. In humans, miR-122 is a liver-specific miRNA and translationally represses CAT1, an amino-acid transporter involved in argentine hydrolysis. During liver regeneration, CAT1 activity needs to be up-regulated to provide sufficient amino acids for protein synthesis [61]. HuR has been shown to bind to 3'UTR of CAT1 RNA during amino acid starvation or other kind of stress, and prevents CAT1 inhibition by miR-122 [62].

An alternative to miRNA regulation is to mimic the target transcript and remove the miR from the cytoplasm. In *Arabidopsis*, Induced by phosphate starvation1 (IPS1) has a 23-nt miR-399 complementary sequence. However, IPS1 cannot be degraded by miR-399 due to mismatches at the critical positions in the target site. Instead, by mimicking the miR-399 target site, overexpression of IPS1 soaks up miR-399 and allow its targets to accumulate [63].

The siRNA pathway

The siRNA pathway (known as the classical RNAi pathway) was the first small RNA-mediated pathway to be uncovered. This pathway led to cleavage events caused by the introduction of exogenous dsRNAs targeting endogenous genes. These exogenous dsRNAs were processed into siRNAs, which are 21-25 nt dsRNAs (dsRNAs) that form a 19 base-pair overlap, with 2 nt overhanging at the 3' ends. siRNAs can also be derived from long dsRNAs from exogenous transposons and viruses [10,64-66].

Whatever their source, these long dsRNAs are processed in *Drosophila* into small dsRNAs by the Rnase III enzyme, Dicer-2 (Dcr-2). Mammals lack Dcr-2, with the mammalian Dcr-1 able to generate both miRNA and siRNAs. The RNA binding protein, R2D2, functions as a partner of Dcr-2, and forms a complex that distinguishes the active strand (guide strand) and non-active strand (passenger strand) of the siRNA. This is achieved by sensing the thermodynamic stability of the siRNA duplexes, with R2D2 binding to more thermostable end and Dcr-2 binding to the other end [67]. The Dcr-2/R2D2 complex then recruits Ago2 through the interacting PAZ domains of Dcr-2 and Ago2. This complex then loads the siRNAs, preventing the siRNAs from freely diffusing in the cytoplasm. Once associated with siRNAs, Ago2 cleaves the passenger strand, with the guide strand directing Ago2 to the target RNA.

Endogenous siRNAs

Endogenous siRNAs (Endo-siRNAs) were first discovered in plants and *C. elegans*, with recent studies demonstrating their existence in a broader spectrum of animals, including *Drosophila* and mammals. In flies, endo-siRNAs can be detected in cultured cells, somatic and germline tissues [68-74]. These endo-siRNAs are ~21nt and are derived from longer dsRNA precursors.

The endo-siRNA pathway shares several common components with the exogenous siRNA pathway. For example, depletion of Dcr-2 and Ago2 caused substantial reductions of endo-siRNA levels in S2 cells and flies, with Ago2 shown (by immunoprecipitation) to associate with the endo-siRNAs [69,71]. Depletion of other small RNA associated factors, including Drosha, Pasha, Dcr-1, or Ago1, does not affect siRNA levels significantly. In

dcr-2 and ago-2 *Drosophila* mutants, mRNA expression of endogenous transposons increases, suggesting a role for the endo-siRNA pathway in their silencing [70,71,73].

Interestingly, the processing of endo-siRNAs depends on the dsRNA-binding protein Loquacious (Loqs), which was previously identified as the co-factor for Dcr-1 in the miRNA pathway. While *loqs* mutant flies show only modest defects in the maturation of miRNAs, they show strong reductions in endo-siRNA levels. In contrast, depletion of the canonical Dcr-2 partner, R2D2, appears to have only minor effects on endo-siRNAs [71].

In *Drosophila*, endo-siRNAs can arise from bidirectionally transcribed loci and structured loci [69-71]. Bidirectionally transcribed loci generate regions of complementarity where two transcripts overlap, leading to dsRNA formation. These loci can overlap either at the transcriptional start sites, 3'UTR sites or in coding regions, with 3' UTR overlaps being most common. The structured loci give rise to long transcripts that contain extended inverted repeats. These are capable of forming hairpins of up to 400nt and of giving rise to siRNAs from the one genomic strand. There are three major structured loci in the fly genome; esi-1, esi-2, and esi-4. esi-2 has high sequence complementary to the DNA-damage response gene, mutagen-sensitive-308 (mus308), and its activity leads to a reduction of mus308 mRNA. The guide strands of *Drosophila* endo-siRNAs have 2'-*O*-methyl modifications at their 3' ends, generated by the *S*-adenosyl methionine-dependent methyltransferase HEN1 (also known as piRNA methyltransferase, PIMET). [70]

One interesting observation is that a highly expressed siRNA loci, CG7739, has sequences complementary to dAgo2 [71]. Thus far, there is no clear evidence that Ago2 is controlled by this siRNA, but as shown by let-7 downregulation of mammalian Dicer, it is possible that the endo-siRNA pathway could be regulated by its own products.

So far, mammalian endo-siRNAs have been only found in mouse oocytes [75,76]. Their origin has been traced to transposons, genic and intergenic regions. Interestingly, some endo-siRNAs appear to originate from antisense pseudogene transcripts. These were shown to have the capacity to pair with the sense mRNA transcripts to form long dsRNAs, which can be cleaved by Dicer to make endo-siRNAs. Loss of mammalian Dicer leads to an upregulation of genes, with the degree of increase corresponding roughly to the number of endo-siRNAs found targeting the mRNA [75]. The most intriguing observation is the abundance of endo-siRNAs targeting microtubule-related genes, as Dicer-depleted oocytes show spindle organization defects [77]. This suggests a possible role for endo-siRNAs in contributing to the mutant phenotype in Dicer-deficient oocytes.

In *C. elegans* and plants, the biogenesis of endo-siRNAs depends on RNA-dependent RNA polymerase (RdRP). RdRP recognizes 3' ends of cleaved primary siRNAs, and produces secondary siRNAs [78-80] downstream of the primary siRNAs. This produces a pool of 22nt siRNAs that are antisense to the original target transcript and capped by 5' triphosphates [80,81]. These siRNAs are then loaded into secondary Argonaute (SAGO) [82], which lacks amino acid residues that are conserved in the cleavage active site, and might act in a cleavage-independent manner [83]. Thus far, RdRP and SAGOs are not found in other animals, suggesting that this endo-siRNA biogenesis pathway might be specific to *C. elegans* and plants.

siRNA-mediated cleavage

In contrast to most miRNAs, siRNAs regulate gene expression post-transcriptionally by directing sequence-specific endonuclease cleavage of the target mRNA, known as "slicer" activity. During a slicing event, one strand of the double-stranded duplex (the guide strand) directs the binding of effector complex to the target site. The target RNA is cleaved at the complementary position between the tenth and eleventh base from the 5' end of the guide [84]. The PIWI domain has been shown to be involved in target cleavage, and has Rnase-H-like fold and an anchor site for the 5' phosphate of the RNA guide strand [85]. Similar to other Rnase-H like enzymes, the cleavage products generated from slicer contain 3'-OH and 5'-phosphate groups. *In vitro* analyses of human Ago1, Ago2, Ago3 and Ago4 demonstrated that only Ago2 possesses this cleavage activity [6,86,87], and that an siRNA-Ago2 complex is sufficient to recapitulate slicer function [88]. Mutations in the RnaseH domain of hAgo2 prevent siRNA-mediated cleavage, and this observation is further supported by similar mutations that are intrinsic in hAgo1 and hAgo4 (both are non-cleaving). In contrast, cleavage activity is more common among different *Drosophila* Ago proteins, withAgo1, Ago2 and Piwi all showing slicer activity [89-91].

Crossing the miRNA and siRNA boundary

Although the miRNA and siRNA pathways appear to have distinct mode of biogenesis and effector actions, there is increasing evidence that substrate from one pathway could be recruited into the other. Several studies have revealed that by changing

the degree of complementarity between the small RNA and its target, the effector mechanisms of siRNAs and miRNAs are interchangeable [92-94]. Experiments have demonstrated that the structure of small RNA duplex dictates their loading partner protein and mechanism of activity. If the duplex contains bulges, mismatches, or GU wobble pairs, they are preferentially loaded into Ago1 and functions in miRNA pathway. In contrast, perfectly matched duplexes are loaded into Ago2, and fed into the siRNA pathway. This has been shown to be due to the higher affinity of Ago2/R2D2 for duplexes with high complementarity compared with those possessing mismatches at the center.

The interchange between the miRNA and siRNA pathways has been shown in both *Drosophila* and mammals. In mammalian tissue culture, exogenous siRNAs can repress the translation of (instead of cleaving) a target mRNA if the target site has partially complementary binding sites in its 3' UTR [93] In *Drosophila*, let-7 and bantam have typical miRNA structure with mismatches, and are predominantly loaded into Ago1. In contrast, miR-277 duplex has high complementarity at central region, resembling an siRNA structure. Consequently, miR-277 is preferentially loaded into Ago2 [95].

The miRNA (Ago1) and siRNA (Ago2) pathways are likely to be competing against each other for small RNAs, leading to the preferential sorting of dsRNA duplexes with differing complementarity into different complexes. It has been shown that loading of dsRNA into one pathway lead to decreased association with the other pathway. Since for efficient endonuclease cleavage, base-pairing at the nucleotide position 10 and 11 is important, this may act as a mechanism for deciding which mechanism of action is chosen, and therefore which Ago is loaded. miRNAs and siRNAs complexes might also have similar components to mediate RNAi gene silencing [92]. Considering this, common

components like RNA binding proteins or RNA helicases, would be included in both siRNA and miRNA complexes, allowing them to perform the other's function [11,92,96]. Although dAgo1 possesses endonucleolytic cleavage activity toward perfectly matched target RNAs, the efficiency of cleavage is much less than is observed with dAgo2 [95,97,98]. The preference of binding small RNA partner and cleavage efficiency might have led to the specialized function of Ago1 and Ago2.

Questions and purpose of the experiment

Small RNA mediated silencing decreases levels of RNAs or proteins through a myriad of mechanisms. However, it is difficult to identify the steps most critical to the operation of each small RNA pathway. There are several steps in each pathway where slight changes in efficiency could translate into observable silencing effects. These include;

(1) Transcription of small RNA precursors or uptake of long dsRNA

(2) RNA processing in the nucleus (miRNA)

(3) RNA transport

(4) Dicer Cleavage

(5) Ago/RISC loading

(6) Translation inhibition, RNA exo-nucleolytic cleavage, endo-nucleolytic cleavage

The list is not exhaustive, and although a great deal of progress has been made in deciphering small RNA-based regulatory networks, it is clear that there are many additional components awaiting identification and functional characterization. Even for the steps listed above, there are many unanswered questions. What are the different components doing at each step? How do these components co-operate at different stages? What are the

differences between the siRNA, endo-siRNA and miRNA pathways and their components?

Genome-wide screens for components of siRNA or miRNA pathways identified several common components [99-104] but none have addressed endo-siRNA pathways, nor have they attempted comparative studies in the same experimental model. Here, I report the results from several comparative and comprehensive RNAi screens that identify components of the Argonaute-dependent small RNA pathways (siRNA, miRNA, and endo-siRNA) in cultured *Drosophila* cells.

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Chapter 2

Comparative Analysis of Argonaute-Dependent Small RNA Pathways in Drosophila

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Introduction

Despite similarities in their form and overall function, small RNAs that bind Argonaute (Ago) proteins in *Drosophila* arise from compartmentalized biogenesis pathways and join effector complexes with specialized properties [105]. *Drosophila* small interfering RNAs (siRNAs) are most often generated from exogenously introduced double-stranded RNAs (dsRNAs), though the replication products of RNA viruses can enter this pathway [106]. Double-stranded RNA can also be produced from the *Drosophila* genome itself, either from loci encoding extensively structured RNAs or by hybridization of convergently transcribed mRNAs [69-72]. These bind Ago2 to form a complex that can efficiently cleave complementary targets. miRNAs are generated via a two-step processing pathway from endogenously transcribed primary miRNAs (pri-miRNAs). miRNAs guide Ago1 via a 5' "seed" sequence to mRNA targets, which are primarily repressed at the translational level [17].

A great deal of progress has been made in deciphering small RNA-based regulatory networks; however, it is clear that many additional components are pending identification and functional characterization. Genome-scale screens for components of siRNA or miRNA pathways have been carried out, with some overlap between components identified [99-104]. However, none of these screens have addressed endo-siRNA pathways, nor have they attempted comparative studies in the same experimental model. Here, we report comparative and comprehensive RNAi screens that identify components of the Argonaute-dependent small RNA pathways (siRNA, miRNA, and endo-siRNA) in cultured *Drosophila* cells.

Results

Assay systems to monitor the siRNA/miRNA pathways

We constructed robust assay systems that allowed us to interrogate the miRNA and siRNA pathways individually. For probing the siRNA pathway, we created an S2 cell line (RZ-14) stably expressing both the Renilla luciferase and a 688 bp perfect inverted repeat that directs Renilla silencing (Figure S1A available online). To identify components of the miRNA pathway, we embedded an artificial miRNA sequence (CXCR4) into the Bantam pri-miRNA (Figure S1B). This construct was transiently introduced into S2 cells together with an expression construct for a Renilla luciferase gene with multiple imperfect CXCR4 complementary sites in its 3′ UTR [93]. In both assays, an expression construct for the firefly luciferase gene served as a normalization control. To prevent the half-life of reporter proteins from confounding our analysis, all transgenes were expressed from the inducible metallothionein promoter.

Both assays systems performed as expected upon knockdown of known components of either pathway. Silencing of Dcr-2 or Ago2 caused significant derepression of siRNA reporters, whereas dsRNAs against *Drosha*, *Dcr-1*, or *Ago1* had no effect (Figure S1C). Conversely, depletion of *Drosha* or *Ago1* led to a marked decrease in miRNA-mediated gene silencing, while treatment with dsRNAs against LacZ, *Dcr-2*, or *Ago2* had no effect (Figure S1D).

Comprehensive identification of siRNA/miRNA pathway components

We screened a collection of 21,000 dsRNAs for those that impacted the siRNA and miRNA pathways. To assess reproducibility, dsRNAs targeting each positive emerging

from the two primary screens were resynthesized and tested multiple times using both assay systems. To minimize potential off-target effects, we also generated additional independent dsRNAs targeting each gene and assessed their impacts on the siRNA and miRNA pathways. Only genes represented by two or more independent consistently scoring dsRNAs were selected as final candidates. We found that *Dcr-2* and *Ago2* were among the siRNA pathway genes, whereas *Drosha* and *Ago1* were among the miRNA pathway candidates (Figure 1C and Table S1), providing an internal validation of each screen.

Recently, an extensive collection of endogenous siRNAs has been characterized in *Drosophila* [69-72]. The biogenesis and function of these endo-siRNAs depend upon canonical RNAi pathway components, including Dcr-2 and Ago2. In some cases, endo-siRNA production depends much more heavily on Loquacious rather than on the canonical Dcr-2 partner, R2D2 [69,71]. We tested all candidates emerging from the miRNA and siRNA pathway screens for their impacts on a sensor for an abundant endo-siRNA, esi-2.1 (Figure S1E). In summary, 177 candidates were identified that affected at least one of the three pathways when knocked down (Figure 2.1A). Among the 116 candidates for the siRNA pathway, 84 also altered the endo-siRNA pathway, and 70 altered the miRNA pathway. Significant overlap (69) was also observed between the endo-siRNA (132) and miRNA pathway (98) candidates. Notably, 54 candidates affected all three pathways.

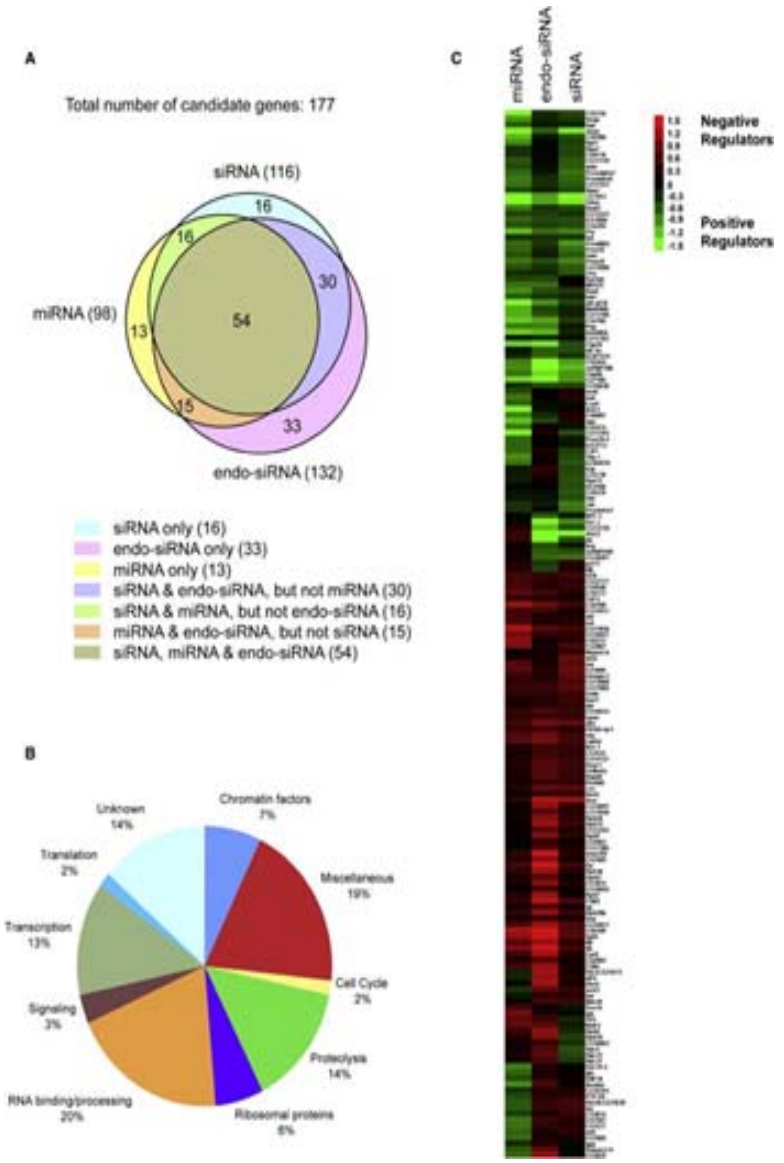

Figure 2.1. Candidates identified from the screens

(A) A Venn diagram showing the impact of the candidates on the siRNA, endo-siRNA, and miRNA assays.
(B) Candidates were sorted into various categories based on their annotated/verified function.
(C) A heat map of the candidates and their scoring patterns in multiple assays. The scores assigned to individual genes in a given assay reflect the relative activity of the pathway upon knockdown of candidate gene expression. Red indicates an increase in silencing, and green indicates a decrease. As each candidate is represented by multiple independent dsRNAs, presented are the average scores from all independent dsRNAs targeting a given gene. Scores for individual dsRNAs are shown in Table S1.

Candidate genes identified from the screens

Based on their annotations, candidates that affect small RNA pathways could be assigned to several functional categories. Notably, genes encoding RNA-binding/processing factors and translation factors were enriched by 10-fold (Figure 2.1B), since only 2% of all *Drosophila* genes belong to this class [107]. These included splicing factors, RNA helicases, and proteins involved in polyadenylation. Splicing factors could have scored in the siRNA pathway screen because the artificial hairpin transcript, which triggers silencing, carries an intron. However, neither the miRNA expression construct nor the endo-siRNA triggers contain intronic sequences, yet most splicing factors still impacted their function. Moreover, in a screen for RNAi pathway components in *C. elegans*, a number of splicing factors, as well as the ortholog of the U1-associated factor, PSI, were identified [101].

Many candidates emerging from the screens showed direct protein-protein interactions, fit into functional modules, or joined known multiprotein complexes (Tables S1 and S2). For example, six U2 snRNP proteins displayed a similar scoring pattern in the screen. We also observed common behavior for two U1 snRNP proteins, snRNP70K and CG5454, and for PSI, which physically and functionally interacts with snRNP70K [108,109].

Knockdown of several ribosomal proteins impacted small RNA pathways. While this effect could be indirect, some ribosomal proteins have been implicated in the siRNA pathway. For example, RpL11 and RpL5 have been shown to reside in a protein complex containing FMR, Dmp68, and Ago2 in S2 cells [110].

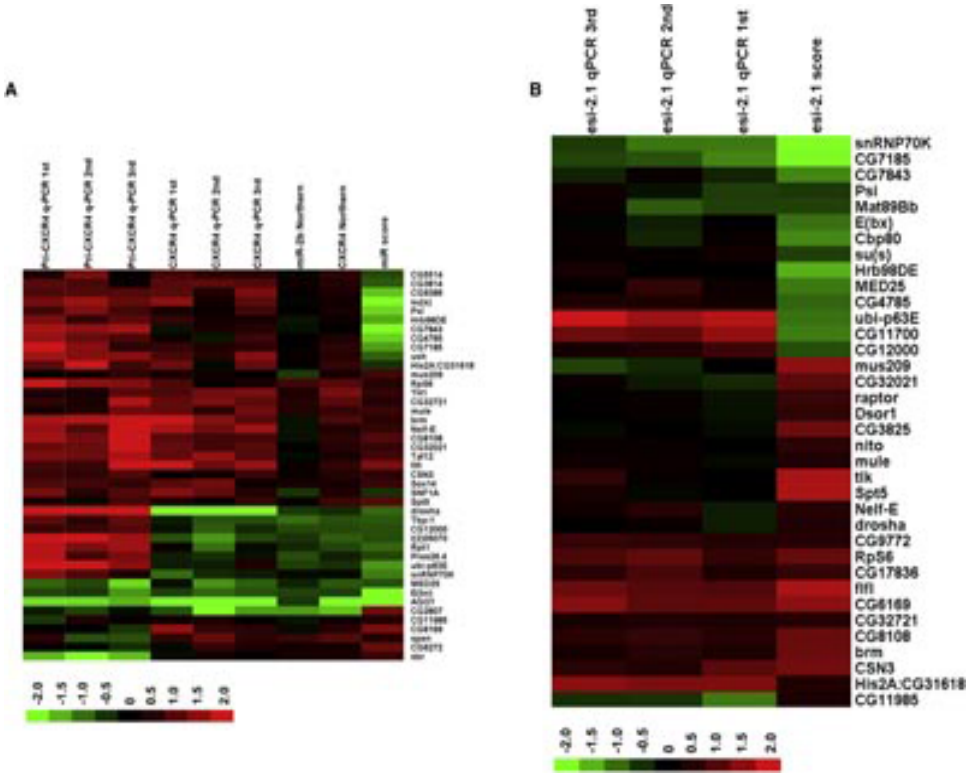

Figure 2.2. Mapping candidates along the small RNA pathway
(A) A heat map of steady-state pri-miRNA and miRNA levels upon knockdown of candidates. Steady-state levels of CXCR4 and those of endogenous miR-2b upon knockdown of 43 selected candidates were examined by northern blotting and semiquantitative RT-PCR (q-PCR). miRNA levels were quantified and normalized first against U6 RNA levels and then against the average of multiple controls (cells treated with dsRNA against LacZ). Also shown are steady-state levels of pri-CXCR4 measured by q-PCR and the relative miRNA pathway activities associated with the representative dsRNA. Red indicates an increase in RNA levels or an increase in the activity of the miRNA pathway, and green indicates a decrease. Presented are the average from two to four independent northern blotting experiments and results from individual independent q-PCR assays. The scores associated with each candidate were from experiments involving one representative dsRNA targeting that gene (Table S3).

(B) A heat map of endo-siRNA levels upon knockdown of candidates. Steady-state levels of esi-2.1 upon knockdown of 36 selected candidates were examined by q-PCR using multiple independent RNA samples. Quantification was performed as described in (A). Also shown are the relative esi-2.1-mediated gene silencing activities. The scores associated with each candidate were from experiments involving one representative dsRNA (Table S4).

Silencing a number of proteasomal components consistently impacted all three small RNA pathways. Indeed, a regulatory subunit, Pros45, has previously been

implicated in RNAi [104]. We find that knockdown of Tbp-1, CG12000, or l(2)05070 led to a decrease in miRNA-mediated silencing and a concomitant reduction in miRNA levels (Figure 2.2A). It is not yet clear whether these effects are direct or indirect.

Placing candidates within the siRNA/miRNA pathway

Candidates affecting the miRNA pathway could impact miRNA biogenesis/stability or miRNA effector functions. To map candidates along the pathway, we examined the effects of their knockdown on steady-state levels of the CXCR4 miRNA mimetic, on endogenous miR-2b, and on those of pri-CXCR4. Knockdown of several candidates led to consistent alterations in mature miRNA levels that paralleled effects on miRNA-mediated silencing (Figure 2.2A and Table S3). For example, Drosha and Ago1 silencing reduced both miRNA levels and miRNA function, and Drosha silencing also led to a coincidental accumulation of pri-CXCR4. This class of positives likely affects miRNA processing and/or stability. Unexpectedly, silencing of Ago1 also had an impact on pri-miRNA levels. While this could represent a feedback mechanism, it is also possible that a miRNA indirectly regulates the metallothionein promoter used to express the pri-CXCR4 in this study. Another set of candidates (such as *CG5514*, *CG3814*, and *CG2807*) impacted miRNA-mediated repression without corresponding effects on mature miRNA levels. These positives most likely impact directly or indirectly effector steps within the pathway.

We also examined steady levels of esi-2.1 upon knockdown of candidates. As shown in Figure 2.2B, knockdown of a group of candidates (e.g., *CG7185* and *snRNP70K*) caused a decrease in esi-2.1-mediated gene silencing, which correlates with a

reduction in esi-2.1 levels. This suggests involvement in siRNA production and/or stability. In contrast, knockdown of *ubi-p63E*, *CG11700*, or *CG12000* caused a change in esi-2.1 activity that did not correlate with alterations in esi-2.1 RNA levels. Considered together, our analyses of the steady-state levels of small RNAs and their precursors following candidate knockdown provide clues on the placement of candidates within small RNA pathways.

Validation of Belle as a bona fide RNAi pathway component

belle (bel) emerged from our screen and from other studies as an RNAi pathway candidate [101,104]. It encodes a DEAD-box RNA helicase, which is required for viability and in the germ line [111]. We chose to validate bel both in animals and through biochemical approaches.

In order to assess RNAi efficiency in flies, we used transgenics carrying an inverted repeat of the *white (w)* gene under the control of the eye-specific *GMR* promoter (*GMR-wIR*; Figure 2.3). These flies display a pale eye color due to strong suppression of white. Because *bel* is essential, we used an eye-specific mitotic recombination system to generate mosaics in which we could assay the impact of a *bel* allele (bel^6) on w silencing in clones [112]. As previously described, eye cells homozygous for a Dcr-2 mutation, $Dcr\text{-}2^{fsL811X}$, display a dark red color, indicating loss of silencing (Figure 2.3D) [19]. Eyes predominantly homozygous for bel^6 are rough and small, suggesting that *bel* is required for cell viability. Importantly, patches of cells with increased pigmentation are observed in homozygous bel^6 clones (Figure 2.3G, arrowheads). We examined *hsc70-4* mutant eyes, which are also small and rough, and found pigmentation to be unaffected,

suggesting that the *bel*^{6} phenotype is specific (Figure 2.3H). These observations suggest that *bel*^{6} mutant clones are defective in RNAi.

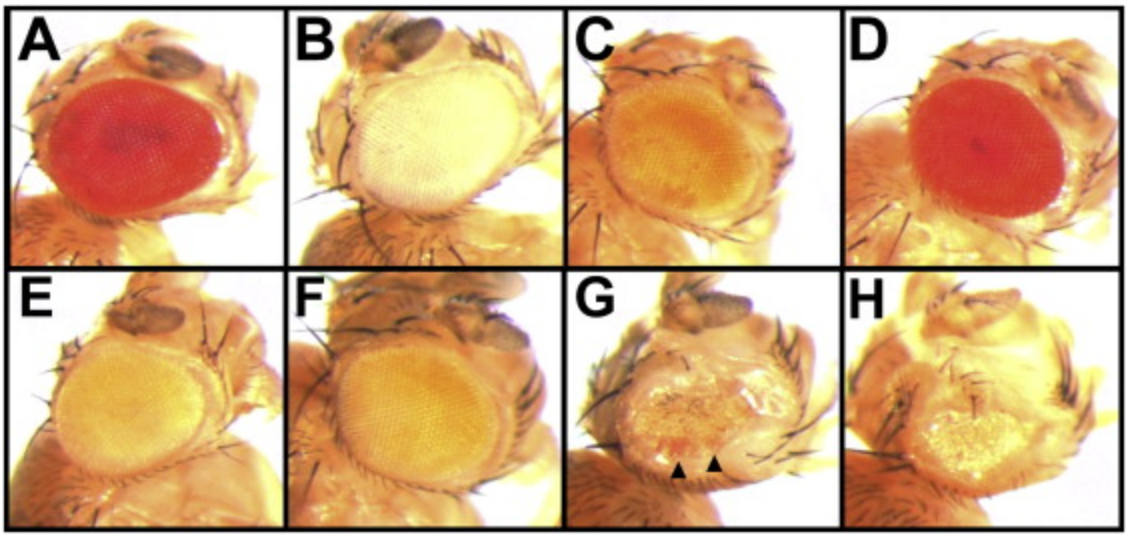

Figure 2.3. *bel*^{6} flies are defective in RNAi
Eye phenotypes of the corresponding genotypes are shown.
(A) *Ore*^{R}
(B) *w*^{1118}
(C) *w+,GMR-wIR/yw; dcr-2*^{L811fsX}*,FRT42D/CyO; EGUF/+*
(D) *w+,GMR-wIR/yw; dcr-2*^{L811fsX}*,FRT42D/FRT42D,GMR-hid,Cl; EGUF/+*
(E) *w+,GMR-wIR/yw; EGUF/+; FRT82B/FRT82B,Cl,GMR-hid*
(F) *w+,GMR-wIR/yw; EGUF/+; bel*^{6}*,FRT82B/TM2*
(G) *w+,GMR-wIR/yw; EGUF/+; bel*^{6}*,FRT82B/FRT82B,Cl,GMR-hid*
(H) *w+,GMR-wIR/yw; EGUF/+; hsc70-4*$^{54.1}$*,FRT82B/FRT82B,Cl,GMR-hid*

Next, we tested whether Bel associates with RNAi components. Cytoplasmic extract was prepared from S2 cells and fractionated by gel filtration. Individual fractions were immunoblotted using antibodies against Bel and components of RISC, including VIG, FMR, Ago2, or Ago1. The majority of Bel cofractionates with Ago1, whereas a smaller fraction coelutes with Ago2, FMR, and VIG, components of the siRISC (Figure 2.4A). We also immunoprecipitated FLAG-tagged Bel from S2 cells and found robust coprecipitation of Ago2, FMR, or VIG (Figure 2.4B). RNase treatment reduced interaction with Ago2 and VIG, but not as substantially with FMR (Figure 2.4B).

To test interactions of Bel with small RNAs, we expressed FLAG-tagged Bel or known components of the RISC together with an artificial siRNA (CXCR4) generated from a perfectly complementary hairpin expression construct. As expected, robust CXCR4 signals could be detected in the Ago2 or VIG complexes (Figure 2.4C). Importantly, CXCR4 was also present in the Bel immunoprecipitate, as was esi-2.1, though they are probably bound directly to another protein in the complex. We conclude that Bel likely acts directly as part of the RNAi machinery, as it resides in a complex that also contains both protein and RNA components of RISC. Interestingly, more CXCR4 siRNA was present in the Ago1 immunoprecipitate than was detected in the Ago2 sample (Figure 2.4C). While this could be attributed to some intrinsic characteristics of the CXCR4 siRNA mimetic, it is also possible that the coupling between miRNA processing and loading steps accounts for this observation.

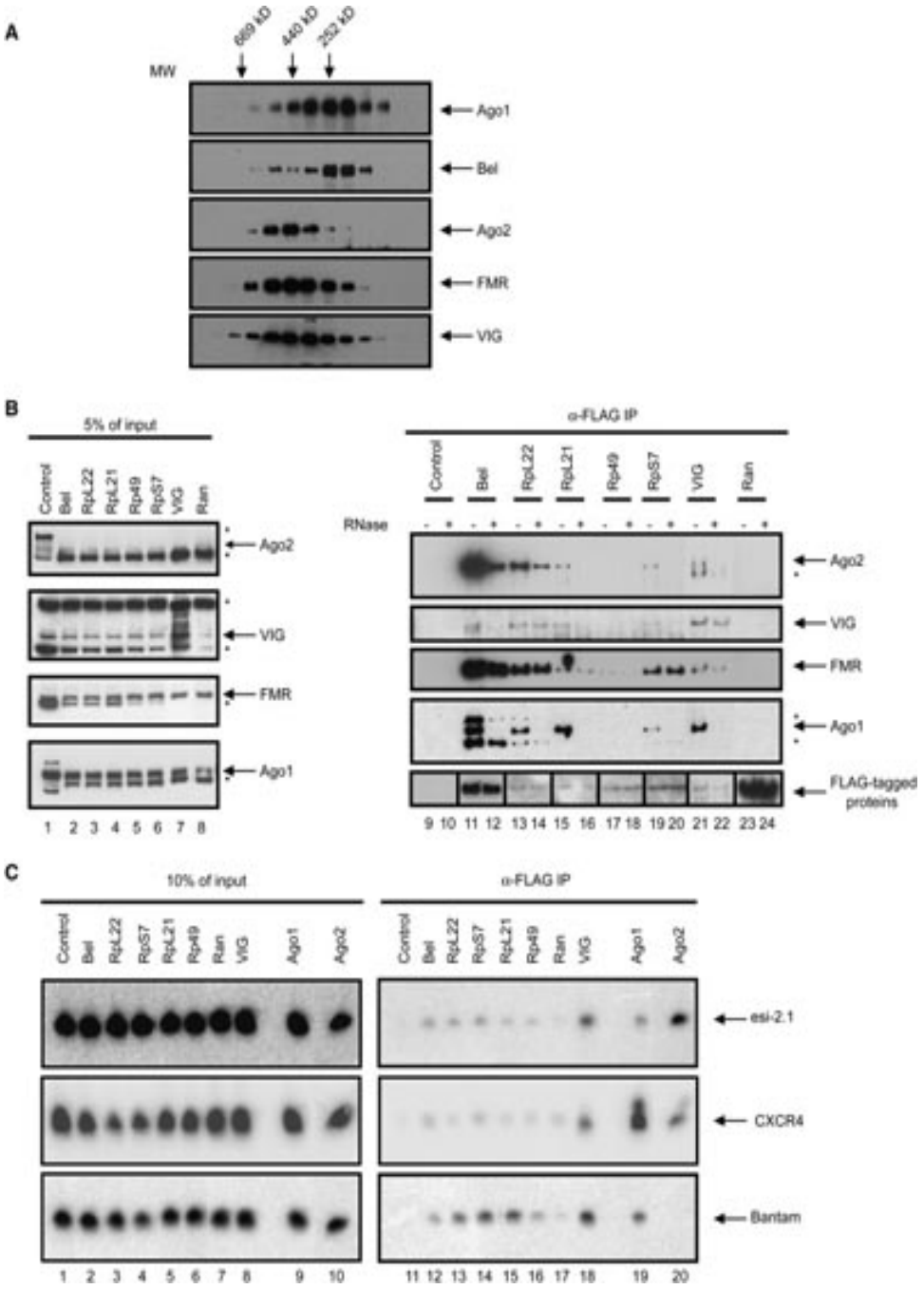

Figure 2.4. Bel and RpL22 interact with components of the RISC

(A) Cytoplasmic extract from S2-NP cells was fractionated by size exclusion chromatography, and the fractions were immunoblotted using antibodies against Bel and components of the RNAi pathway, as indicated. The elution profile of molecular markers is shown on top of the panel.

(B) FLAG-tagged proteins (as labeled above the panel) were expressed in S2-NP cells, and anti-FLAG immunoprecipitates were prepared. The samples were evenly split, and one set was treated with RNase, while the other served as control. The samples were immunoblotted sequentially using antibodies against VIG, Ago2, FMR, Ago1, and FLAG.

A small amount of cell extract was processed in parallel as input control. Nonspecific bands are marked with an asterisk. Note that the images shown in lanes 9 through 12 and 23 through 24 of the anti-FLAG panel were from films that were subjected to different exposure times from the rest due to differences in expression levels.

(C) Small RNAs are enriched in the Bel and ribosomal protein immunoprecipitates. Expression constructs for FLAG-tagged proteins together with that for the CXCR4 siRNA mimetic were transfected into S2-NP cells. Total RNA was extracted from FLAG immunoprecipitates and subjected to northern blotting using probes against Bantam, esi-2.1, and CXCR4. To avoid signal saturation, 25% of the RNA recovered from the Ago1 and Ago2 samples was loaded compared to the rest of the samples. Total RNAs recovered from 10% of the cell extracts were processed in parallel as input control.

Ribosomal proteins associate with the RNAi machinery

We found that RpL22, a candidate from the screen, could coimmunoprecipitate components of the siRISC, including VIG, FMR, and Ago2, suggesting either a direct or indirect interaction. Similar behavior was observed for several other ribosomal proteins (Figure 2.4B). These interactions seemed to display different degrees of dependence on RNA. For example, RNase treatment caused a moderate decrease in the levels of Ago2 in the RpL22 immunoprecipitate, whereas the levels of FMR and VIG remained unaffected (Figure 2.4B). Similar observations were also made for the RpS7 and RpL21 samples. Moreover, both CXCR4 and esi-2.1 are present in the immunoprecipitates of a number of ribosomal proteins, including RpL22, RpS7, RpL21, and Rp49 (Figure 2.4C). These observations indicate that these ribosomal proteins reside in large RNA protein complex(es) that also contain core components of the RNAi machinery.

Discussion

In Drosophila, siRNA and miRNA pathways have been viewed as being biochemically compartmentalized. However, the boundary between these pathways has

been blurred by recent observations that, depending on the configuration of their precursors, miRNAs (and possibly siRNAs) can be partitioned between Ago1 and Ago2 [22,95]. Moreover, Loquacious plays roles both in miRNA biogenesis and in the production of some endo-siRNAs [69,71]. Intimate connections between siRNA and miRNA pathways are also suggested by the observation that knockdown of Ago2 leads to more pronounced silencing by miRNAs (Figure 2.1C), possibly by increasing the access of Ago1 to miRNAs or other limiting components that were previously bound by Ago2.

Analysis of miRNA and siRNA screens shows extensive overlap between genes that impact these pathways. Suppression of many candidates reduces the efficiency of the target small RNA pathways, indicating that those genes might be components of siRNA- or miRNA-mediated responses. Silencing of a roughly equal number of genes increases silencing, indicating that they encode negative regulators of small RNA pathways. These so-called "enhancer of RNAi" phenotypes might indicate attractive targets for genetic manipulation or small molecule inhibitors that could increase the activity of RNAi in either experimental or therapeutic settings. It is worth noting that our analysis of steady-state levels of small RNAs upon candidate knockdown revealed that, for some, enhanced silencing is correlated with increased levels of the small RNA silencing trigger, as is the case for the miRNA pathway candidates such as *CG32721*, *mule*, *TH1*, or *flfl* (Figure 2.2A). In contrast, while knocking down *CG2807* led to markedly enhanced silencing by the miRNA mimetic CXCR4, the steady-state levels of both CXCR4 and miR-2b significantly decreased. While these effects on the small RNA pathways could be indirect, these observations suggest that some of these negative regulators of RNAi are primarily

involved in the biogenesis and/or stability of the small RNA silencing trigger, while others are implicated in the downstream effector steps.

Each pathway was uniquely or differentially affected by a number of genes (Figure 2.1C). For example, knockdown of one class of genes (*caf1*, *CG17293*, and genes encoding ribosomal proteins L5, L21, L22, and S15) led to decreased silencing by exogenous siRNAs but enhanced silencing by endo-siRNAs. Suppression of such a class of genes might enhance the production or loading of endo-siRNAs into RISC, thereby depleting the pool available for products of exogenously introduced dsRNAs, a model that has been previously proposed for some loci in C. elegans [113]. Knockdown of another group of genes (*hsc70-4*, *CG3825*, and *CG2577*) decreased silencing by miRNAs but enhanced silencing by endo-siRNAs. A number of possibilities, including effects on small RNA sorting, might account for these observations.

We validated Bel as a bona fide component of the RNAi pathway. Bel most likely functions at step(s) downstream of siRNA processing and loading, as neither steady-state levels of esi-2.1 nor the levels of Ago2-bound esi-2.1 are affected by Bel knockdown (Figures S2 and S3). Interestingly, Ago1 and Bantam are also present in the Bel immunoprecipitate, consistent with the cofractionation of Bel with miRISC (Figures 2.4B and 2.4C). Thus, Bel may also participate in the miRNA pathway. While none of the *bel* dsRNAs met the scoring criteria in the miRNA assay, they did trend consistently (Table S1).

Ago1 and Bantam were present in a number of ribosomal protein immunoprecipitates, and the association between Ago1 and these ribosomal proteins was abolished by RNase treatment (Figures 2.4B and 2.4C). These observations are consistent

with the notion that miRISC associates with the translation machinery. Both protein and RNA components of the siRISC are also present in these immunoprecipitates, and that knockdown of a number of ribosomal proteins consistently leads to enhanced silencing by endo-siRNAs (Table S1). Thus, the integrity and function of the translational machinery as a whole may be impacting the small RNA pathways.

In summary, our comparative genome-wide screens (Table S5) generate a rich resource for further study of the three Argonaute-dependent small RNA regulatory pathways in Drosophila. These studies not only point to extensive overlap and interplay among small RNA directed silencing machineries in flies but also highlight specific players in each of the three pathways.

Experimental procedures

DNA constructs and cell culture

Detailed description of DNA constructs can be found in the Supplemental Experimental Procedures. S2-NP cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% FBS and 1% pen-strep (Invitrogen). To generate the RZ-14 stable cell line, S2-NP cells were transfected with pRmHa-3-Firefly-long, pRmHa-3-Renilla, and pRmHa-3-Renilla-hairpin together with pHS-neo, using Effectene (QIAGEN). Transfected cells were selected and maintained in growth medium supplemented with 400 μg/ml G418.

RNAi screening

Detailed description of the RNAi screening and bioinformatic analysis can be found in the Supplemental Experimental Procedures.

RNA isolation, Northern blotting, and q-PCR Assays

S2-NP cells were transiently transfected with DNA constructs for the miRNA assay (pMT-Renilla-CXCR4-6B, pMT-D05, and pRmHa-3-Firefly-long). Two days after transfection, 3 × 106 cells were incubated in 1.5 ml serum-free Schneider's medium containing 10 μg of candidate dsRNAs in 6-well plates, and 3 ml serum-containing medium was added 45 min later. After 3 days of dsRNA treatment, cells were induced with 200 mM $CuSO_4$ for 24 hr. A small aliquot of cells were subjected to luciferase assays to examine the effect of dsRNA treatment on reporter activity. Total RNAs were extracted from the rest of the samples using Trizol (Invitrogen). Northern blotting was performed as previously described [71]. miRNA signals were normalized against that of the U6 RNA. A score was assigned to each sample based on the average results from two or four independent experiments. To quantify steady-state levels of pri- and mature miRNAs, semiquantitative RT-PCR (q-PCR) assays were performed (Applied Biosystems) using primers specific for pri-Bantam, CXCR4, and U6.

Immunoprecipitation of proteins and RNAs

Cells were transfected with expression constructs for epitope-tagged proteins, induced with 500 μM $CuSO_4$ 2 days after transfection, and harvested another 24 hr later. Immunoprecipitation was performed as described [71]. For RNase treatment, the immunoprecipitates were split into two sets, and one was incubated in lysis buffer with 100 μg/ml RNase A at 4°C for 15 min prior to washing. For RNA immunoprecipitation, cells were transfected with protein expression constructs together with pMT-F12, an expression vector for CXCR4 derived from a perfectly base-paired precursor, and induced with 500 μM $CuSO_4$ 2 days after transfection. Cells were harvested and cell

lysates prepared 24 hr later. About 80% of the cell lysates were subject to immunoprecipitation using M2 agarose beads. After washing, 10% of the immunoprecipitates were analyzed by western blotting to verify the expression of the epitope-tagged proteins. Total RNAs were extracted from the remaining immunoprecipitates and cell lysates and analyzed by northern blotting.

Size exclusion chromatography

S2-NP cells were lysed in hypotonic buffer (20 mM HEPES [pH 7.0], 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM DTT) and spun at 30,000 × g for 20 min. The supernatant was spun at 200,000 × g for 2.5 hr, and the resulting pellet was subsequently resuspended in buffer A (20 mM HEPES [pH 7.0], 2 mM $MgCl_2$, 0.2 mM $CaCl_2$,1 mM DTT, 0.5% octyl glucoside, 400 mM KCl) and spun at 200,000 × g for another 2.5 hr. The supernatant was fractionated on Superose-6 HR10/10 (Pharmacia).

Fly stocks

bel mutant flies (*bel*^{6}) were from the Bloomington stock center and from Dr. Paul Lasko; *EGUF* flies were from the Bloomington stock center and from Drs. Thomas Schwarz and Stephen Stowers; and the *GMR-wIR* and *dcr-2*^{L811fsX} flies were from Drs. Richard Carthew and Sara Cherry.

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Supplemental data

Supplemental experimental procedures

DNA constructs

A 688-bp DNA fragment was PCR-amplified from a *Renilla* luciferase cDNA and cloned into pWIZ using compatible XbaI/AvrII sites to generate pWIZ-*Renilla*-Reverse. Subsequently, an XbaI/T4/EcoRI fragment from pWIZ-*Renilla*-Reverse was cloned into pRmHa-3 using BamHI/T4/EcoRI sites to generate pRmHa-3-*Renilla*-Reverse. Finally, the same *Renilla* PCR product was cloned into pRmHa-3-*Renilla*-Reverse using compatible XbaI/NheI sites to generate pRmHa-3-*Renilla*-hairpin. A SalI/BglII fragment from pGL3-Basic (Promega) was cloned to pRmHa-3 using SalI/BamHI sites to generate pRmHa-3-Firefly-long. A DNA fragment containing the coding region of the *Renilla* luciferase gene was amplified by PCR and cloned into pRmHa-3 using BamHI/EcoRI sites to generate pRmHa-3-*Renilla*. A pair of oligos containing two perfect binding sites for esi-2.1 were annealed and cloned into pRmHa-3-*Renilla* using BamHI/SalI sites to generate sensor constructs for esi-2.1.

The pMT-D05 construct carries a DNA fragment derived from a modified short pri-Bantam (pri-banCX) where the secondary structure remains the same as pri-Bantam except that the stem sequence has been modified to generate CXCR4. The pri-banCX fragment was amplified by PCR from a template made by ligation of synthesized DNA fragments, and cloned into pMT (Invitrogen) using EcoRI and XhoI restriction sites. The pMT-F12 construct was generated using a similar strategy except that the CXCR4 region in the pri-banCX is perfectly basepaired.

Template sequence for pMT-D05:

CGCTCAGATGCAGATGTTGTTGATGTTATAAAGACCACGATCGAAAGAGGA

AAAACGGAAAACGAACGAAAAGCGTTTGTAACTCCAATGATTTGACTACGAG

ACGTTTTCACTCGTGCTAACTACTGTTTTTCATACAAGTTGTTAGCTGGAGTG

AAAACTTCTTGTCAATGAATACCACATTCCACATTCCGATGGTCGATTTTTGT

TGTTTGGTGTGCCTACACAGATATAGCGATATCGTGTAACAAAGCTTTGAAC

TTATGCCGACCGATC

Template sequence for pMT-F12:

CGCTCAGATGCAGATGTTGTTGATGTTATAAAGACCACGATCGAAAGAGGA

AAAACGGAAAACGAACGAAAAGCGTTTGTAACTCCAATGATTTGACTACGAG

AAGTTTTCACTCCAGCTAACAACTGTTTTTCATACAAGTTGTTAGCTGGAGTG

AAAACTTCTTGTCAATGAATACCACATTCCACATTCCGATGGTCGATTTTTGT

TGTTTGGTGTGCCTACACAGATATAGCGATATCGTGTAACAAAGCTTTGAAC

TTATGCCGACCGATC

Cloning Primers:

Bantam-CXCR4 EcoRI fwd

TTCACGTGAATTC CGCTCAGATGCAGATGTTGTTGATG

Bantam-CXCR4 XhoI rev

TTCACGTCTCGAG GATCGGTCGGCATAAGTTCAAAGCTTTG

The *Renilla* luciferase reporter construct carrying CXCR4 target sites (pMT*Renilla*-CXCR4-6B) was subcloned from the RL-CXCR-6-bulge reporter plasmid into pMT using EcoRI and XhoI sites [93].

RNAi screening

The screens were performed in 384-well plates. To screen for components of the siRNA pathway, 10 μL of RZ-14 cells (suspended in serum-free Schneider's medium at a density of $2X10^6$ cells/mL) were dispensed to each well containing 5 μL dsRNA (~150-250 ng in total), incubated at room temperature for 45 minutes, then supplemented with 30 μL growth medium containing FBS. Three days after dsRNA treatment, cells were induced with 25 μM $CuSO_4$ and luciferase assays were performed another two days later using Dual-Glo luciferase reagents (Promega). To screen for components of the miRNA pathway, a transient transfection approach was employed. Approximately 100ng plasmid DNA (a mixture of 5 ng pMT-*Renilla*-CXCR4-6B, 93 ng pMT-D05 and 2 ng pRmHa-3-Firefly-long) was mixed with 0.8 μl Enhancer in 15 μl EC (Qiagen) and incubated at room temperature for 5 minutes. Then 0.35 μl of Effectene reagent was added and the mixture was immediately dispensed into each well containing ~80-120 ng of dsRNAs. After incubation at room temperature for 10 minutes, 40 μl S2-NP cells (106 cells/mL) were added to each well. Cells were induced with 200 mM $CuSO_4$ 3 days after transfection, and luciferase assays were performed 24 hours later. For each well, the ratio of *Renilla* luciferase to firefly luciferase, referred to as relative luciferase unit (RLU), was calculated. For each individual plate, the mean RLU (RLUmean) was calculated, and each well received a score based on the ratio of RLUsample/RLUmean. For the siRNA pathway primary screen, cutoff values of 1.5/0.6 were used to select candidates, whereas cutoff values of 1.6/0.6 were used for the miRNA pathway primary screen. For the secondary screen, candidate dsRNAs were re-synthesized using the MegaScript kit (Ambion) and arrayed in 384-well plates. Each assay plate also contains 24-48 wells

containing *LacZ* dsRNAs as controls. The plates were subsequently assayed in 3 to 4 independent experiments using the corresponding siRNA/miRNA assay systems. Each sample was assigned a score as fold change in RLU relative to the average of RLUs derived from the control samples (cells treated with dsRNA against LacZ). The cutoff values for the secondary screens were 1.2/0.8 for the siRNA pathway screen, and 1.4/0.75 for the miRNA pathway screen, respectively. To minimize potential off-target effects associated with long dsRNAs, for candidate dsRNAs that scored in the secondary screen, one or more additional independent dsRNAs were synthesized and tested in a similar setting. Only genes represented by two or more independent consistently scoring dsRNAs were selected as final candidates.

To screen for dsRNAs that impacted the endogenous siRNA pathway, a sensor construct for esi-2.1 was employed to screen the composite collection of candidate dsRNAs emerging from the siRNA and miRNA primary screens. The experimental setting was the same as described in the miRNA primary screen except that a mixture of 98 ng pRmHa-3-*Renilla*-esi-2.1-sensor and 2 ng pRmHa-3-Firefly-long was transfected into each well. The cutoff values for the endosiRNA pathway screen were 1.2/0.8.

Bioinformatic analysis of the screening results

To generate Figure 1C, the average fold change in reporter activity for multiple independent dsRNAs targeting the same gene was calculated, and the reciprocals of the average fold change in reporter activity were log(2) transformed. The resulting values reflect the relative activities of each small RNA pathway in a given sample. The values were subjected to Hierarchical clustering (average linkage analysis) using the Cluster software. As for Figure 2A, the miRNA pathway activity (determined by the procedure

described above) associated with selected dsRNAs targeting a given gene, the relative CXCR4 and miR-2b RNA levels (as an average from 2 to 4 independent Northern blotting experiments), and the relative levels of mature and pri-CXCR4 (from 3 independent q-PCR experiments) were subject to Hierarchical clustering. The resulting image was re-arranged to generate the final figure. Similar procedure was followed to generate Figure 2B.

Supplemental figure legends:

Figure S1. Assay systems for monitoring the siRNA, miRNA, and endosiRNA pathways in cultured *Drosophila* cells

(A) Schematic representation of the RNAi pathway reporter system (RZ-14 cells) in which a perfectly complementary hairpin induces *Renilla* luciferase silencing.

(B) Schematic representation of the miRNA pathway reporter system. An artificial miRNA (CXCR4) embedded in pri-Bantam silences a *Renilla* luciferase transcript carrying multiple imperfect CXCR4-binding sites in the 3' UTR. **(C)** RZ-14 cells were treated with indicated dsRNAs by soaking, and gene expression was induced with CuSO4 3 days after dsRNA treatment. Luciferase activity was measured after another 48 hours. The ratio of *Renilla* luciferase/Firefly luciferase (Relative luciferase unit, or RLU) was calculated and normalized against RLU from cells treated with dsRNA against *LacZ*. Error bars represent standard deviation ($n \geq 4$). **(D)** S2-NP cells were transfected with the CXCR4 miRNA expression vector and reporter plasmids together with various dsRNAs, and treated with $CuSO_4$ 3 days after transfection to induce transgene expression.

Luciferase assays were conducted after another 24 hours and analyzed and plotted as described in **C**. **(E)** Schematic representation of the endo-siRNA pathway reporter system. An endogenous siRNA (esi-2.1) silences a *Renilla* luciferase transcript carrying two perfect esi-2.1-binding sites in the 3' UTR.

Figure S2. Knockdown of Bel does not affect steady-state levels of esi-2.1

S2-NP cells were treated with various dsRNAs (as labeled on top of the panel). Total RNAs were extracted and subjected to Northern blotting analysis using probes against Bantam and esi-2.1. 2S RNA serves as loading control.

Figure S3. Knockdown of Bel does not affect levels of Ago2-bound esi-2.1

(A) S2-NP cells were first transfected with an expression construct for FLAGtagged Ago2, then treated with various dsRNAs (as labeled on top of the panel). Cells were harvested and cell lysates were prepared. Approximately the same amount of total cell lysates were subjected to immunoprecipitation using anti-FLAG agarose beads. Total RNAs were subsequently extracted from the immunoprecipitates and subjected to Northern blotting analysis using a probe against esi-2.1. RNAs purified from an equivalent of ~25% of the cell lysates employed in the immunoprecipitation experiment were processed in parallel to serve as control for input material. **(B)** Approximately 1% of the input cell lysates and 5% of the immunoprecipitates were subjected to anti-FLAG immunoblotting to show the relative amount of the input material, and the membrane carrying cell lysates was re-probed with the Bel antibody to show effective knockdown.

Reference

1. Doench, J. G., Petersen, C. P. & Sharp, P. A. siRNAs can function as miRNAs. *Genes Dev* **17**, 438-42 (2003).

Table S1. List of candidate genes identified from the screen. Candidate genes, corresponding dsRNAs (listed as amplicons), and their scores in the siRNA, endo-siRNA, and miRNA assays are shown. For any gene to be considered a candidate in a given assay, at least two independent dsRNAs have to score. A "−1" represents negative regulators of a given pathway, whereas a "1" represents positive regulators and "0" means "did not score." Information in the columns labeled as "Eukaryotic clusters" and "Eukaryotic orthologous groups" were based on annotations in the COG database(http://www.ncbi.nlm.nih.gov/COG/). Each candidate gene was also assigned to a functional category based on the information from the COG database and the Flybase (http://www.flybase.org/). Detailed information on individual amplicons is listed at the DRSC website (http://www.flyrnai.org/).

Amplicon	Gene	Fold change siRNA reporter	Fold change endo-siRNA reporter	Fold change miRNA reporter	siRNA candidate	endo-siRNA candidate	miRNA candidate	Eukaryotic clusters	Eukaryotic orthologous groups	Brief description from the flybase and the COG database	Category
DRSC05912	AGO1	1.036	1.381	4.999	0	0	1	ACDH-P-	KOG1041	miRISC component	RNA binding/processing
DRSC30761	AGO1	0.994	1.033	5.592							
DRSC30762	AGO1	0.749	0.812	4.197							
DRSC10847	AGO2	3.109	9.145	0.763	1	1	-1	ACDH-P-	KOG1041	siRISC component	RNA binding/processing
DRSC31768	AGO2	3.164	7.321	0.599							
DRSC31769	AGO2	3.214	8.159	0.743							
DRSC16555	bel	1.273	1.781	1.270	1	0	0	ACDHYPE	KOG0335	ATP-dependent RNA helicase	RNA binding/processing
DRSC31929	bel	1.213	1.110	1.357							
DRSC31930	bel	1.477	0.964	1.226							
DRSC27666	Bx42	1.051	1.003	0.598	-1	-1	0	ACDHYP-	KOG2441	mRNA splicing factor/probable chromatin binding snw family nuclear protein	RNA binding/processing
DRSC32295	Bx42	0.580	0.646	1.090							

DRSC32296	Bx42	0.574	0.545	0.950							
DRSC16601	Cbp20	1.601	2.473	1.748	1	1	1	ACDHYPE	KOG0121	Nuclear cap-binding protein complex, subunit CBP20 (RRM superfamily)	RNA binding/processing
DRSC31999	Cbp20	1.269	2.683	2.434							
DRSC32000	Cbp20	1.530	2.674	3.003							
DRSC18450	Cbp80	2.133	2.300	1.781	1	1	1	ACDHYP-	KOG1104	Nuclear cap-binding complex, subunit NCBP1/CBP80	RNA binding/processing
DRSC32001	Cbp80	1.735	2.323	1.593							
DRSC32002	Cbp80	1.963	2.898	1.721							
DRSC34871	CG10630	1.283	0.998	0.979	1	1	0		None	Double-stranded RNA binding	RNA binding/processing
DRSC34872	CG10630	1.653	1.208	1.396							
DRSC09757	CG10630	1.353	2.205	1.467							
DRSC14460	CG11985	0.671	0.775	0.735	-1	-1	-1	ACDH-P-	KOG3485	Splicing factor 3B subunit 10	RNA binding/processing
DRSC31771	CG11985	0.713	0.620	0.661							
DRSC31770	CG11985	0.359	0.719	0.696							
DRSC36192	CG13900	0.525	0.627	0.829	-1	0	0	ACDHYPE	KOG1898	Splicing factor 3b, subunit 3, U2 snRNP component	RNA binding/processing
DRSC08370	CG13900	0.753	0.853	0.650							
DRSC15166	CG16941	0.703	0.821	0.664	-1	-1	-1	ACDHYP-	KOG0007	Splicing factor 3a, subunit 1, U2 snRNP component	RNA binding/processing
DRSC31838	CG16941	0.759	0.658	0.403							
DRSC31839	CG16941	0.733	0.622	0.484							
DRSC21723	CG17293	1.319	0.969	3.663	1	-1	1	ACDHYPE	KOG1446	Histone H3 (Lys4) methyltransferase complex and RNA cleavage factor II complex, subunit SWD2	RNA binding/processing
DRSC34968	CG17293	1.972	0.707	3.166							
DRSC34969	CG17293	1.225	0.793	2.890							
DRSC02680	CG18591	2.009	1.775	0.760	1	1	0	ACDHYPE	KOG1774	Small nuclear ribonucleoprotein E	RNA binding/processing
DRSC31784	CG18591	1.383	1.225	0.993							
DRSC31785	CG18591	1.740	2.001	0.954							
DRSC06787	CG1874	0.670	0.472	0.825	-1	-1	0	ACDHYPE	KOG1831	CCR4-Not complex component, Not1	RNA binding/processing
DRSC32356	CG1874	0.771	0.398	0.640							
DRSC3	CG187	0.998	0.473	0.790							

2357	4										
DRSC0 6794	CG188 4	0.742	0.601	0.713	-1	-1	0	ACDH YPE	KOG1 831	CCR4-Not complex component, Not1	RNA binding/proc essing
DRSC3 0775	CG188 4	0.844	0.466	0.935							
DRSC3 0776	CG188 4	0.767	0.419	1.067							
DRSC0 0535	CG280 7	0.934	0.873	0.426	-1	0	-1	ACDH YPE	KOG0 213	Splicing factor 3b, subunit 1	RNA binding/proc essing
DRSC3 1841	CG280 7	0.587	0.718	0.474							
DRSC3 1840	CG280 7	0.369	0.869	0.555							
DRSC0 0619	CG360 5	0.827	0.916	0.611	-1	-1	-1	ACDH YPE	KOG2 330	Splicing factor 3b, subunit 2, U2 snRNP component	RNA binding/proc essing
DRSC3 1846	CG360 5	0.348	0.773	0.517							
DRSC3 1847	CG360 5	0.446	0.602	0.632							
DRSC1 5804	CG545 4	1.946	4.163	1.471	1	1	1	ACDH YPE	KOG3 454	U1 snRNP-specific protein C	RNA binding/proc essing
DRSC3 0973	CG545 4	2.022	3.391	2.087							
DRSC3 0974	CG545 4	2.065	3.863	1.871							
DRSC1 0597	CG616 9	0.616	0.353	0.396	-1	-1	-1	ACDH YPE	KOG2 937	Decapping enzyme complex, predicted pyrophosphatase DCP2	RNA binding/proc essing
DRSC3 4890	CG616 9	0.685	0.303	0.423							
DRSC3 4891	CG616 9	0.581	0.256	0.415							
DRSC1 0781	CG718 5	2.052	3.906	2.577	1	1	1	-CDH- --	KOG4 849	mRNA cleavage factor I subunit/CPSF subunit	RNA binding/proc essing
DRSC3 4892	CG718 5	2.279	4.012	2.656							
DRSC3 4893	CG718 5	1.608	4.740	2.095							
DRSC0 6992	Dcr-2	1.447	4.322	0.904	1	1	0	ACDH -P-	KOG0 701	dsRNA-specific nuclease Dicer and related ribonucleases	RNA binding/proc essing
DRSC3 2999	Dcr-2	2.052	3.841	1.207							
DRSC3 3000	Dcr-2	2.178	4.737	1.035							
DRSC0 7607	drosha	0.977	0.740	1.955	0	-1	1	- CDHY P-	KOG1 817	Ribonuclease	RNA binding/proc essing
DRSC3 5708	drosha	0.923	0.528	1.848							
DRSC2 7807	glo	0.876	0.753	1.657	0	-1	1	ACDH ---	KOG4 211	Splicing factor hnRNP-F and related RNA-binding proteins	RNA binding/proc essing
DRSC1 6145	glo	0.924	0.989	1.886							
DRSC3	glo	1.040	0.783	1.341							

4656											
DRSC17135	gw	0.972	0.997	0.509	0	-1	-1		None	RNA recognition motif, RNP-1	RNA binding/processing
DRSC34234	gw	1.228	0.709	0.476							
DRSC34235	gw	0.890	0.711	0.564							
DRSC16709	Hrb98DE	1.494	2.789	2.697	1	1	1	ACDHYPE	KOG0118	RRM domain	RNA binding/processing
DRSC34920	Hrb98DE	0.927	1.727	2.432							
DRSC34919	Hrb98DE	1.264	2.075	2.059							
DRSC07502	hrg	1.122	1.691	0.946	0	1	0	ACDHYPE	KOG2245	Poly(A) polymerase and related nucleotidyltransferases	RNA binding/processing
DRSC31961	hrg	1.143	1.278	0.900							
DRSC05943	nito	0.650	0.682	0.626	-1	-1	-1	-CDH--	KOG01	Large RNA-binding protein (RRM superfamily)	RNA binding/processing
DRSC35891	nito	0.470	0.356	0.690							
DRSC26152	noi	0.859	0.926	0.495	-1	0	-1	ACDHYPE	KOG2636	Splicing factor 3a, subunit 3, U2 snRNP component	RNA binding/processing
DRSC31860	noi	0.421	0.717	0.598							
DRSC31861	noi	0.468	0.882	0.684							
DRSC23350	pcf11	1.121	1.709	0.666	-1	1	0	ACDHYPE	KOG2071	mRNA cleavage and polyadenylation factor I/II complex, subunit Pcf11	RNA binding/processing
DRSC34272	Pcf11	0.567	1.297	0.811							
DRSC34273	Pcf11	0.586	1.038	0.869							
DRSC07519	Psi	2.239	1.491	3.341	1	1	1	ACDH---	KOG1676	K-homology type RNA binding proteins	RNA binding/processing
DRSC34932	Psi	2.046	2.251	2.304							
DRSC34931	Psi	1.157	1.268	1.157							
DRSC16223	Rrp6	1.658	1.751	1.739	1	1	1	ACDHY-E	KOG2206	Exosome 3'-5' exoribonuclease complex, subunit PM/SCL-100 (Rrp6)	RNA binding/processing
DRSC32600	Rrp6	1.075	1.298	1.928							
DRSC32601	Rrp6	1.379	1.415	2.073							
DRSC20368	sbr	1.043	0.808	0.625	-1	-1	-1	-CDHYP-	KOG3763	mRNA export factor TAP/MEX67	RNA binding/processing
DRSC29803	sbr	1.055	1.150	0.612							
DRSC33351	sbr	0.610	0.629	0.588							

DRSC3 3352	sbr	0.537	0.774	0.823							
DRSC0 9800	snRNP 69D	1.732	1.610	0.762	1	1	0	ACDH YPE	KOG3 428	Small nuclear ribonucleoprotein SMD1 and related snRNPs	RNA binding/processing
DRSC3 1709	snRNP 69D	1.214	1.536	0.985							
DRSC0 3612	snRNP 70K	1.016	1.219	1.221	1	1	1	ACDH YP-	KOG0 113	U1 small nuclear ribonucleoprotein (RRM superfamily)	RNA binding/processing
DRSC2 8803	snRNP 70K	1.989	3.610	1.674							
DRSC2 2071	snRNP 70K	2.650	4.087	2.169							
DRSC3 4939	snRNP 70K	1.969	3.981	2.301							
DRSC3 4940	snRNP 70K	3.532	4.261	1.996							
DRSC0 0839	spen	0.870	0.819	0.732	-1	-1	-1	ACDH YPE	KOG0 123	Polyadenylate-binding protein (RRM superfamily)	RNA binding/processing
DRSC3 4301	spen	0.630	0.561	0.689							
DRSC3 4302	spen	0.631	0.723	0.806							
DRSC1 8839	su(s)	2.605	1.546	6.541	1	1	1	ACDH YPE	KOG1 040	Polyadenylation factor I complex, subunit, Yth1 (CPSF subunit)	RNA binding/processing
DRSC3 4942	su(s)	2.543	0.991	6.063							
DRSC3 4941	su(s)	2.838	2.264	4.592							
DRSC0 6445	U2A	0.495	0.908	0.541	-1	-1	-1	ACDH YP-	KOG1 644	U2-associated snRNP A' protein	RNA binding/processing
DRSC3 1825	U2A	0.844	0.631	0.661							
DRSC3 1826	U2A	0.629	0.600	0.738							
DRSC0 3704	RpL21	1.589	0.671	0.732	1	-1	0	ACDH YPE	KOG1 732	60S ribosomal protein L21	Ribosomal protein
DRSC3 2240	RpL21	1.376	0.655	0.818							
DRSC3 2241	RpL21	1.078	0.590	1.017							
DRSC1 8707	RpL22	1.555	0.716	0.938	1	-1	0	ACDH YPE	KOG3 434	60S ribosomal protein L22	Ribosomal protein
DRSC2 5025	RpL22	1.369	0.443	0.736							
DRSC0 3801	RpL5	1.294	0.622	1.024	1	-1	0	ACDH YPE	KOG0 875	60S ribosomal protein L5	Ribosomal protein
DRSC3 2562	RpL5	1.738	0.610	0.729							
DRSC0 3419	RpS13	0.883	0.407	0.667	-1	-1	0	ACDH YPE	KOG0 400	40S ribosomal protein S13	Ribosomal protein
DRSC3 2575	RpS13	0.424	0.470	1.210							
DRSC3	RpS13	0.760	0.421	0.913							

2576											
DRSC07151	RpS15	1.059	0.533	0.553	0	-1	0	ACDHYPE	KOG0898	40S ribosomal protein S15	Ribosomal protein
DRSC32256	RpS15	1.726	0.448	1.084							
DRSC03420	RpS26	1.039	0.516	0.632	-1	-1	0	ACDHYPE	KOG1768	40s ribosomal protein S26	Ribosomal protein
DRSC30693	RpS26	0.629	0.491	1.006							
DRSC30694	RpS26	0.557	0.441	0.941							
DRSC18258	RpS28b	0.834	0.450	0.643	0	-1	0	ACDHYPE	KOG3502	40S ribosomal protein S28	Ribosomal protein
DRSC32590	RpS28b	0.547	0.434	0.795							
DRSC26355	RpS30	0.985	0.473	0.635	0	-1	0	ACDHYPE	KOG0009	Ubiquitin-like/40S ribosomal S30 protein fusion	Ribosomal protein
DRSC32262	RpS30	0.585	0.389	0.862							
DRSC18712	RpS6	0.956	0.452	0.697	0	-1	-1	ACDHYPE	KOG1646	40S ribosomal protein S6	Ribosomal protein
DRSC37989	RpS6	1.532	0.458	0.706							
DRSC15394	RpS7	1.081	0.408	0.806	0	-1	0	ACDHYPE	KOG3320	40S ribosomal protein S7	Ribosomal protein
DRSC28468	RpS7	1.050	0.508	0.688							
DRSC32264	RpS7	0.970	0.351	0.871							
DRSC32263	RpS7	0.673	0.488	0.792							
DRSC11273	RpS9	0.884	0.444	0.725	-1	-1	0	ACDHYPE	KOG3301	Ribosomal protein S4	Ribosomal protein
DRSC32598	RpS9	0.741	0.430	0.818							
DRSC32599	RpS9	0.632	0.560	1.194							
DRSC08463	ago	1.039	1.968	2.919	-1	0	1	ACDHYPE	KOG0274	Cdc4 and related F-box and WD-40 proteins	Proteolysis
DRSC30817	ago	0.579	1.182	1.928							
DRSC30818	ago	0.771	1.119	1.968							
DRSC23384	CG11700	0.876	1.012	1.441	1	1	1	ACDHYPE	KOG0001	Ubiquitin and ubiquitin-like proteins	Proteolysis
DRSC17794	CG11700	1.434	2.165	2.412							
DRSC31545	CG11700	1.285	1.296	2.548							
DRSC12186	CG12000	1.640	1.692	1.701	1	1	1	ACDHYPE	KOG0185	20S proteasome, regulatory subunit beta type PSMB4/PRE4	Proteolysis
DRSC32030	CG12000	1.521	0.915	1.511							
DRSC3	CG120	1.531	1.277	1.345							

2029	00										
DRSC02603	CG17331	1.781	1.604	1.681	1	0	1	ACDHYPE	KOG0177	20S proteasome, regulatory subunit beta type PSMB2/PRE1	Proteolysis
DRSC31562	CG17331	1.494	1.107	1.880							
DRSC31563	CG17331	1.452	1.164	1.643							
DRSC28203	CG9772	0.820	0.710	0.565	-1	-1	-1	-CDH--	KOG2120	SCF ubiquitin ligase, Skp2 component	Proteolysis
DRSC12329	CG9772	0.834	0.585	0.518							
DRSC33294	CG9772	0.685	0.643	0.609							
DRSC33295	CG9772	0.753	0.656	0.640							
DRSC11859	CSN3	0.810	0.414	0.678	-1	-1	-1	ACDH-P-	KOG2582	COP9 signalosome, subunit CSN3	Proteolysis
DRSC32997	CSN3	0.500	0.262	0.667							
DRSC32998	CSN3	0.584	0.474	0.877							
DRSC06808	CSN7	0.860	0.458	0.558	0	-1	-1	A-DH-P-	KOG3250	COP9 signalosome, subunit CSN7	Proteolysis
DRSC06807	CSN7	0.723	0.386	0.668							
DRSC34904	CSN7	0.902	0.491	0.728							
DRSC16971	hyd	1.216	1.083	1.493	1	0	1	-CDH--	KOG0943	Predicted ubiquitin-protein ligase/hyperplastic discs protein, HECT superfamily	Proteolysis
DRSC34921	hyd	1.176	1.269	1.688							
DRSC34922	hyd	1.204	1.068	1.486							
DRSC07159	l(2)05070	1.396	1.136	1.668	1	0	1	ACDHYPE	KOG0174	20S proteasome, regulatory subunit beta type PSMB6/PSMB9/PRE3	Proteolysis
DRSC32140	l(2)05070	1.423	0.800	2.011							
DRSC32139	l(2)05070	1.807	0.858	1.595							
DRSC00828	lwr	0.665	0.844	1.111	-1	-1	0	ACDHYPE	KOG0424	Ubiquitin-conjugating enzyme, E2	Proteolysis
DRSC34570	lwr	0.721	0.766	1.183							

DRSC34571	lwr	0.950	0.660	1.138							
DRSC10509	mule	0.961	0.737	0.608	-1	-1	-1	ACDH-P-	KOG1865	Ubiquitin carboxyl-terminal hydrolase	Proteolysis
DRSC30860	mule	0.590	0.693	0.706							
DRSC30861	mule	0.496	0.777	0.867							
DRSC11256	Pros26	1.981	1.680	1.855	1	1	1	ACDHYPE	KOG0179	20S proteasome, regulatory subunit beta type PSMB1/PRE7	Proteolysis
DRSC32168	Pros26	2.073	1.671	1.664							
DRSC32169	Pros26	1.488	1.063	1.699							
DRSC25012	Pros26.4	1.139	1.139	1.613	0	0	1	ACDHYPE	KOG0726	26S proteasome regulatory complex, ATPase RPT2	Proteolysis
DRSC32051	Pros26.4	1.424	0.869	1.859							
DRSC32052	Pros26.4	0.892	0.972	1.476							
DRSC03401	Pros35	1.512	1.261	1.451	1	1	1	ACDHYPE	KOG0863	20S proteasome, regulatory subunit alpha type PSMA1/PRE5	Proteolysis
DRSC32172	Pros35	1.622	1.170	1.859							
DRSC32173	Pros35	1.938	1.255	1.543							
DRSC07516	Prosalpha7	1.681	1.238	1.570	1	1	1	ACDHYPE	KOG0184	20S proteasome, regulatory subunit alpha type PSMA3/PRE10	Proteolysis
DRSC32176	Prosalpha7	2.068	1.269	1.771							
DRSC32177	Prosalpha7	1.396	0.880	1.502							
DRSC16801	Prosbeta3	1.791	1.019	1.121	1	0	0	ACDHYPE	KOG0180	20S proteasome, regulatory subunit beta type PSMB3/PUP3	Proteolysis
DRSC32180	Prosbeta3	1.519	0.855	1.388							
DRSC32181	Prosbeta3	1.802	1.293	1.415							

DRSC07517	Prosbeta5	1.806	1.312	1.605	1	1	1	ACDHYPE	KOG0175	20S proteasome, regulatory subunit beta type PSMB5/PSMB8/PRE2	Proteolysis
DRSC32182	Prosbeta5	1.619	1.400	1.701							
DRSC32183	Prosbeta5	1.454	1.101	2.004							
DRSC07514	ProsMA5	1.637	1.327	1.487	1	1	1	ACDHYPE	KOG0176	20S proteasome, regulatory subunit alpha type PSMA5/PUP2	Proteolysis
DRSC32184	ProsMA5	2.271	1.157	1.680							
DRSC32185	ProsMA5	2.022	1.541	1.829							
DRSC03422	Rpn11	1.651	1.168	1.347	1	0	0	ACDHYPE	KOG1555	26S proteasome regulatory complex, subunit RPN11	Proteolysis
DRSC32195	Rpn11	1.366	0.666	1.028							
DRSC16839	Rpn2	1.622	1.217	1.663	1	1	1	ACDHYPE	KOG2062	26S proteasome regulatory complex, subunit RPN2/PSMD1	Proteolysis
DRSC32198	Rpn2	1.456	1.281	1.152							
DRSC32199	Rpn2	1.216	1.086	1.511							
DRSC16841	Rpn7	1.732	1.074	1.147	1	0	0	ACDHYPE	KOG0687	26S proteasome regulatory complex, subunit RPN7/PSMD6	Proteolysis
DRSC32204	Rpn7	1.164	0.916	1.429							
DRSC32205	Rpn7	1.398	1.094	1.339							
DRSC07542	Rpt1	1.627	1.166	1.934	1	0	1	ACDHYPE	KOG0729	26S proteasome regulatory complex, ATPase RPT1	Proteolysis
DRSC30799	Rpt1	1.018	1.058	1.979							
DRSC30800	Rpt1	1.502	0.840	1.780							

DRSC03611	smt3	0.640	0.476	1.027	-1	-1	0	ACDHYPE	KOG1769	Ubiquitin-like proteins	Proteolysis
DRSC33355	smt3	0.713	0.695	1.929							
DRSC33356	smt3	0.774	0.565	1.018							
DRSC26269	Tbp-1	1.588	0.965	1.782	1	0	1	ACDHY-E	KOG0652	26S proteasome regulatory complex, ATPase RPT5	Proteolysis
DRSC16842	Tbp-1	1.584	0.917	1.611							
DRSC33360	Tbp-1	1.162	0.812	2.076							
DRSC33361	Tbp-1	1.377	0.847	2.202							
DRSC33366	ubi-p63E	1.286	2.060	3.428	1	1	1	ACDHYPE	KOG0001	Ubiquitin and ubiquitin-like proteins	Proteolysis
DRSC08703	ubi-p63E	1.568	1.805	2.481							
DRSC19337	Bap60	0.675	0.594	0.602	-1	-1	0	ACDHYPE	KOG2570	SWI/SNF transcription activation complex subunit, Brahma complex subunit	Chromatin factors
DRSC32656	Bap60	0.671	0.719	0.975							
DRSC32657	Bap60	0.783	0.496	0.951							
DRSC03488	barr	1.236	1.668	1.484	1	0	1	A-DHYPE	KOG2328	Chromosome condensation complex Condensin, subunit H	Chromatin factors
DRSC34869	barr	1.170	1.116	1.717							
DRSC34870	barr	1.227	1.130	1.468							
DRSC11330	brm	0.553	0.434	0.689	-1	-1	-1	ACDHYP-	KOG0386	Chromatin remodeling complex SWI/SNF, component SWI2 and related ATPases (DNA/RNA helicase superfamily), Brahma complex subunit	Chromatin factors
DRSC30901	brm	0.399	0.365	0.890							

DRSC30902	brm	0.366	0.376	0.638							
DRSC16596	Caf1	1.401	0.795	1.473	1	-1	1	ACDHYPE	KOG0264	Nucleosome remodeling factor, subunit CAF1/NURF55/MSI1	Chromatin factors
DRSC30994	Caf1	1.392	0.871	1.704							
DRSC30995	Caf1	1.408	0.751	1.701							
DRSC04559	dom	0.742	0.670	0.562	-1	0	0	AC-HYP-	KOG0391	SNF2 family DNA-dependent ATPase	Chromatin factors
DRSC31605	dom	0.409	0.910	0.860							
DRSC31606	dom	0.453	1.053	1.002							
DRSC08583	E(bx)	1.000	2.057	4.447	1	1	1	ACDH---	KOG1473	Nucleosome remodeling factor, subunit NURF301/BPTF	Chromatin factors
DRSC08509	E(bx)	2.783	2.495	3.393							
DRSC34907	E(bx)	2.736	2.009	4.993							
DRSC34908	E(bx)	2.871	1.752	4.545							
DRSC12515	gpp	1.339	0.884	1.134	0	-1	0	-CDHY--	KOG3924	Putative protein methyltransferase involved in meiosis and transcriptional silencing (Dot1)	Chromatin factors
DRSC34915	gpp	0.878	0.698	1.264							
DRSC34916	gpp	0.937	0.748	1.369							
DRSC21264	His2A:CG31618	0.938	0.735	1.414	0	-1	1	AC-HYPE	KOG1756	Histone 2A	Chromatin factors
DRSC31121	His2A:CG31618	0.365	0.683	1.676							
DRSC21267	His3:CG31613	0.861	0.413	1.260	0	-1	0	ACDHYPE	KOG1745	Histones H3 and H4	Chromatin factors
DRSC27099	His3:CG31613	0.850	0.403	1.153							
DRSC16703	His4r	0.885	0.377	1.273	0	-1	0	ACDHYPE	KOG3467	Histone H4	Chromatin factors
DRSC31002	His4r	0.844	0.462	0.816							

DRSC07446	Iswi	1.840	1.119	1.518	1	0	1	ACDHY-E	KOG0385	Chromatin remodeling complex WSTF-ISWI, small subunit	Chromatin factors
DRSC34923	Iswi	1.317	1.230	1.472							
DRSC34924	Iswi	1.506	1.103	1.497							
DRSC22386	Nipped-A	0.575	1.012	0.897	-1	-1	0	ACDHYPE	KOG0889	Histone acetyltransferase SAGA, TRRAP/TRA1 component, PI-3 kinase superfamily	Chromatin factors
DRSC04883	Nipped-A	0.774	1.152	1.093							
DRSC31823	Nipped-A	0.752	0.672	0.969							
DRSC31824	Nipped-A	0.943	0.693	0.928							
DRSC16873	Su(var)3-9	0.803	0.775	1.305	0	-1	0	ACDH-P-	KOG1082	Histone H3 (Lys9) methyltransferase SUV39H1/Clr4, required for transcriptional silencing	Chromatin factors
DRSC13081	Su(var)3-9	1.216	0.499	1.967							
DRSC27837	Su(var)3-9	0.949	0.457	1.326							
DRSC22858	Cka	1.690	1.181	0.889	1	1	0	-CDHYP-	KOG0642	Cell-cycle nuclear protein, contains WD-40 repeats	Cell Cycle
DRSC34540	Cka	1.549	1.339	1.752							
DRSC34541	Cka	1.356	1.204	1.175							
DRSC03296	CycE	0.759	0.302	0.689	-1	-1	-1	-CDH---	KOG0655	G1/S-specific cyclin E	Cell Cycle
DRSC30675	CycE	0.841	0.302	0.630							
DRSC30676	CycE	0.743	0.658	0.722							
DRSC17071	stg	0.959	0.526	0.481	-1	-1	-1	ACDHYPE	KOG3772	M-phase inducer phosphatase	Cell Cycle
DRSC32775	stg	0.477	0.577	0.645							

DRSC32776	stg	0.603	0.501	0.713							
DRSC19863	CG2577	0.881	0.733	2.516	0	-1	1	-CDH--	KOG1163	Casein kinase (serine/threonine/tyrosine protein kinase)	Signaling
DRSC26322	CG2577	0.861	0.643	1.520							
DRSC32890	CG2577	0.615	0.857	1.116							
DRSC20230	CkIIbeta	0.631	0.560	0.654	-1	-1	0	ACDHYPE	KOG3092	Casein kinase II, beta subunit	Signaling
DRSC33296	CkIIbeta	0.793	0.618	0.774							
DRSC33297	CkIIbeta	0.674	0.659	1.008							
DRSC18462	Dsor1	0.852	0.654	0.677	-1	-1	-1	ACDHYP-	KOG0581	Mitogen-activated protein kinase kinase (MAP2K)	Signaling
DRSC31052	Dsor1	0.606	0.452	0.644							
DRSC31051	Dsor1	0.600	0.666	1.090							
DRSC16682	Gprk2	0.846	0.386	0.555	0	-1	0	-CDH--	KOG0986	G protein-coupled receptor kinase	Signaling
DRSC31000	Gprk2	1.010	0.709	0.902							
DRSC31001	Gprk2	0.729	0.746	1.073							
DRSC18359	raptor	0.758	0.610	0.574	-1	-1	-1	ACDHYP-	KOG1517	Regulatory associated protein of TOR	Signaling
DRSC31049	raptor	0.645	0.453	0.599							
DRSC31050	raptor	0.592	0.470	0.574							
DRSC23624	Sos	0.839	0.931	0.680	0	-1	0	-CDHYPE	KOG3417	Ras1 guanine nucleotide exchange factor	Signaling
DRSC30698	Sos	0.925	0.677	0.990							
DRSC30697	Sos	0.785	0.623	0.873							
DRSC07584	bic	0.853	0.789	1.938	0	-1	0	ACDHYP-	KOG2240	RNA polymerase II general transcription factor BTF3 and related proteins	Transcription

DRSC37934	bic	0.513	0.615	1.165							
DRSC04085	CG10955	0.795	0.603	0.904	0	-1	0	ACDHYPE	KOG2402	Paf1/RNA polymerase II complex, RTF1 component (involved in regulation of TATA box-binding protein)	Transcription
DRSC30728	CG10955	1.002	0.789	0.958							
DRSC26715	CG12252	0.829	0.576	0.745	0	-1	0	ACDHYPE	KOG0323	TFIIF-interacting CTD phosphatases, including NLI-interacting factor	Transcription
DRSC37944	CG12252	0.756	0.630	1.154							
DRSC28718	CG17836	0.810	0.632	0.275	-1	-1	-1		None	HMG-I and HMG-Y, DNA-binding	Transcription
DRSC32680	CG17836	0.483	0.521	0.402							
DRSC32681	CG17836	0.526	0.630	0.579							
DRSC15283	CG17836	0.887	0.631	0.315							
DRSC17734	CG32721	0.945	0.714	0.711	0	-1	-1		None	negative regulation of transcription from RNA polymerase II promoter	Transcription
DRSC34513	CG32721	0.524	0.780	0.631							
DRSC12567	CG33097	0.779	0.377	1.140	-1	-1	0	ACDH-P-	KOG0155	Transcription factor CA150	Transcription
DRSC34879	CG33097	0.737	0.452	0.780							
DRSC34880	CG33097	0.821	0.524	0.989							
DRSC10612	CG6272	0.882	0.972	0.440	0	0	-1	-CDH--	KOG3119	Basic region leucine zipper transcription factor	Transcription
DRSC37966	CG6272	0.642	0.920	0.481							

DRSC22786	Deaf1	1.623	1.715	2.569	1	0	0	-CDH--	KOG4333	Nuclear DEAF-1 related transcriptional regulator (suppressin) and related SAND domain proteins	Transcription
DRSC34906	Deaf1	1.253	0.877	0.994							
DRSC16931	dei	0.811	0.749	0.629	0	-1	0		None	Helix-loop-helix DNA-binding	Transcription
DRSC37977	dei	0.645	0.798	1.080							
DRSC15675	ear	0.893	0.793	0.623	0	0	-1	ACDHYPE	KOG3149	Transcription initiation factor IIF, auxiliary subunit	Transcription
DRSC37978	ear	0.679	0.865	0.661							
DRSC07423	Elongin-C	0.744	0.916	0.628	-1	0	0	ACDHYP-	KOG3473	RNA polymerase II transcription elongation factor Elongin/SIII, subunit elongin C	Transcription
DRSC29393	Elongin-C	0.414	0.669	0.764							
DRSC16127	Ets96B	0.791	0.636	0.733	-1	-1	0	-CDH--	KOG3806	Predicted transcription factor	Transcription
DRSC37979	Ets96B	0.691	0.778	1.012							
DRSC11182	HLH106	1.301	1.091	0.991	1	0	0	-CDHYP-	KOG2588	Predicted DNA-binding protein	Transcription
DRSC31626	HLH106	1.496	0.882	1.112							
DRSC31627	HLH106	1.608	0.834	1.019							
DRSC16977	kay	1.528	0.752	1.212	1	-1	0	ACDHYP-	KOG1414	Transcriptional activator FOSB/c-Fos and related bZIP transcription factors	Transcription
DRSC31021	kay	1.243	0.796	1.160							

DRSC31022	kay	1.487	0.674	1.065							
DRSC20346	l(1)10Bb	1.937	1.791	1.775	1	1	1	ACDHYPE	KOG3404	G10 protein/predicted nuclear transcription regulator	Transcription
DRSC31943	l(1)10Bb	1.203	1.356	1.567							
DRSC31944	l(1)10Bb	1.281	1.163	1.541							
DRSC22981	MED25	1.175	2.143	1.701	0	1	1		None	RNA polymerase II transcription mediator	Transcription
DRSC32496	MED25	0.663	1.540	1.790							
DRSC32497	MED25	0.827	1.327	1.636							
DRSC11217	MTF-1	1.157	1.654	0.994	0	1	0		None	Zinc finger, C2H2-type/integrase, DNA-binding	Transcription
DRSC21341	MTF-1	1.100	1.475	1.067							
DRSC10567	Nelf-E	0.917	0.709	0.637	0	-1	-1		None	negative regulation of transcription from RNA polymerase II promoter	Transcription
DRSC34575	Nelf-E	1.393	0.504	0.499							
DRSC27025	Sox14	0.976	0.757	0.613	0	0	-1	-CDHYP-	KOG0527	HMG-box transcription factor	Transcription
DRSC37990	Sox14	1.295	1.071	0.747							
DRSC07556	Spt5	0.672	0.295	0.500	-1	-1	-1	ACDHYPE	KOG1999	RNA polymerase II transcription elongation factor DSIF/SUPT5H/SPT5	Transcription
DRSC31819	Spt5	0.637	0.278	0.431							
DRSC31820	Spt5	0.469	0.248	0.418							
DRSC26497	Su(Tpl)	0.916	1.216	1.389	0	1	0	-CDH---	KOG4796	RNA polymerase II elongation factor	Transcription
DRSC10954	Su(Tpl)	1.094	1.651	1.441							

DRSC28181	Taf12	0.800	1.099	0.580	-1	-1	-1	ACDHYPE	KOG1142	Transcription initiation factor TFIID, subunit TAF12 (also component of histone acetyltransferase SAGA)	Transcription
DRSC33205	Taf12	1.005	0.570	0.572							
DRSC33204	Taf12	0.776	0.673	1.014							
DRSC34600	TH1	1.267	0.738	0.547	1	-1	-1		None	negative regulation of transcription from RNA polymerase II promoter	Transcription
DRSC34601	TH1	1.576	0.580	0.556							
DRSC20289	TH1	0.874	0.772	0.647							
DRSC03526	eIF-4a	1.230	1.306	0.938	1	1	0	ACDHYPE	KOG0327	Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	Translation
DRSC34260	eIF-4a	1.715	1.789	1.293							
DRSC20165	eIF5	0.701	0.552	1.333	0	-1	0	ACDHYPE	KOG2767	Translation initiation factor 5 (eIF-5)	Translation
DRSC34909	eIF5	0.836	0.368	1.102							
DRSC34910	eIF5	0.867	0.416	1.221							
DRSC06854	l(2)01424	1.151	1.467	0.945	1	1	0	ACDHYPE	KOG0401	Translation initiation factor 4F, ribosome/mRNA-bridging subunit (eIF-4G)	Translation
DRSC33055	l(2)01424	1.301	1.207	1.235							
DRSC33056	l(2)01424	1.371	2.163	1.454							
DRSC23280	caz	0.749	0.612	0.695	-1	-1	-1	ACDH---	KOG1995	Conserved Zn-finger protein	Miscellaneous
DRSC29957	caz	0.603	0.654	0.703							

DRSC31434	caz	0.517	0.502	0.852							
DRSC31435	caz	0.482	0.483	0.856							
DRSC24394	CG10344	0.893	0.998	1.771	0	-1	0	ACDHYPE	KOG1311	DHHC-type Zn-finger proteins	Miscellaneous
DRSC32807	CG10344	0.923	0.652	1.029							
DRSC32806	CG10344	0.566	0.707	1.062							
DRSC08145	CG10863	0.836	0.682	0.540	0	-1	0	ACDHYPE	KOG1577	Aldo/keto reductase family proteins	Miscellaneous
DRSC37937	CG10863	1.740	0.773	1.150							
DRSC17804	CG12102	1.104	1.224	1.317	0	1	0	ACDHYPE	KOG0393	Ras-related small GTPase, Rho type	Miscellaneous
DRSC28919	CG12102	1.069	1.340	1.066							
DRSC15175	CG17119	1.792	0.913	1.427	1	0	1	ACDHY--	KOG3145	Cystine transporter Cystinosin	Miscellaneous
DRSC33257	CG17119	1.274	1.144	1.533							
DRSC33258	CG17119	1.570	1.061	1.632							
DRSC08562	CG2469	0.957	0.441	0.714	0	-1	0	ACDHYPE	KOG2002	TPR-containing nuclear phosphoprotein that regulates K(+) uptake	Miscellaneous
DRSC37953	CG2469	0.764	0.505	0.848							
DRSC28677	CG31917	1.008	0.675	0.463	0	-1	0	ACDHYP-	KOG3362	Predicted BBOX Zn-finger protein	Miscellaneous
DRSC31149	CG31917	0.689	0.658	0.861							
DRSC06853	CG3814	0.920	1.101	1.808	-1	0	1	ACDHYP-	KOG2322	N-methyl-D-aspartate receptor glutamate-binding subunit	Miscellaneous
DRSC37961	CG3814	0.696	0.815	1.483							
DRSC37962	CG3814	0.478	0.625	0.837							
DRSC02774	CG4738	1.561	0.834	1.101	1	0	0	ACDH---	KOG4521	Nuclear pore complex, Nup160 component	Miscellaneous
DRSC34518	CG4738	1.463	0.647	1.085							

DRSC34519	CG4738	1.364	0.843	1.269							
DRSC34888	CG4963	0.799	0.780	1.070	0	-1	0	ACDHYP-	KOG0760	Mitochondrial carrier protein MRS3/4	Miscellaneous
DRSC34889	CG4963	0.971	0.654	0.834							
DRSC29623	CG7597	0.739	0.679	1.207	0	-1	0	ACDHYPE	KOG0600	Cdc2-related protein kinase	Miscellaneous
DRSC23323	CG7597	0.834	0.678	2.296							
DRSC03159	CG9226	1.442	1.251	1.218	1	0	0	A-DH-P-	KOG2919	Guanine nucleotide-binding protein	Miscellaneous
DRSC34900	CG9226	1.636	0.899	0.974							
DRSC07402	Dp	0.703	0.268	0.603	-1	-1	-1	ACDH--E	KOG2829	E2F-like protein	Miscellaneous
DRSC30793	Dp	0.905	0.289	0.678							
DRSC30792	Dp	0.447	0.257	0.726							
DRSC16667	FK506-bp1	1.030	0.884	0.601	-1	-1	0	A-D-YP-	KOG0552	FKBP-type peptidyl-prolyl cis-trans isomerase	Miscellaneous
DRSC33030	FK506-bp1	0.733	0.564	0.785							
DRSC33029	FK506-bp1	0.577	0.684	0.797							
DRSC16474	flfl	0.817	0.299	0.385	-1	-1	-1	ACDHYP-	KOG2175	Protein predicted to be involved in carbohydrate metabolism	Miscellaneous
DRSC30990	flfl	0.772	0.536	0.714							
DRSC30991	flfl	0.694	0.491	0.675							
DRSC00353	hoe1	0.918	0.731	0.574	-1	-1	0	A-DH--	KOG2639	Sodium sulfate symporter and related arsenite permeases	Miscellaneous
DRSC30623	hoe1	0.606	0.838	0.982							
DRSC30624	hoe1	0.503	0.758	0.936							
DRSC29729	Hsc70-4	0.895	0.644	1.397	0	-1	1	ACDHYPE	KOG0101	Molecular chaperones HSP70/HSC70, HSP70 superfamily	Miscellaneous

DRSC16711	Hsc70-4	0.905	0.635	1.987							
DRSC32137	Hsc70-4	1.005	0.779	2.156							
DRSC32138	Hsc70-4	1.103	1.038	1.812							
DRSC03563	l(2)37Cc	0.951	1.108	1.723	0	0	1	ACDHYP-	KOG3083	Prohibitin	Miscellaneous
DRSC27403	l(2)37Cc	1.299	0.855	1.489							
DRSC25329	Lcp4	0.967	1.141	1.552	0	0	1		None	structural constituent of chitin-based cuticle	Miscellaneous
DRSC25194	Lcp4	1.169	0.990	1.649							
DRSC07466	Mdr49	0.954	0.769	1.428	-1	-1	0	ACDHYP-	KOG0055	Multidrug/pheromone exporter, ABC superfamily	Miscellaneous
DRSC33062	Mdr49	0.533	0.632	0.828							
DRSC33061	Mdr49	0.558	0.583	0.854							
DRSC07653	mus209	0.812	0.353	0.571	0	-1	-1	ACDHYPE	KOG1636	DNA polymerase delta processivity factor (proliferating cell nuclear antigen)	Miscellaneous
DRSC33327	mus209	0.838	0.366	0.820							
DRSC33328	mus209	0.684	0.328	0.743							
DRSC16184	Nrx-1	0.929	0.759	0.473	-1	-1	0	-CDH--	KOG3514	Neurexin III-alpha	Miscellaneous
DRSC32755	Nrx-1	0.592	0.615	1.017							
DRSC32756	Nrx-1	0.634	0.623	0.916							
DRSC11096	nudC	1.004	1.050	1.407	0	0	1	ACDH-PE	KOG2265	Nuclear distribution protein NUDC	Miscellaneous
DRSC35899	nudC	0.617	0.920	1.490							
DRSC11252	Pep	1.337	1.628	2.347	0	1	0		None	Zinc finger, C2H2-type	Miscellaneous
DRSC37986	Pep	0.970	1.990	1.205							
DRSC06178	pk	1.381	4.137	0.817	1	1	0	-CDHYP-	KOG1704	LIM domain	Miscellaneous
DRSC34930	pk	1.573	4.515	0.618							
DRSC23325	PTP-ER	0.989	0.948	1.776	-1	-1	0	ACDHYP-	KOG0789	Protein tyrosine phosphatase	Miscellaneous

DRSC30738	PTP-ER	0.473	0.642	1.341							
DRSC30737	PTP-ER	0.767	0.754	1.201							
DRSC20364	ran	2.303	1.363	1.234	1	0	0	ACDHYPE	KOG0096	GTPase Ran/TC4/GSP1 (nuclear protein transport pathway), small G protein superfamily	Miscellaneous
DRSC31106	ran	1.260	0.977	1.355							
DRSC16852	Sbf	1.455	0.884	1.117	1	0	0	-CDH--	KOG1090	Predicted dual-specificity phosphatase	Miscellaneous
DRSC34937	Sbf	1.539	1.129	1.125							
DRSC34938	Sbf	1.369	1.329	1.153							
DRSC11285	Snap	1.406	1.242	1.795	1	0	1	ACDHYP-	KOG1586	Protein required for fusion of vesicles in vesicular transport, alpha-SNAP	Miscellaneous
DRSC31261	Snap	1.306	1.155	2.128							
DRSC28055	SNF1A	0.952	0.898	1.426	0	-1	1	ACDHYPE	KOG0583	Serine/threonine protein kinase	Miscellaneous
DRSC31402	SNF1A	1.139	0.714	1.560							
DRSC31401	SNF1A	0.777	0.724	1.388							
DRSC22926	tlk	0.662	0.300	0.496	-1	-1	-1	ACDH---	KOG1151	Tousled-like protein kinase	Miscellaneous
DRSC37991	tlk	0.665	0.220	0.491							
DRSC20382	up	0.969	0.585	0.473	0	-1	0	-CDH--	KOG3634	Troponin	Miscellaneous
DRSC28798	up	0.721	0.691	1.203							
DRSC00843	ush	0.888	0.795	1.885	-1	0	1	ACDHYPE	KOG1721	Zinc finger, C2H2-like	Miscellaneous
DRSC32226	ush	0.618	0.820	1.456							
DRSC32227	ush	0.724	0.826	1.513							
DRSC18852	wds	1.391	0.993	1.620	1	0	1	ACDHYP-	KOG0266	WD40 repeat-containing protein	Miscellaneous

DRSC34944	wds	1.685	1.110	1.508							
DRSC34945	wds	1.657	1.112	1.198							
DRSC23606	CG10915	0.854	0.807	0.724	-1	-1	-1	-CDH--	KOG1103	Predicted coiled-coil protein	Unknown
DRSC32810	CG10915	0.556	0.512	0.801							
DRSC32811	CG10915	0.739	0.582	0.657							
DRSC14405	CG11848	0.728	0.432	0.450	0	-1	0	-CDH--	KOG4335	FERM domain-containing protein KRIT1	Unknown
DRSC37940	CG11848	0.816	0.795	1.372							
DRSC01920	CG13243	1.314	1.609	1.869	1	0	0		None		Unknown
DRSC33879	CG13243	1.264	1.126	0.955							
DRSC08329	CG13720	1.593	3.421	0.749	1	0	0		None		Unknown
DRSC33933	CG13720	1.324	1.005	0.832							
DRSC10142	CG14137	0.875	0.616	0.575	0	-1	0		None		Unknown
DRSC25376	CG14137	0.537	0.677	1.052							
DRSC06664	CG17385	0.956	0.702	0.696	0	-1	0		None		Unknown
DRSC34977	CG17385	0.708	0.602	1.396							
DRSC23926	CG32021	0.755	0.479	0.624	-1	-1	-1		None		Unknown
DRSC37958	CG32021	0.473	0.391	0.666							
DRSC15493	CG3532	0.876	0.658	0.701	0	-1	0		None		Unknown
DRSC35289	CG3532	0.610	0.726	0.989							
DRSC26013	CG3825	0.963	0.333	1.621	0	-1	1		None		Unknown
DRSC04422	CG3825	1.097	0.431	1.600							
DRSC32067	CG3825	1.045	0.501	1.666							
DRSC32068	CG3825	1.262	0.494	1.762							
DRSC04460	CG4294	1.429	1.130	1.844	1	1	0		None		Unknown
DRSC34516	CG4294	1.323	1.572	1.335							
DRSC34517	CG4294	1.578	1.424	1.352							
DRSC00668	CG4785	1.597	1.921	3.260	1	1	1		None		Unknown
DRSC34884	CG4785	1.569	3.159	2.295							
DRSC34885	CG4785	0.936	1.126	1.603							
DRSC24273	CG5514	1.328	0.937	1.656	0	0	1		None		Unknown
DRSC32938	CG5514	1.079	0.870	1.587							
DRSC3	CG551	0.919	0.914	2.053							

2939	4										
DRSC25145	CG5726	1.602	1.291	4.100	1	1	1		None		Unknown
DRSC06946	CG5726	1.813	1.319	5.018							
DRSC34394	CG5726	1.002	1.258	1.203							
DRSC10744	CG6897	0.977	1.345	1.749	0	0	1		None		Unknown
DRSC37968	CG6897	0.611	1.153	1.866							
DRSC24716	CG6903	0.961	0.642	0.708	0	-1	0	A-DH--	KOG4683	Uncharacterized conserved protein	Unknown
DRSC37969	CG6903	0.793	0.559	0.931							
DRSC18405	CG6903	0.895	0.718	0.777							
DRSC18420	CG7065	1.092	0.856	1.756	0	0	1		None		Unknown
DRSC35474	CG7065	0.577	0.830	1.570							
DRSC04893	CG7843	3.792	2.280	5.829	1	1	1	ACDH-P-	KOG2295	C2H2 Zn-finger protein	Unknown
DRSC34894	CG7843	3.208	2.412	7.087							
DRSC34895	CG7843	3.101	1.440	4.376							
DRSC25065	CG8108	0.755	0.442	0.507	-1	-1	-1		None	Zinc finger, C2H2-type	Unknown
DRSC09675	CG8108	0.572	0.913	0.322							
DRSC30844	CG8108	0.725	0.380	0.458							
DRSC30845	CG8108	0.580	0.395	0.431							
DRSC07242	CG8710	1.325	1.156	1.177	1	0	0		None		Unknown
DRSC34896	CG8710	1.378	1.151	1.299							
DRSC34897	CG8710	1.563	0.788	1.379							
DRSC16485	CG9386	1.670	1.412	3.040	1	0	1	-CDHYP-	KOG3790	Uncharacterized conserved protein	Unknown
DRSC34902	CG9386	1.298	0.874	1.446							
DRSC35612	CG9548	0.630	0.650	0.727	0	0	1	ACDHYP-	KOG1705	Uncharacterized conserved protein, contains CXXC motifs	Unknown
DRSC03261	CG9548	0.803	0.959	0.557							
DRSC00708	lilli	0.779	1.265	0.604	0	1	0		None		Unknown
DRSC32745	lilli	0.822	1.587	0.951							
DRSC32746	lilli	0.801	1.435	0.888							

DRSC16733	Mat89Bb	1.324	1.578	2.457	0	1	1	-CDH--	KOG3711	Uncharacterized conserved protein	Unknown
DRSC36476	Mat89Bb	1.062	1.251	1.818							
DRSC22747	noe	1.000	1.173	1.875	0	0	1		None	unknown	Unknown
DRSC35896	noe	0.567	0.756	1.616							

Table S2. Protein-protein interaction and functional interaction among candidate gene products. These conclusions were based on annotations from the BioGRID database (http://www.thebiogrid.org/). The following annotations were considered as indications of protein-protein interactions: affinity capture-western; cofractionation; affinity capture-MS; reconstituted complex or two-hybrid. Phenotypic enhancement or phenotypic suppression were considered as an indication of genetic interaction.

Physical Interaction		**Genetic Interaction**	
Protein 1	**Protein 2**	**Gene 1**	**Gene 2**
su(s)	CG17293	*Dsor1*	*PTP-ER*
RpL22	RpL22	*eIF-4a*	*Dp*
CkIIbeta	dei	*CycE*	*Dp*
Bap60	brm	*CycE*	*ago*
lwr	mus209	*CycE*	*brm*
CG4785	eIF-4a	*CycE*	*Hsc70-4*
snRNP70K	Psi	*CycE*	*stg*
Pros35	Prosalpha7	*CycE*	*spen*
Iswi	Caf1	*Dp*	*brm*
Iswi	E(bx)	*Dp*	*spen*
mus209	mus209	*brm*	*dom*
CG7185	Cbp20	*Su(var)3-9*	*His3:CG31613*
AGO2	AGO2	*stg*	*His3:CG31613*
CSN3	noi		
noi	CG16941		
noi	RpS7		
noi	RpL5		
Caf1	E(bx)		

Table S3. Steady-state pri-miRNA and miRNA levels upon knockdown of candidate genes. Steady-state levels of CXCR4 and those of endogenous miR-2b upon knockdown of 43 selected candidate genes were examined by northern blotting and semiquantitative RT-PCR (q-PCR). For the northern blotting experiment, miRNA levels were quantified and normalized first against U6 RNA levels and then against the average results from multiple control samples (cells treated with dsRNA against LacZ). The results were subsequently log(2) transformed. As for the q-PCR assays, to measure steady-state levels of mature CXCR4, miR-2b, and pri-CXCR4, the ΔΔ Ct values ([CtU6 − CtmiRNA]sample − [CtU6 − CtmiRNA]control) are shown. Results from northern blotting assays represent average values from two to four independent batches of RNA samples, whereas results from independent q-PCR experiments using the same batch of RNAs are shown. The reciprocals of the "fold change of miR reporter" values were log(2) transformed to generate the "miR scores." One representative dsRNA for each candidate gene was employed in the assay.

Gene	Amplicon	miR score	CXCR4 Northern	miR-2b Northern	Pri-CXCR4 qPCR 1st	Pri-CXCR4 qPCR 2nd	Pri-CXCR4 qPCR 3rd	CXCR4 qPCR 1st	CXCR4 qPCR 2nd	CXCR4 qPCR 3rd
AGO1	DRSC05912	-2.32	-1.99	-0.55	-1.80	-1.78	-1.51	-1.97	-2.70	-2.07
brm	DRSC11330	0.54	0.91	-0.18	1.74	1.85	2.11	2.07	1.45	1.03
CG11985	DRSC14460	0.44	0.22	-0.23	-0.56	0.44	0.08	0.19	0.36	-0.02
CG12000	DRSC12186	-0.77	-0.71	-0.71	0.69	0.71	0.99	-0.18	-0.86	-0.43
CG2807	DRSC00535	1.23	-1.61	-1.34	-0.27	-0.66	-0.13	-0.77	-2.00	-1.36
C G3 20 21	DRSC23926	0.68	0.50	-0.01	1.15	1.20	2.22	1.17	0.79	1.01
CG32721	DRSC17734	0.49	1.03	0.47	0.57	0.37	1.81	1.11	1.36	1.06
CG3814	DRSC06853	-0.85	0.46	0.19	1.02	0.91	0.16	1.03	1.09	0.95
CG4785	DRSC00668	-1.70	0.41	0.01	1.57	1.18	0.87	0.18	-0.23	0.73
CG5514	DRSC24273	-0.73	0.57	-0.07	0.40	1.36	0.09	0.86	0.58	0.55
CG6169	DRSC10597	1.34	0.50	-0.11	-0.01	0.01	-0.52	1.54	0.64	0.56
CG6272	DRSC10612	1.19	0.29	0.27	0.09	-0.08	-0.48	-0.08	0.64	0.41
CG7185	DRSC10781	-1.37	0.58	0.06	2.26	1.45	0.56	0.65	0.42	0.54
CG7843	DRSC04893	-2.54	0.01	-0.16	1.72	1.52	1.69	-0.18	0.35	0.47
CG8108	DRSC25065	0.98	0.55	-0.17	1.67	1.28	2.04	1.00	0.80	0.81
CG9386	DRSC16485	-1.60	0.39	0.08	0.89	0.95	0.99	1.02	0.39	1.35
CSN3	DRSC11859	0.56	0.47	0.16	0.71	0.59	0.92	0.03	0.27	0.72
drosha	DRSC07607	-0.97	-0.44	-0.58	3.34	2.47	3.45	-2.23	-2.21	-2.81
E(bx)	DRSC08583	-2.15	-0.62	-0.67	-0.68	-0.58	-1.31	-0.62	-0.94	-0.77
flfl	DRSC16474	1.38	0.63	0.11	0.83	0.71	2.96	2.73	1.21	1.59
His2A: CG31618	DRSC21264	-0.50	0.65	0.04	1.05	2.07	0.80	1.22	-0.07	1.17
Hrb98DE	DRSC16709	-1.43	0.16	-0.20	1.34	0.64	1.06	0.66	0.26	0.31
l(2)05070	DRSC07159	-0.74	-0.87	-0.52	2.11	2.13	1.64	-0.78	-1.32	-0.69
MED25	DRSC22981	-0.77	-0.84	-0.36	-0.94	-0.71	-1.75	-0.17	-1.27	-0.89

mule	DRSC10509	0.72	0.66	0.47	0.26	0.45	1.21	1.10	0.53	1.25
mus209	DRSC07653	0.81	0.31	-0.59	0.12	0.23	0.61	0.33	0.21	0.07
Nelf-E	DRSC10567	0.65	0.43	-0.19	1.56	0.95	2.23	1.71	1.23	1.65
Pros26.4	DRSC25012	-0.69	-0.35	-0.75	1.85	0.84	1.32	-0.31	-0.49	-0.17
Psi	DRSC07519	-1.74	0.31	0.05	0.80	1.38	0.81	0.37	0.13	0.78
RpS6	DRSC18712	0.52	1.01	0.77	2.43	1.49	1.43	0.92	0.06	0.35
Rpt1	DRSC07542	-0.95	-0.67	-0.41	1.87	1.37	1.65	-0.16	-1.03	-0.11
sbr	DRSC29803	0.71	0.33	0.32	-1.55	-2.14	-1.59	-0.13	0.25	0.00
SNF1A	DRSC28055	-0.51	0.38	-0.54	1.31	0.54	0.96	1.45	1.14	0.46
snRNP70K	DRSC22071	-1.12	-0.09	-0.10	1.44	0.96	0.36	-0.99	-0.43	-0.29
Sox14	DRSC27025	0.71	0.31	0.29	0.92	0.81	0.63	1.02	1.25	0.20
spen	DRSC00839	0.45	0.96	0.69	0.54	-0.78	-0.78	0.60	1.15	0.55
Spt5	DRSC07556	1.00	0.75	0.12	0.32	-0.08	0.83	0.44	0.16	0.69
su(s)	DRSC18839	-2.71	0.22	-0.05	1.29	1.62	1.13	1.50	0.24	1.07
Taf12	DRSC28181	0.78	0.35	-0.08	1.35	1.21	1.77	1.10	1.56	0.96
Tbp-1	DRSC26269	-0.83	-0.77	-1.00	1.09	0.56	1.10	-0.30	-0.78	-0.75
TH1	DRSC20289	0.63	1.34	0.38	0.39	0.31	1.00	0.92	1.24	1.36
ubi-p63E	DRSC08703	-1.31	-0.48	-0.62	3.46	3.21	1.45	-0.32	-0.30	0.45
ush	DRSC00843	-0.91	0.48	0.07	1.69	1.42	1.46	0.89	0.48	1.45

Table S4. Steady-state esi-2.1 levels upon knockdown of candidate genes. Steady-state levels of esi-2.1 upon knockdown of 36 selected candidate genes were examined by semiquantitative RT-PCR (q-PCR). The ΔΔCt values ([CtU6 − Ctesi-2.1]sample − [CtU6 − Ctesi-2.1]control) are shown. The reciprocals of the "fold change of endo-siRNA reporter" values were log(2) transformed to generate the "esi-2.1 scores." One representative dsRNA for each candidate gene was employed in the assay.

Gene	Amplicon	esi-2.1 sensor score	esi-2.1 qPCR 1st	esi-2.1 qPCR 2nd	esi-2.1 qPCR 3rd
brm	DRSC11330	1.20	0.42	0.51	0.30
Cbp80	DRSC18450	-1.20	0.12	-0.38	0.10
CG11700	DRSC17794	-1.11	1.54	1.09	1.22
CG11985	DRSC14460	0.37	-1.09	-0.52	-0.49
CG12000	DRSC12186	-0.76	0.74	0.43	0.26
CG17836	DRSC28718	0.66	0.41	0.87	0.52
CG32021	DRSC23926	1.06	-0.43	-0.27	0.15
CG32721	DRSC17734	0.49	0.40	0.43	0.35
CG3825	DRSC04422	1.21	-0.11	-0.01	-0.09
CG4785	DRSC00668	-0.94	0.22	0.41	0.40
CG6169	DRSC10597	1.50	1.09	1.06	1.44
CG7185	DRSC10781	-1.97	-1.18	-0.80	-0.68
CG7843	DRSC04893	-1.19	-0.38	0.12	-0.38
CG8108	DRSC25065	1.18	0.50	0.64	0.52
CG9772	DRSC28203	0.49	0.47	0.59	0.68
CSN3	DRSC11859	1.27	1.05	0.45	0.56
drosha	DRSC07607	0.43	-0.31	0.13	0.01
Dsor1	DRSC18462	0.61	-0.17	0.09	0.02
E(bx)	DRSC08583	-1.04	0.01	-0.31	-0.03
flfl	DRSC16474	1.74	0.96	1.00	1.20
His2A:CG31618	DRSC21264	0.44	1.38	1.34	1.47
Hrb98DE	DRSC16709	-1.48	0.02	0.00	0.31
Mat89Bb	DRSC16733	-0.66	-0.63	-0.86	0.13
MED25	DRSC22981	-1.10	0.17	0.63	0.04
mule	DRSC10509	0.44	-0.08	0.15	0.18
mus209	DRSC07653	1.50	-0.01	-0.40	-0.66
Nelf-E	DRSC10567	0.50	-0.29	0.48	0.21
nito	DRSC05943	0.55	-0.01	0.14	0.12
Psi	DRSC07519	-0.58	-0.62	-0.18	0.21
raptor	DRSC18359	0.71	-0.18	0.09	0.03
RpS6	DRSC18712	1.15	0.62	1.11	0.90
snRNP70K	DRSC22071	-2.03	-1.09	-1.07	-0.60
Spt5	DRSC07556	1.76	0.01	-0.06	0.32
su(s)	DRSC18839	-0.63	0.21	0.07	0.06
tlk	DRSC22926	1.74	0.01	0.11	0.60
ubi-p63E	DRSC33366	-1.04	1.83	1.62	2.06

Table S5. List of candidate genes that were also identified in published genome-wide RNAi screens. Candidate genes and the corresponding references are listed.

Gene	Citations
AGO1	(Eulalio *et al*., 2007)
AGO2	(Kim *et al*., 2005; Saleh et al., 2006; Ulvila *et al*., 2006; Dorner *et al.*, 2006)
bel	(Kim *et al*., 2005; Ulvila *et al*., 2006)
Caf1	(Kim *et al*., 2005)
Cbp20	(Kim *et al*., 2005; Parry et al., 2007)
Cbp80	(Parry *et al*., 2007)
CG16941	(Kim *et al*., 2005)
CG17293	(Parry *et al*., 2007)
CG33097	(Kim *et al*., 2005)
CG7597	(Parry *et al*., 2007)
CkIIbeta	(Kim *et al*., 2005)
Dcr-2	(Kim *et al*., 2005; Parry et al., 2007; Saleh *et al*., 2006; Ulvila *et al*., 2006)
drosha	(Eulalio *et al*., 2007)
gw	(Eulalio *et al*., 2007)
His4r	(Parry *et al*., 2007)
Hsc70-4	(Dorner *et al*., 2006)
Nipped-A	(Parry *et al*., 2007)
Psi	(Kim *et al*., 2005)
Rrp6	(Parry *et al*., 2007)

Chapter 3

Overall perspective

Overview of screen results

By comparing our results with other small RNA screens generated by other groups, we found that there are genes that overlapped among those studies. For example, Ruvkun and colleagues performed an RNAi screen by using GFP sensor in *C. elegans*. Several genes, including Dcr-2, Caf1 and CKII beta, also scored in their screen. In addition, the functional profile from their screen shows a similar pattern to our results. It includes chromatin factors, signaling, and transcription factors [101]. From this, we predict that there are conserved function groups for small RNA pathway in different organisms. Although these screens were done only in *Drosophila* or *C.elegans*, based upon the high level of conservation of major small RNA components (Ago, Drosha, and Dicer) in mammals, it is likely that many of candidates identified in C. elegans and Drosophila might have similar functions in mammalian small RNA pathways.

The major advantage of our screen compared with previous studies is that it is very comprehensive, and the use of a common platform, library and biological systems allows us to compare between the miRNA and siRNA pathways. Many of the previous small RNA pathway analyses were focused on one complex or one phenomenon. However, these decisions limited such studies to contributing to our understanding of only a subset of the small RNA-mediated pathways. While Dicer and Argonaute are key factors in the small RNA pathways, they cannot account for all the functions of small RNA systems. To understand the full complexity of the small RNA pathways, it is important to cover the components that work at the various stages of the pathway in different cellular locations.

The results from our screens identifies a variety of the functional groups as possible players in small RNA pathways, including proteolysis, ribosomal, and splicing factors, with

the majority acting similarly within each group. Therefore, we raise the possibility that the functional group, rather than the individual genes, dictate the effect on the small RNA pathways.

In most small RNA-mediated gene regulation studies, "suppression" of the pathway tends to be studied to a greater extent that "enhancement". However, our screening results include both enhancers and inhibitors of small RNA pathways, with a similar number of each. This suggests that a tight and balanced control of small RNA pathways might be essential to proper gene regulation. As shown by the example of Drosha and DGCR8, where Drosha can control DGCR8 level [52], small RNA components can regulate each other to maintain balance in the pathway. This mutual regulation might occur not only at the single molecule level but also among multiple functional groups. Each function group might regulate other functional groups and ensure the equilibrium state is maintained. Only when certain gene or protein needs to be repressed, then the system is shifted into an "active" state, leading to the silencing of the gene.

After screening ~21,000 dsRNAs targeting *Drosophila* genes, we identified 177 candidates that affect the function of siRNA, endo-siRNA, and miRNA pathways. These candidates could be categorized into several different groups; those that enhanced silencing in all three pathways, those that suppressed silencing in all three pathways, and those that show differential effects depending on the pathway being analyzed.

Enhancers of small RNA-mediated silencing

Knock down of most proteasome components caused decreased silencing in small RNA-mediated pathways. This observation was supported by another knockdown screen

where Ubi-p63E was added to the cells to cause RNAi-mediated cell lethality. In this screen, the lethality is normally rescued by addition of an RNAi related genes, Dicer-2, but a proteasome component, pros45 was also able to rescue the lethal phenotype [104].

Previous biochemical analyses argue against protein degradation of miRNA targets as the likely mode of action. As discussed in the introduction, pulse-chase experiments did not show any polypeptides from an miRNA reporter after miRNA-mediated silencing [47]. Even when the reporter is directed (during translation) to endoplasmic reticulum (ER) to prevent protease from accessing to target, the reporter activity was still repressed [41]. With these results, it is hard to connect reduced protein levels and posttranslational degradation or proteasome activity within small RNA pathways.

Another possibility is that the proteasome might regulate the turnover of RNAi components rather than small RNA targets. In flies, synaptic mRNA and protein synthesis are regulated by components of the siRNA pathway, including the SDE helicase, Armitage. Strong transgenic expression of Armitage can reduce the level of CaMK II expression. However, when CaMK II expression was increased by exposure of acetylcholine or nicotine (agonist of nicotinic acetylcholine receptors), transgenic armitage expression was suppressed. This supression of armitage was caused by proteolysis, and could be blocked by proteolytic inhibitor, lactacystin [114]. Further analyses of proteasome components from our screens might help to understand how proteasome activity can get involved in silencing pathway.

Inhibitors of small-RNA-mediated silencing

Suppression of many candidates in our screens increases small-RNA mediated silencing, suggesting that they encode negative regulators of small RNA pathway responses. Knock down of some transcription factors and signaling factors decreased small RNA-mediated silencing effects. In Drosophila, heterochromatic silencing of tandemly repeated genes or transgenes depends on RNAi components. Loss of RNAi components (piwi, aubergine, or spindle-E), as well as heterochromatin protein1, HP1 and the H3K9 methyltransferase, SU(VAR)3-9, lead to the de-repression of transgenes that are inserted in pericentromeric heterochromatin [115] Mutations in aubergine and spindle-E have can up-regulate tandemly repeated Stellate genes, while mutations in piwi block the silencing of *Alcohol dehydrogenase* (*Adh*) transgenes. These observations suggest that they are positive regulator of silencing.

However, our results reveal that knock down of many transcription/ chromatin related candidate genes (including the chromatin factor, dom, and transcription factor, Ets96B) leads to enhancement of silencing of the reporter used in the screen. This raises the possibility that there are different mechanisms to regulate target genes through small RNA pathways. For example, oncogenic transcription factor, c-Myc, is known to both up and down regulate microRNAs. c-Myc binds directly to the promoter of a cluster of six miRNAs (miR-17-5p, miR-18, miR-19, miR-20, miR-92 and miR-106) on human chromosome 13 and activates their expression transcriptionally [116]. However, myc can also negatively regulate the expression of miR-23a and miR23b in human P-493 B lymphoma cells and PC3 prostate cancer cells, with Q-PCR analysis showing that miRNA-23a/b levels

increased when Myc expression is diminished [117]. Therefore, each transcription factors might have different function to depending on the species of small RNAs.

Another possibility is that the negative regulation and positive regulation pathways are not totally separated in small RNA mediated silencing, rather, those regulators might compete against each other. A candidate from screen, Spt5 is regulator of transcription, and it binds to the large subunit of RNA polymerase II *in vitro*. In yeast, transcriptional arrest can trigger ubiquitin-mediated proteolysis of RNA polymerase II (RNAPII). This ubiquitilation can be reversed by Ubiquitin protease3 (UBP3) complex, which includes UBP3, RNAPII, Spt5 and TFIIF [118]. Our screening data implies that proteasome is positive regulator of silencing, and therefore, this complex might function to repress silencing and counteract proteasome function.

Suppression of most splicing factors, including CG11985, CG2807 and CG16941, lead to an increase in small RNA-mediated silencing. Since reporter which was used for screening does not contain an intron, the possibility of splicing acting on the target reporter can be excluded. Another possibility is that those splicing factors cause changes in the splicing of small RNA pathway components. When the activity of small RNA pathway needs to be down regulated, this altered splicing might promote the production of dominant negative forms of RNAi factors. Splicing factors, SR proteins, including SF2/ASF, SC35, SRp40, are capable of enhancing NMD when mRNAs contains premature termination codons [119]. The alternatively spliced RNAi components RNA could be targeted to this NMD event.

Measuring effects on small RNA-mediated silencing can offer insights into the functions of the genes emerging from our screen. However, even when levels of silencing

are similarly affected in different pathways, it does not mean that the component always acts in the same way. For example, knock down of Bel results in a similar reduction in silencing in all pathways, and Flag-tagged Bel coimmunoprecipitated Ago2, FMR, VIG and Ago1. However, fractionation of the protein showed differential association, with the majority of Bel co-fractionating with Ago1 and minute amounts with Ago2. This result implies that Bel might participate in both Ago1 and Ago2 pathway but Bel might function in different way in each pathway.

Differential regulators of small RNA pathways

From our screens, we found several groups of genes that show different responses depending on the small RNA pathway. These include the Ago1/Drosha like response group, and the Ago2/Dcr-2 like response group. Suppression of Ago1 and Drosha show decreased silencing in the miRNA pathway but either increases or had no effect on the activity of the siRNA/endo-siRNA pathway. In contrast, knock down of Ago2 and Dcr-2 decrease silencing in siRNA/endo-siRNA pathway but have different effects in the miRNA pathway. Since Ago1/Drosha and Ago2/Dcr-2 are widely accepted as "miRNA specific" and "siRNA specific" components respectively, genes that follow their patterns of regulation might be specific to either the miRNA or siRNA pathways.

miRNA-specific regulators

The Ago1/Drosha group genes include diverse functional groups, including proteolysis, transcription factors and RNA binding proteins. Protein kinases/phosphatases,

including SNF1A, PTP-ER, CG7597 and CG2577, are slightly more represented, and there might be miRNA specific kinase/phosphatase mediated signal transduction systems.

It is worth noting that CCR4-NOT complex component, CG1874, does not belong to this group. CCR4-NOT promotes mRNA deadenylation and causes miRNA-mediated RNA degradation in miRNA dependent manner. However, knock down of CG1874 increases siRNA and endo-siRNA mediated silencing and also slightly increases miRNA mediated silencing. In addition, suppression of the de-capping enzyme, CG6169, also increases silencing in all three pathways. Recent study implied that deadenylation may not be required for silencing. In *D. melanogaster*, mRNAs without a poly(A) tail could be still silenced by miRNA [41,48]. Therefore, de-capping and de-adenylation proteins might have additional impacts on small RNA pathways.

siRNA-specific regulators

The study of the candidates that affect siRNA pathway might provide important insights into understanding viral immune responses. The RNA interference pathway has been shown to protect adult flies from viral infection. Mutations in Dcr-2 and R2D2 lead to rapid virus accumulation after FHV virion injection, resulting in death [106].

Knock down of siRNA component candidates in combination with viral infection of the cells might reveal other viral response factors that help defend against the infection. By enhancing immune response gene, the protection against viral infection might be increased, and this method could be utilized for therapeutic purposes.

Blurring the line between the RNA pathways

Previous analysis of the Ago1/Ago2 pathways suggests that the Ago2-associated siRNA pathway and Ago1-associated miRNA pathway have distinct functions and can be functionally separated. Mutants of siRNA pathway are mostly normal and fertile, whereas mutants in miRNA pathway are lethal during larval development. Most miRNAs are conserved, whereas most siRNAs are poorly conserved [69,71]. These results imply that siRNA and miRNA have different roles in gene silencing.

However, the observations from combinatorial mutations showed that some previously miRNA or siRNA-specific factors have functions in both pathways. Although R2D2 is implicated as a Dcr2 partner in siRNA pathway, *Drosophila* r2d2 mutants showed developmental defects and female infertility, whereas Dicer-2 mutants have no fertility defects [120]. Furthermore, the loss of single copy of dcr-1, when combined with r2d2 mutant, enhanced female infertility defect. This suggests that R2D2 might have additional functions beyond the Dcr-2/siRNA pathway. Similarly, LOQs is a partner of Dicer-1 in the miRNA pathway but biochemical and genetic analyses reveal an interaction with Dicer-2, and plays an important role in endo-siRNA pathway. From this data, it's clear that the previous distinct small RNA pathways might utilize the same combination of factors depending on the situation. Therefore, it is a distinct possibility that the endo-siRNA and miRNA pathways compete for LOQs or R2D2.

Competition between RNA pathways

Some of the candidates from the screens show decreased silencing in one pathway but an increased effect in the other pathway. This might be explained as the result of competition between miRNA, siRNA and endo-siRNA pathways, where enhancements to a pathway might impair the others. For example, a knock down of Drosha decreased miRNA-mediated silencing but enhanced silencing by endogenous siRNA. However, Drosha is known only as a processor of precursor of miRNA, and does not affect siRNAs or endo-siRNAs levels. A logical conclusion is that there are Drosha-interacting proteins that are shared between the miRNA and siRNA pathways, leading to competition. The loss of Drosha or the reduction in miRNAs would free up the common component, and enhance the activity of the siRNA pathway.

Cross talk with piRNA pathway

Regardless of the different functions of miRNAs and siRNAs, there are many overlapped genes from screen in both pathways. This fact suggests the possibility that piRNAs pathway also might have overlapped components with miRNAs and siRNAs pathway.

Current studies showed that endo-siRNAs and piRNAs pathway are functionally linked together. In Drosophila germ line, both piRNA and endo-siRNA systems functions in the transposon-silencing pathway. In addition, both piRNAs and siRNAs can be mapped to piRNAs clusters with overlapping regions [121]. In ovaries, *Ago2* or *Dcr-2* mutation causes derepression of several transposons that can be potentially targeted by siRNAs.

Although piRNAs pathway was not analyzed in this study, it might be possible (based on the previous experiment results) to predict which components of the miRNA and siRNA pathways are shared with the piRNA pathway, and which ones are distinct. However, these predicted candidates would require validation, possibly using a piRNA-targeted reporter knockdown screen.

Previous data have shown that biogenesis factors would be different between the piRNA and the miRNA/siRNA pathways, with the RNase III protein-family members Drosha and Dicer unnecessary for piRNA biogenesis in Drosophila [121,122]. However, chromatin factors and transcription factors could be the candidates of shared components, with a recent study showing that *Drosophila* Heterochromatin protein-1a (HP1a) can interact with N-terminus of PIWI, and co-localize in pericentric heterochromatin regions [123]. Mutations that prevents PIWI from binding to HP1 fail to rescue silencing in PIWI-depleted animals, implying that PIWI may be function in heterochromatin formation. With many chromatin factors (ex. Iswi, CG17293) scoring on our screen, some of them could be the candidates in piRNA-mediated gene regulation. Knock down of these candidate genes might lead to changes in piRNAs mediated repression, and will give valuable insights into understanding piRNAs mediated gene regulation.

In summary, comparative genome-wide screens provide wide range of information regarding to miRNA, siRNA, and endo-siRNA pathway in *Drosophila*. The comparison of each pathway aids in the identification of the common components, and determining which components are unique to each pathway. It is likely that the selected candidates include

many key components of the small RNA pathway, and analysis of those components will open further aspects of small RNA-mediated silencing mechanisms.

The mechanisms of small RNA pathways are not still clear, and there are still many aspects of small RNA pathways that are not well analyzed. However, at least, our screen results provide some clues to which types of cellular functions need to be focused upon and which components should be analyzed.

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Appendix

Drosophila Ago1 binding protein purification

Ago1 Immunoprecipitation

Although the small RNA screens were useful in finding important components within small RNA pathways, a potential problem is the presence of false negative results in the system. This might be due to inefficient knock down by dsRNA, or redundancy. For example, Dcr-1 and Pasha are important components in the miRNA pathway but failed to show a significant effect in the screen when knocked down. There might be more genes that do not affect repression level in our system but still function in the small RNA pathway. One way to address this is to use biochemical approaches to compliment the screen, both to validate the selected candidates, as well as finding "hidden" components.

In the miRNA pathway, one of the most important components, Ago1, was used as the vehicle for analyzing interacting proteins that might also function in the pathway. Flag-Ago1 was stably transfected into *Drosohila* S2 cells, and S10 cell lysate was used for Flag-immunoprecipitation. The precipitated proteins were eluted by 8M Urea, and analyzed by Multidimensional Protein Identification Technology (MudPIT) sequence system.

Ago1 co-precipitated proteins include the small RNA components, Dcr-1, Loquacious, and Fmr1. (Table. A1) The precipitated proteins were categorized into several functional groups, including signal transduction, proteolysis and splicing factors. Those groups are also present in the candidate genes in miRNA screen. The co-precipitation of ribosomal proteins supports previous data that Flag-tagged RpL22, RpL21 or RPs7 were co-immunoprecipitated with Ago1, and ribosomal proteins, RpL22,

Rps7, RpL21 and Rp49 co-precipitates with bantam, a miRNA. (Figure 4B and 4C) These results indicate that Ago1 and microRNAs are in ribosomal complex, and function together.

There are eight genes that were found in both in miRNA pathway screen and immunoprecipitation result (e.g. RpS28b, snRNP69D, RpL21). However, the overall overlap between the two sets of results was lower than expected. There are several reasons for this. One reason might be the transient or weak indirect interaction between certain proteins and Ago1, thus preventing a successful co-immunoprecipitation for further analyses. Ago1-independent complexes or processes might also explain the difference between the two techniques, such as the case of Drosha in processing miRNA precursors. These components would not be precipitated by Ago1 immunoprecipitation but could affect miRNA-mediated repression.

Although Dcr-1, Loquacious, and Fmr1 are important small RNA pathway components, the knock down of those genes does not show big changes in target suppression. However, since these proteins were co-immunoprecipitated with Ago1, we argue that even if the genes did not show up either in screen or immunoprecipitation result, they still might have a significant role. The combined screen and biochemistry results should compliment each other and allow for a more comprehensive analysis of the miRNA pathway. Although siRNA components were not analyzed in this study, purification of Ago2 binding protein will no doubt offer the same advantage to the study of the siRNA pathway.

Table A1. Proteins precipitated with Drosophila Ago1

	Uniprot kb ID	CG number	Flybase name
RNAi related	Q8INM7	CG6203	Fmr1
	Q9VCU9	CG4792	Dcr-1
	Q9VJY9	CG6866	Loquacious
Cytoskeleton	P06605	CG2512	alphaTub84D
	P08840	CG9359	betaTub85D
	Q9W0W8	CG15792:	zip
	Q9VQ78	CG726	CG726
	P29742	CG9012	Chc
	O62621	CG6699	beta'Cop
	Q00174	CG10236	LanA
	P11046	CG7123	LanB1
	P15215	CG3322	LanB2
Signal transduction	P92177	CG31196	14-3-3epsilon
	Q9VZ23	CG1404	ran
	Q9VWS1	CG6617	CG6617
	P36179	CG17291	Pp2A-29B
	O18640	CG7111	Rack1
	Q9VGP4	CG5252	Ranbp9
Heat shock proteins	P02517	CG4183	Hsp26
	P02516	CG4463	Hsp23
	P82910	CG31366	Hsp70Aa
	Q9VFV9	CG8863	CG8863
	P29844	CG4147	Hsc70-3
	Q24276	CG12019	Cdc37

RNA binding	Q9VVI0	CG6322	CG6322
	Q8MRG6	CG7757	CG7757
	P40796	CG10922	La
	Q94901	CG8597	lark
	Q9VN21	CG14648	growl
	P07909	CG9983	Hrb98DE
	Q9VU02	CG10753	snRNP69D
	Q9VHP0	CG9748	bel
Ribosomal Protein	Q9W1B9	CG3195	RpL12
	Q9W237	CG4046	RpS16
	Q9W334	CG2998	RpS28b
	Q9VWG3	CG14206	RpS10b
	Q9VTP4	CG7283	RpL10Ab
	Q9VBU9	CG10423	RpS27
	Q9VA91	CG1883	RpS7
	Q9V9M7	CG12775:	RpL21
	Q7PL86	CG17420	RpL15
	Q9W3W8	CG3203	RpL17
	P46222	CG7726	RpL11
	P41093	CG6510	RpL18A
	P48159	CG3661	RpL23
	Q24154	CG10071	RpL29
	P04359	CG7939	RpL32
	P14130	CG1524	RpS14a
	P17704	CG3922	RpS17
	P48588	CG6684	RpS25
	P31009	CG5920	sop

	P41042	CG11276	RpS4
	Q8IMB0	CG2168	RpS3A
	Q8SYL1	CG12792	l(2)09851
	Q9VBN5	CG4759	RpL27
	Q9VS34	CG8615	RpL18
	Q9VNE9	CG1475	RpL13A
	O61231	CG17521	Qm
	Q9VZS5	CG12740	RpL28
	P08570	CG4087	RpLP1
	P05389	CG4918	RpLP2
	P80455	CG11271	RpS12
	Q06559	CG6779	RpS3
Proteainase	Q95029	CG6692	Cp1
	Q9VN93	CG12163	CG12163
	Q9VW19	CG9372	CG9372
Other proteins	P41073	CG6143	Pep
	Q9VT61	CG8108	CG8108
	Q9VHI7	CG11982	CG11982
	Q9W153	CG4622	CG4622
	Q9VNA3	CG11999	CG11999
	Q9VR80	CG17068	CG17068
	Q9VRP3	CG5495	Txl
	Q9VK59	CG5787	CG5787
	Q9VIT9	CG10363	TepIV
	Q8IPZ5	CG9641	CG9641
	O97365	CG6378	BM-40-SPARC
	P17336	CG6871	Cat

	Q9V405	CG16916	Rpt3
	Q9VLT5	CG14472	poe
	Q9VXD5	CG18572	r
	Q9W362	CG12058	mxc
	P14199	CG10360	ref(2)P
	Q9XZ21	CG3231	snama
	Q9VRV1	CG10289	CG10289
	Q9W4C1	CG15784	CG15784
	Q9VTC4	CG7949	CG7949
	Q9VK69	CG5525	CG5525
	Q9VPQ2	CG4164	CG4164
	Q8MT18	CG5028	CG5028
	Q9VLX0	CG7144	CG7144

Method

FLAG-Ago1 stably expressing S2R cells were lysed in hypotonic buffer and spun at 30,000*g*. The lysate was supplemented with 150mM NaCl and 0.1% NP40, and incubated with FLAG beads. The beads were washed and eluted with 8M Urea. The eluted samples were analyzed by MUDPIT sequence system.