Cargo sorting to lysosome-related organelles regulates siRNA-mediated gene silencing

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Mammals lacking BLOC-3 have impaired formation of melanosomes, a type of lysosome-related organelle (LRO), and, in earlier work, we found that a subunit of the BLOC-3 complex inhibits loading of Argonaute (Ago) proteins with small ribonucleic acids (RNAs) in Drosophila melanogaster cells. Small RNAs such as small interfering RNAs (siRNAs) direct Ago proteins to repress the stability of messenger RNA transcripts. In this paper, we show that BLOC-3 is required for biogenesis of Drosophila LROs called pigment granules. Other complexes that sort cargo to pigment LROs also negatively regulate siRNA activity. However, regulation is not obligately linked to biogenesis of LROs but instead to specific cargo-sorting processes. Negative regulation is also not linked to sorting into all LROs but only a specific class of pigment LRO. Thus, regulation of siRNA activity is tied to sorting of specific types of cargo to particular LROs.

Introduction

Posttranscriptional gene silencing by small noncoding RNAs is a widespread mechanism to regulate gene expression in eukaryotes. siRNAs inhibit virus replication, transposon replication, and select protein-coding genes (Carthew and Sontheimer, 2009). Biogenesis of siRNAs requires the Dicer RNase III enzyme, which generates 21–23-nt siRNA duplexes from longer double-stranded RNA (dsRNA) precursors (Bernstein et al., 2001; Elbashir et al., 2001). Small RNA duplexes are then loaded from Dicer into an Argonaute (Ago) protein, where they unwind and only one RNA strand remains associated (Schwarz et al., 2003; Pham et al., 2004; Tomari et al., 2004; Kim et al., 2007). This ribonucleoprotein complex can potentially form base pair interactions between the guide siRNA strand and cytoplasmic mRNA transcripts. If base pairing is perfect across the center of the guide RNA, Ago cleaves the mRNA transcript (Schwarz et al., 2004). This is the primary mechanism by which siRNAs inhibit their target genes.

In one sense, Ago activity is highly dynamic in that different types of siRNAs can load or unload, generating complexes of considerable diversity and specificity. However, it is not as clear how Ago activity is regulated at the cellular level. Surprisingly, this can occur in a manner linked to membrane trafficking. Mammalian Dicer and Ago2 are found to be associated with intracellular membranes (Cikaluk et al., 1999; Lugli et al., 2005; Gibbings et al., 2009), and Ago2 was initially characterized as a Golgi- and ER-associated protein (Cikaluk et al., 1999). Further evidence has come from studies in Drosophila melanogaster. A genetic screen identified Drosophila HPS4 (dHPS4) as an antagonist of siRNA-mediated silencing (Lee et al., 2009). Loss of dHPS4 results in enhanced loading of siRNAs into Ago2 and increased Ago2 activity. Mammalian HPS4 protein is a subunit of the BLOC-3 complex, which mediates sorting of cargo proteins to lysosome-related organelles (LROs) such as melanosomes (Nguyen et al., 2002; Suzuki et al., 2002; Chiang et al., 2003; Martina et al., 2003; Nazarian et al., 2003). Genetic studies of mouse models for Hermansky-Pudlak syndrome (HPS), a human disorder associated with albinism, have implicated BLOC-3 as an important mediator of melanosome formation (Nguyen et al., 2002; Suzuki et al., 2002).

Functional LROs are generated by a multistep process in which an immature organelle forms and then matures by acquiring specialized cargo proteins. Proteins destined for residence in LROs flow from the trans-Golgi network and early endosomes to their final destination via transport vesicles that pinch...
off of donor organelles and fuse with acceptor organelles (Raposo et al., 2007). Vesicles carry a specific set of cargo proteins as part of the process. Delivery of cargo uses transport vesicles coated with the AP-3 adapter complex, which functions to sort cargo from early endosomes to LROs (Bonifacino and Glick, 2004; Raposo et al., 2007). Additional sorting complexes mediate sorting of LRO cargo. BLOC-1 and BLOC-2 sort cargo from early endosomes to melanosomes in a pathway that is distinct from the AP-3 pathway (Raposo and Marks, 2007). BLOC-3 appears to function independently of AP-3 and the other biogenesis of LRO complexes (BLOCs) in sorting protein to melanosomes. However, BLOC-3 function is not limited to LRO biogenesis; BLOC-3-deficient fibroblasts exhibit abnormal lysosome distribution, suggesting a role in lysosome motility (Falcón-Pérez et al., 2005). Rab GTPases are well-known regulators of vesicular transport, and Rab32 and Rab38 have been directly implicated in trafficking to LROs (Raposo and Marks, 2007). They are highly homologous proteins that are expressed in a limited set of cell types, including melanocytes (Di Pietro and Dell’Angela, 2005; Huizing et al., 2008). Ubiquitous Rab proteins also appear to regulate LRO biogenesis (Hirosaki et al., 2002; Jordens et al., 2006). GTP-bound Rabs can associate with the homotypic fusion and vacuole protein sorting (HOPS) protein complex, which promotes lysosome membrane fusion events by interacting with the SNARE machinery (Raposo and Marks, 2007). The HOPS complex is also required for melanosome biogenesis, though its mechanism is not well understood.

We previously found that dHPS4 inhibits siRNA loading of Ago2 and its consequent efficacy for gene silencing (Lee et al., 2009). Cytoplasmic distribution of the Ago protein was also dependent on dHPS4, prompting us to hypothesize that its effects on Ago distribution and activity were possibly caused by lysosome motility. However, it was also possible that its effects were a result of a connection with cargo trafficking to Drosophila LROs. Drosophila orthologues for BLOC-1, BLOC-2, AP-3, Rab38, and HOPS proteins are required for biogenesis of a class of LROs called pigment granules (Sevrioukov et al., 1999; Krämer, 2002; Ma et al., 2004; Falcón-Pérez et al., 2007; Syrzycka et al., 2007; Cheli et al., 2010). These observations suggest an evolutionarily conserved role for these complexes in the biogenesis of LROs. Here, we test the model that cargo sorting to LROs is the primary mechanism by which Ago2 is negatively regulated. Specifically, we wanted to know whether Drosophila BLOC-3 is involved in biogenesis of LROs, whether other sorting complexes regulate the siRNA silencing pathway, and, if so, how sorting is coupled to Ago2 activity.

Results

Role of dHPS4 in pigment LRO biogenesis

Eye pigment cells of Drosophila contain specialized LROs called pigment granules, which can be classified into two distinct types based on pigment composition and cell type (Nolte, 1961; Lloyd et al., 1998). Type I granules contain ommochrome pigment and are found in primary, secondary, and tertiary pigment cells. Type II granules contain drosopentin pigment and are only found in secondary and tertiary pigment cells. Mutations that disrupt biogenesis of one granule type have no effect on the other type, suggesting that their formation is independent of one another (Nolte, 1961; Shoup, 1966). Consistent with this idea, biogenesis of ommochrome LROs precedes drosopentin LROs by many hours during cell differentiation (Shoup, 1966). Genetic loss of either BLOC-1, BLOC-2, AP-3, Rab38, or the HOPS complexes results in aberrant formation of both types, indicating common pathways of cargo sorting to both LROs (Sevrioukov et al., 1999; Krämer, 2002; Ma et al., 2004; Falcón-Pérez et al., 2007; Syrzycka et al., 2007; Cheli et al., 2010). However, each LRO type is differentially dependent on certain types of complexes.

It was of interest to determine whether the Drosophila BLOC-3 complex is also required for pigment LRO biogenesis. We measured ommochrome pigment level in a dHPS4 mutant, and the mutant showed a significant decrease in ommochrome compared with that of wild type (Fig. 1 A). Drosopentin pigment levels were modestly reduced (15%) in the mutant (Fig. 1 B). Other dHPS4 alleles showed similar effects (unpublished data). The mouse BLOC-3 complex functions in an independent pathway from the AP-3 and BLOC-2 pathways. We wished to determine whether BLOC-3 also has an autonomous relationship with other sorting complexes in Drosophila pigment cells. Therefore, we generated double mutants of dHPS4 and other HPS genes and assayed levels of ommochrome pigment in the double mutants (Fig. 1 A). The garnet (g) and ruby (rb) genes encode two subunits of the AP-3 complex (Krämer, 2002). Ommochrome levels in g dHPS4 and rb dHPS4 double mutant flies were lower than those in the single mutants alone, indicating that AP-3 and BLOC-3 independently regulate ommochrome LRO biogenesis. The pink (p) gene encodes for a subunit of BLOC-2. The p dHPS4 double mutants also showed less ommochrome than that of the single mutants alone. Similar additive interactions were observed between dHPS4 and claret (ca), encoding a guanine nucleotide exchange factor for Rab GTPases, and between dHPS4 and carnation (car), encoding a core subunit of the HOPS complex (Fig. 1 A). Interestingly, loss of dHPS4 had no effect on ommochrome levels when the Rab38 gene lightoid (lid) was mutated. This result indicates that lid is epistatic to dHPS4, and, therefore, Rab38 and BLOC-3 function in a common pathway with Rab38 downstream of BLOC-3.

We also examined drosopentin pigment levels in the double mutants (Fig. 1 B). Additive interactions were detected between dHPS4 and g, rb, ca, and lid. In contrast, when either the ca subunit of HOPS or the p subunit of BLOC-2 was mutant, loss of dHPS4 had no effect on drosopentin levels. This suggests that HOPS, BLOC-2, and BLOC-3 act in a common sorting pathway for drosopentin LROs. Collectively, these results show a complex hierarchy of interactions between dHPS4 and other HPS proteins, consistent with BLOC-3 functioning in biogenesis of both classes of pigment LROs.

AP-3 and BLOC-2 negatively regulate the siRNA pathway

Because dHPS4 participates in pigment LRO biogenesis, we wished to know whether its effects on siRNA activity were
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was modestly enhanced when rb or p was mutant and was much more enhanced in a ca mutant background (Fig. 2, D–G). None of the mutants had any effect on eye patterning in the absence of argos siRNAs (unpublished data). We also expressed a hairpin RNA against the brown (bw) gene, resulting in a fivefold reduction in bw mRNA levels (Fig. 2 H). This reduction was enhanced in a g, p, or ca mutant background. Loss of rb had no effect on bw silencing. Similar effects were seen with RNAi knockdown of the purple (pr) gene (Fig. 2 H). The stronger dependence of white and argos RNAi on rb is countered with stronger dependence of bw and pr RNAi on g. These data suggest that AP-3 subunits might contribute differentially to gene targeting in some unknown way.

We also tested the effect of the HOPS complex on siRNA activity. We did so by assaying silencing activity in a car mutant, which inactivates the core VPS22A subunit of HOPS. Interestingly, the car mutant did not enhance silencing of white by GMR-wIR (Fig. 2, A and B). This result indicates that pigment LROs themselves are not the key determinants of siRNA regulation, as the car mutant allele significantly impairs biogenesis of both types of pigment LROs (Fig. 1; Sevrioukov et al., 1999). Regulation appears to be tied to specific cargo-sorting processes that depend on BLOC-2, BLOC-3, and AP-3 but not the HOPS complex.

Ommochrome LROs specifically inhibit siRNA activity

There are two types of pigment LROs, and it is possible that cargo sorting to one type or both types regulates siRNA activity.
bw or pr did not enhance the repressive effect of GMR-wIR but in fact strongly suppressed white (RNAi) (Fig. S1). These results suggest that siRNA activity is specifically inhibited by the ommochrome pathway, whereas the drosopterin pathway potentiates siRNA activity.

To further explore the relationship between the ommochrome LRO pathway and the siRNA pathway, we focused on one cargo protein that acts inside ommochrome LROs. The product of the cd gene converts 3-hydroxykynurenine (3-HOK) into ommochrome pigment (Howells et al., 1977; Mackenzie et al., 2000). We isolated new mutant alleles of cd (Fig. 3 A). Using these new alleles, we positionally localized the gene to CG6969, which encodes a heme peroxidase (Fig. 3 B). We sequenced CG6969 in five independent mutants, and each mutant had base changes that significantly altered the predicted protein product (Fig. 3 B).
Two alleles contained premature stop codons that would produce truncated proteins, lacking an intact peroxidase domain. The two mutations likely represent null alleles. To confirm that CG6969 corresponded to cd, we expressed the CG6969 cDNA in eye cells. This completely rescued the phenotype associated with a homozygous cd-null mutant (Fig. 3 C). A genomic fragment that spans the CG6969 transcription unit (Fig. 3 B) also rescued the cd-null phenotype (not depicted). To confirm that Cd converts 3-HOK to ommochrome, we looked for the accumulation of 3-HOK in the adult eye of cd mutants by taking advantage of the unique blue autofluorescent properties of 3-HOK (Siddiqui and Babu, 1980). We observed strong blue autofluorescence in the eyes of cd mutants (Fig. 3 D).

The Cd protein is predicted to have two functional domains (Figs. 3 B and S2). There is a transmembrane domain located 45 amino acids from the amino terminus, suggesting that the product might be an integral membrane protein. At the carboxy terminus is the heme peroxidase domain. If the amino-terminal domain targets the protein to membranes, we predicted the protein would be membrane localized. Therefore,
we coexpressed Cd::RFP in S2 cells with various markers for cytoplasmic organelles. Cd::RFP protein localization specifically overlapped with organelles marked by Lamp-1::GFP (Fig. 4, E–H). Lamp-1 is a type I integral membrane protein found in the limiting membrane of late endosomes and lysosomes (Lloyd et al., 1998). Cd::RFP protein was not uniformly distributed on lysosomal membranes but rather was localized to subdomains overlapping and interior to Lamp-1::GFP, suggesting that the Cd::RFP carboxy terminus is lumenal. This interpretation is consistent with structural features of Cd that suggest it is a type II membrane protein: there is no amino-terminal signal sequence, there is a proximal transmembrane domain, and there is a strong charge differential between the flanking sequences directed positive to negative.
gene silencing was also seen by monitoring white activity (Fig. S1). To confirm that the cd effect on silencing was mediated by siRNAs, we combined cd with a dicer-2-null mutation. Loss of dicer-2 blocks the production of siRNAs in Drosophila (Lee et al., 2004). The enhanced silencing of white by cd was completely negated by dicer-2; flies had an eye color resembling simple loss of cd (Fig. S3).

We determined whether enhanced gene silencing by cd mutants also applied to other target genes. Silencing of argos in eye

**Cd is an inhibitor of siRNA-mediated silencing.** (A) The level of white mRNA in the presence or absence of GMR-wIR to induce white(RNAi). Levels are shown from flies in a wild-type and cd<sup>F993S</sup> background. RNA values were measured by real-time qPCR and normalized to Rpl32 mRNA. Statistical significance was determined by a Student’s t test: **, P < 0.01. (B–E) Scanning electron microscopy of eyes from wild-type (B) and GMR>argosIR (C–E) flies shown under low magnification in the left column and higher magnification in the right column. Mispattering is worsened in a GMR>Dicer-2 (D) and cd<sup>F993S</sup> mutant (E) background. (F) Silencing of bw head mRNA by eye-specific bw siRNAs made by GMR-bwIR. GMR>Dicer-2 and cd mutant backgrounds are indicated. WT, wild type. (G) Silencing of pr head mRNA by eye-specific pr siRNAs made by GMR>prIR. GMR>Dicer-2 and cd mutant backgrounds are indicated. Errors bars represent SDs. Each sample was performed in quadruplicate. For F and G, statistical significance was determined by a Student’s t test: *, P < 0.05; **, P < 0.01. Bar: (B–E) 100 µm; (B’–E’) 10 µm.
lysates made from dsRNA processing by incubating labeled dsRNA in cytoplasmic lysates from wild-type and cd mutant lysates. Radiolabeled siRNA was incubated with lysate and labeled complexes were detected by native gel electrophoresis. Some label remains adhered to the origin of electrophoresis. The path-
way of Ago2 processing is schematized below. RDI, R2D2–Dicer2 initiator; RLC, RNA-induced silencing complex–loading complex. (C) siRNA-induced mRNA cleavage in wild-type and cd mutant lysates. The 5' labeled mRNA is cleaved upon incubation with lysate and siRNA, accumulating a 5' cleavage product, as indicated after electrophoresis. Molecular weights of RNAs are indicated in nucleotides. The asterisks refer to the schematic of the molecular structure showing where the radioactive label is located.

If more Ago2 is loaded in a cd mutant, more Ago2 should be available to cleave target mRNA. We loaded an unlabeled siRNA into Ago2 by addition of the siRNA to lysate and then assayed Ago2 cleavage activity by incubation with an end-labeled mRNA substrate.

Figure 6. Cd inhibits Ago2 loading. (A) Processing of dsRNA substrate into siRNAs by cytoplasmic lysates from wild-type and cd mutant embryos. Normalized siRNA levels over time are plotted. (B) Ago2 loading in wild-type (WT) and cd mutant lysates. Radiolabeled siRNA was incubated with lysate, and labeled complexes were detected by native gel electrophoresis. Some label remains adhered to the origin of electrophoresis. The pathway of Ago2 loading is schematized below. RDI, R2D2–Dicer2 initiator; RLC, RNA-induced silencing complex–loading complex. (C) siRNA-induced mRNA cleavage in wild-type and cd mutant lysates. The 5' labeled mRNA is cleaved upon incubation with lysate and siRNA, accumulating a 5' cleavage product, as indicated after electrophoresis. Molecular weights of RNAs are indicated in nucleotides. The asterisks refer to the schematic of the molecular structure showing where the radioactive label is located.

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containing a complementary binding site. Cleavage was detected by the appearance of the end-labeled RNA fragment. The cd mutant lysate generated significantly more cleavage product than wild-type lysate (Fig. 6 C). The increase in cleavage activity (threefold) was comparable with the increase in loaded Ago2 (three- to fivefold), suggesting that cd enhances the loading of Ago2 but not its cleavage activity once loaded.

Discussion

The aim of this study is to determine how gene silencing is connected to membrane trafficking. We had observed that loss of dHPS4 protein enhanced siRNA loading into Ago2 and altered the distribution of Ago protein in cells (Lee et al., 2009). Knockdown of human HPS4 in HeLa cells had similar effects. HPS4 is required for LRO biogenesis and also for lysosome motility in cells lacking LROs (Wei, 2006). This raised the question of whether LRO trafficking or some other feature of organelle biology was responsible for siRNA regulation. To explore this question, we have determined that loss of dHPS4 impairs production of eye pigments and affects pigmentation independent of the AP-3 and BLOC-2 cargo-sorting pathways. This observation is consistent with data from a mouse genetic study that found BLOC-3 to independently control melanosome biogenesis and also for lysosome motility in cells lacking LROs (Wei, 2006). This raised the question of whether LRO trafficking or some other feature of organelle biology was responsible for siRNA regulation. To explore this question, we have determined that loss of dHPS4 impairs production of eye pigments and affects pigmentation independent of the AP-3 and BLOC-2 cargo-sorting pathways. This observation is consistent with data from a mouse genetic study that found BLOC-3 to independently control melanosome biogenesis and also for lysosome motility in cells lacking LROs (Wei, 2006).

Intriguingly, dHPS4 acts genetically upstream of ltd, the Drosophila orthologue of Rab38, for ochromochrome LRO production. Therefore, the conserved function of HPS4 for LRO biogenesis makes it plausible that LRO trafficking regulates siRNA activity.

This hypothesis was further tested by measuring siRNA activity when other LRO cargo-sorting complexes are disrupted. We found that disruption of the BLOC-2, AP-3, or Rab38 complexes leads to stronger gene silencing. However, disruption of the HOPS complex has no effect on silencing, which argues that the simple presence of the LRO compartment is not the regulatory determinant, as there are fewer pigment LROs when HOPS activity is compromised (Sveriouk et al., 1999). The precise role of HOPS in LRO formation is not well understood, but other studies indicate that it tethers trafficking vesicles and organelles to late endosomes and lysosomes for fusion (Bonifacino and Glick, 2004; Raposo et al., 2007). If it performs analogous functions in LRO biogenesis, it would act downstream of AP-3 and BLOC-2, which are thought to function in linking transmembrane cargo (through cytoplasmic sorting signals) to vesicle formation on post-Golgi membranes. In melanocytes, AP-3 and BLOC-2 probably function in sorting cargo into vesicles from early endosomes. These results would indicate that the early steps of cargo sorting to the LRO compartment regulate siRNA activity. Ago proteins can physically associate with the exterior of Golgi and endosomes even though they are not transmembrane proteins (Cikaluk et al., 1999; Gibbings et al., 2009; Lee et al., 2009). One possibility is that Ago protein shuttles between organelles using preexisting sorting pathways, and its competence to load small RNAs is modulated by which compartment it is associated with. In this model, Ago2 associated with early endosomes is capable of loading siRNAs, but it is incapable of loading when shuttled away from early endosomes. Loss of AP-3 and other sorting complexes would block the shuttling process, whereas loss of HOPS would presumably not prevent shuttling away from endosomes. Another possibility is that Ago protein distributes between a free cytosolic pool that is capable of loading small RNAs and a membrane-bound pool that is not. The size of this membrane-bound pool might then be regulated by the magnitude and mode of cargo sorting.

Pigment cells contain two different pigment LROs: ochromochrome and drosopterin pigment granules. Remarkably, sorting into ochromochrome LROs inhibits loading of Ago2 with siRNAs, whereas drosopterin LRO sorting has the opposite effect. How do the two LROs differentially regulate silencing? One possibility is that negative regulation only occurs in cells that exclusively contain ochromochrome LROs. However, siRNA silencing of drosopterin gene expression is enhanced by mutants that disrupt ochromochrome LROs. Thus, negative regulation is occurring in cells that contain both types of LROs. Another possibility is that cargo exclusive to the ochromochrome LRO regulates Ago2 in a negative manner. Greater Ago2 loading is observed when the ochromochrome cargo protein Cd is not produced, lending support to this possibility. The Cd protein appears to be anchored to the ochromochrome-limiting membrane, with a cytoplasmic tail of 45 amino acids.

In conclusion, siRNA activity is regulated by cargo sorting to LROs. Regulation occurs by controlling the level of Ago2 loaded with siRNAs and, thereby, the level of active Ago2. Regulation is linked to specific steps in cargo sorting and specific types of cargo. Thus, it is not likely a general property of all cell types but might be particularly significant in certain cell types such as pigment cells and possibly neurons. The reason why this regulation exists is tantalizingly mysterious, but the fact that it is conserved between Drosophila and humans argues that the reason is of general importance.

Materials and methods

Genetics

Mutants in the pigment synthesis pathway and transport pathways were obtained from the Bloomington Stock Center. The alleles used were g1, rb1, p1, ltd1, ca1, car1, cdr, bw1, cn1, a1, kar2, v, p1r, and argos. New cd alleles were isolated from a screen for RNAi enhancer mutations (Lee et al., 2004). Allele designations are according to their amino acid change. The dicer-2L811fs allele and dHPS4W515X are null alleles as described in Lee et al. (2004, 2009). A genomic transgene encompassing CG6969 (cd) was made by subcloning a 3.5 kb fragment into P[acman] (Venken et al., 2006). To generate an upstream activating sequence (UAS) transgene, the coding sequence from a cd cDNA was cloned via Gateway (Carnegie Institution of Washington) into the pPW UAS transgene vector. We obtained the cDNA from the Drosophila Genomics Resource Center Gold collection but found sequence inconsistencies in it compared with the genome sequence. These inconsistencies were corrected in the cDNA before subcloning. The coding sequence was also cloned via Gateway into the pPWG vector to fuse GFP in frame to the last cd codon. P[acman] transgenes were integrated into the Act51D site 1 model System Genomics (Duke University). P element transgenes were integrated by standard procedures.

RNAi assays

The GMR-wr transgene has been described in Lee and Carthew (2003). One copy of the GMR-wr transgene partially silences white and generates a pale orange eye. The other transgenic RNAi lines (UAS-argos, UAS-bw1R, and UAS-sprlR) were previously described in Dietzl et al. (2007), and GMR-Gal4 was used to drive their expression specifically in the compound eye. UAS-Dicer-2 transgenic lines were obtained from the Bloomington Stock Center and were coexpressed with RNAi transgenes to enhance their RNAi effects.
Quantification of eye pigments
The concentration of drososperin and ommochrome pigments in adult heads was measured by the method previously described in Falcón-Pérez et al. (2007). Drososperin was extracted from pools of four freshly dissected females heads (2–5 d old) by homogenization in 250 µl of 30% (volume/volume) ethanol (acidified to pH ~2 with HCl) followed by tumbling overnight at RT. The extracts were cleared twice by centrifugation for 1 min at 13,000 g, and their absorbance at 480 nm was measured against extracts prepared in parallel from ♂♂18 fly heads, which were used as blank controls. To measure drososperin in the presence of the GMR-wIR transgene, the number of fly heads was increased to 30, and the subsequent clarified solution was evaporated using speed vacuum and redissolved in 20 µl. Ommochrome was extracted from pools of eight freshly dissected heads except when in the presence of GMR-wIR, when the number of heads was increased to 30 and the extract was concentrated to 10 µl. Heads were homogenized in 150 µl of 2N HCl using a motorized pestle followed by an addition of 10 µl of 100-g/L sodium metabisulphite (Na2S2O5) and 200 µl n-butanol and by tumbling for 1 h at RT. After centrifugation for 5 min at 4,000 g, the organic phase was washed using 150 µl of 0.66% (weight/volume) sodium metabisulphite, tumbled for 40 µl at RT, and then centrifuged again. Subsequently, 1.45 µl of the washed organic phase was dried down in a speed vacuum. The pellet was dissolved in 10 µl ethanol, and absorbance was immediately measured at 490 nm against distilled water, which was used as a blank control. For both types of pigments, the resulting absorbance values were normalized to those obtained using FRITB2 or Oregon-R.

Real-time quantitative PCR (qPCR)
Real-time qPCR was performed as described in Marques et al. (2010). Total RNA was extracted using TRIZOL reagent according to the manufacturer’s protocol (Invitrogen). 100 ng–1 µg of total RNA was reverse transcribed using random primers and SuperScript Reverse Transcriptase (Invitrogen). The resulting cDNA was used as a template for qPCR reaction using Sybr green (Invitrogen) on an iCycler system (Bio-Rad Laboratories). The relative amount of the indicated RNAs normalized to an internal control was calculated using the ∆Ct method (Rpl32). The primers used were as follows: cd, 5′-TGTCAAGCTGACAGTCTG3′ and 5′-CCGGCCAGACGTTCTGTA-3′; w, 5′-TCTGACACCGACATCCCTTC-3′ and 5′-AAAAACTGCACCTG-3′; sp, 5′-CCGCTTCCAGAAGGCCAGCAGT3′ and 5′-AACCGGCGGTGTGAG-3′; bw, 5′-CAGGGATCGTCTACCA-3′ and 5′-TCGTGACCCAGCTGTTAA-3′; and pr, 5′-GATACGCCGTGCCTGTTA-3′ and 5′-TTTCTGTGCAAGCTGCTGAT-3′.

Cytoplasmic lysate preparation
Embryo lysates were prepared in lysis buffer [30 mM Hepes-KOH, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 5 mM DTT, and Complete Mini Protease Inhibitor (Roche)] from 0–6-h-old embryos as described in Tuschen et al. (1999).

mRNA cleavage assay
Pp-luc mRNA was transcribed with T7 RNA polymerase to generate a 510-nt product. The transcript was then 7-methylguanosine capped using guanylyl transferase (Invitrogen) and γ[32P]GTP (MP Biomedicals) according to the manufacturer’s instructions. The synthetic Pp-luc siRNA used in target mRNA cleavage was identical to that previously described (Pham et al., 2004). Duplex siRNA was prepared by annealing the single-stranded RNAs in annealing buffer for 1 h at 37°C after a 2-min incubation at 95°C. For target mRNA cleavage reactions, unlabelled siRNA was incubated with embryo lysate and labeled mRNA at 25°C as described in Pham et al. (2004). Products were resolved by electrophoresis on a 10% acrylamide-urea gel.

dRNA processing assay
Pp-luc dRNA was prepared by transcribing sense and antisense strands using T7 and SP6 RNA polymerase, respectively. Transcription reactions were performed in the presence of [α-32P]UTP (MP Biomedicals) to give internally radiolabeled products 465 nt (sense) or 460 nt (antisense) long. The products were mixed 1:1 in annealing buffer [30 mM Hepes, pH 7.5, 100 mM potassium acetate, and 2 mM magnesium acetate], heated to 95°C for 2 min, and annealed overnight at 37°C to give duplex RNA. Reactions were performed mixing labeled dRNA and embryo lysate at 25°C as described in Pham et al. (2004). Products were resolved by electrophoresis on a 15% acrylamide-urea gel. To quantitate processing, Phosphor Imager scans (Storm; GE Healthcare) were performed, and radioactive intensities of substrates and products were measured for each time point and subtracted for background.

Ago2 loading assay
Single-stranded Pp-luc siRNA oligonucleotides were 5′ end labeled with γ[32P]ATP (MP Biomedicals) and polynucleotide kinase before annealing to form an siRNA duplex. Reactions were performed by incubating embryo lysate with labeled siRNA in 5-µl RNAi reactions at 25°C for 40 min. The reactions were quenched with 1 µl heparin mix (60 mM potassium phosphate, 3 mM magnesium chloride, 3% PEG8000, 6% glycerol, and 4 mg/ml heparin), yielding a final heparin concentration of 0.67 mg/ml. They were then loaded onto a prechilled 4% (40:1 acrylamide/bisacrylamide) native gel and run at 4°C in 1× Tris/Borate/EDTA at 10 W. The gel was dried and exposed to a Phosphor Imager overnight.

S2 cell experiments
Using Gateway, cd cDNA was used to recombine the cd ORF into the pAWR vector, generating a carboxy-terminally tagged RFP construct for expression in S2 cells. The construct was under Actin5c promoter control. A stable cell line expressing constitutive Lamp-1::GFP was a gift from V. Gelfand (Northwestern University, Chicago, IL). Cells were transfected using Cellfectin (Invitrogen) and harvested 24 h after transfection. Before fixation, cells were transfected onto poly-l-lysine coverslips. Cells were fixed with 2% PFA in PBS at RT. After washes, coverslips were mounted in Vectashield mounting medium (Vector Laboratories).

In situ fluorescence
Pupae carrying GM-R-Gal4; UAS-Cd::GFP transgenes were dissected at 52 h after pupariation at 22°C. Eyes were fixed in 4% PFA/PBS at RT. After washes in PBS, they were mounted in Vectashield.

Microscopy
Adult eyes were examined on intact frozen flies by scanning electron or light microscopy. A scanning electron microscope (S-3400N-II; Hitachi) was used on mounted but noncoated specimens under variable chamber pressure. Images were acquired with the Hitachi software and exported into TIFF format for Photoshop (Adobe). For light microscopy, a binocular microscope (SV-6; Carl Zeiss) was used on specimens, and images were acquired with Axiocam (Carl Zeiss) and AxioVision (Carl Zeiss) software. S2 cells and pupal eye tissue specimens were viewed under a confocal microscope (LSM510; Carl Zeiss) using a 63x objective (NA 1.4 oil immersion) at RT. Laser-scanning microscope acquisition software (Carl Zeiss) was used. Z sections of 0.5-µm thickness were captured as 1,024 × 1,024-pixel images with line averaging. Images were transferred into TIFF format and rendered in Photoshop CS3.

Online supplemental material
Fig. S1 shows how pigmentation genes regulate white(RNAi). Fig. S2 highlights the conserved domains in Cd. Fig. S3 shows that Cd repression of white(RNAi) requires Dicer-2. Fig. S4 does not find a genetic interaction between cd and argos. Fig. S5 shows the mRNA expression of cd in different Drosophila tissues. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201102021/DC1.

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