The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing

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Many metazoan cells can take up exogenous double-stranded (ds) RNA and use it to initiate an RNA silencing response, however, the mechanism for this uptake is ill-defined. Here, we identify the pathway for dsRNA uptake in *Drosophila melanogaster* S2 cells. Biochemical and cell biological analyses, and a genome-wide screen for components of the dsRNA-uptake machinery, indicated that dsRNA is taken up by an active process involving receptor-mediated endocytosis. Pharmacological inhibition of endocytic pathways disrupted exogenous dsRNA entry and the induction of gene silencing. This dsRNA uptake mechanism seems to be evolutionarily conserved, as knockdown of orthologues in *Caenorhabditis elegans* inactivated the RNA interference response in worms. Thus, this entry pathway is required for systemic RNA silencing in whole organisms. In *Drosophila* cells, pharmacological evidence suggests that dsRNA entry is mediated by pattern-recognition receptors. The possible role of these receptors in dsRNA entry may link RNA interference (RNAi) silencing to other innate immune responses.

RNAi is a highly conserved dsRNA-guided mechanism that mediates sequence-specific gene silencing[1]. A number of animal cells can naturally take up exogenous dsRNA and use it to initiate RNAi silencing[2,3]. In some organisms, such as *Drosophila,* certain cells can efficiently take up dsRNA but seem to be unable to transmit this dsRNA to other cells in the body[4]. dsRNA uptake without further transmission to other cells has also been reported for some mammalian cell types[5–7]. Other organisms (such as *C. elegans* or juvenile grasshoppers) can both take up dsRNA and spread it systemically to elicit an RNAi response throughout the entire animal[8,9]. The mechanisms of uptake and spread of dsRNA are poorly understood. It is unclear whether dsRNA enters cells through passive, non-specific mechanisms, or whether there is an active mechanism that controls entry. Genetic analysis to identify genes involved in systemic spread of dsRNA in *C. elegans* isolated several mutants unable to distribute an ingested dsRNA signal from the gut throughout the body[8,10,11]. One of these, SID-1 (also known as RSD-8) is a putative transmembrane protein required for systemic spread[8]. When expressed ectopically in *Drosophila* cells, SID-1 enhanced the RNAi response observed at low dsRNA concentrations[12], raising the possibility that SID-1 may function as a channel on the cell surface for uptake of dsRNA. However, endogenous *sid-1* homologues have not been found in the *Drosophila* genome, yet *Drosophila* S2 cells effectively take up dsRNA and use it to induce gene silencing. As it is difficult to distinguish between uptake and systemic spread mechanisms in whole animal screens, we decided to directly examine the dsRNA-uptake mechanism using cultured *Drosophila* cells. An added advantage of this

approach is that it allowed the detection of essential genes and processes that would be lethal in the context of a whole organism. Here, we report the identification of the dsRNA-uptake pathway in *Drosophila* using a combination of biochemical, cell biological and genomic approaches. Taken together, our results indicate that dsRNA entry and initiation of an RNAi silencing response requires receptor-mediated endocytosis in *Drosophila* S2 cells. A genome-wide RNAi screen implicated numerous components of endocytosis and vesicle-mediated trafficking in dsRNA uptake and, importantly, RNAi silencing. Furthermore, orthologues of these genes are also critical for the RNAi response in *C. elegans*, pointing to conserved evolution of this entry pathway.

RESULTS

Initiation of efficient gene silencing depends on exogenous dsRNA length

Drosophila S2 cells can efficiently take up dsRNA over a wide range of concentrations (see Supplementary Information, Fig. S1a) and use it to initiate an RNAi response. To explore the properties of dsRNA entry pathway, we initially determined whether there was an optimal dsRNA length for efficient entry through the natural uptake machinery of *Drosophila* S2 cells. dsRNAs targeting firefly *luciferase*, and ranging from 21–592 base pairs (bp) in length, were either added to the culture supernatant ('soaking') or forcibly introduced into cells by transfection, as a control (Fig. 1a, b). As expected, transfection of dsRNA resulted in effective firefly *luciferase* silencing irrespective of the dsRNA length

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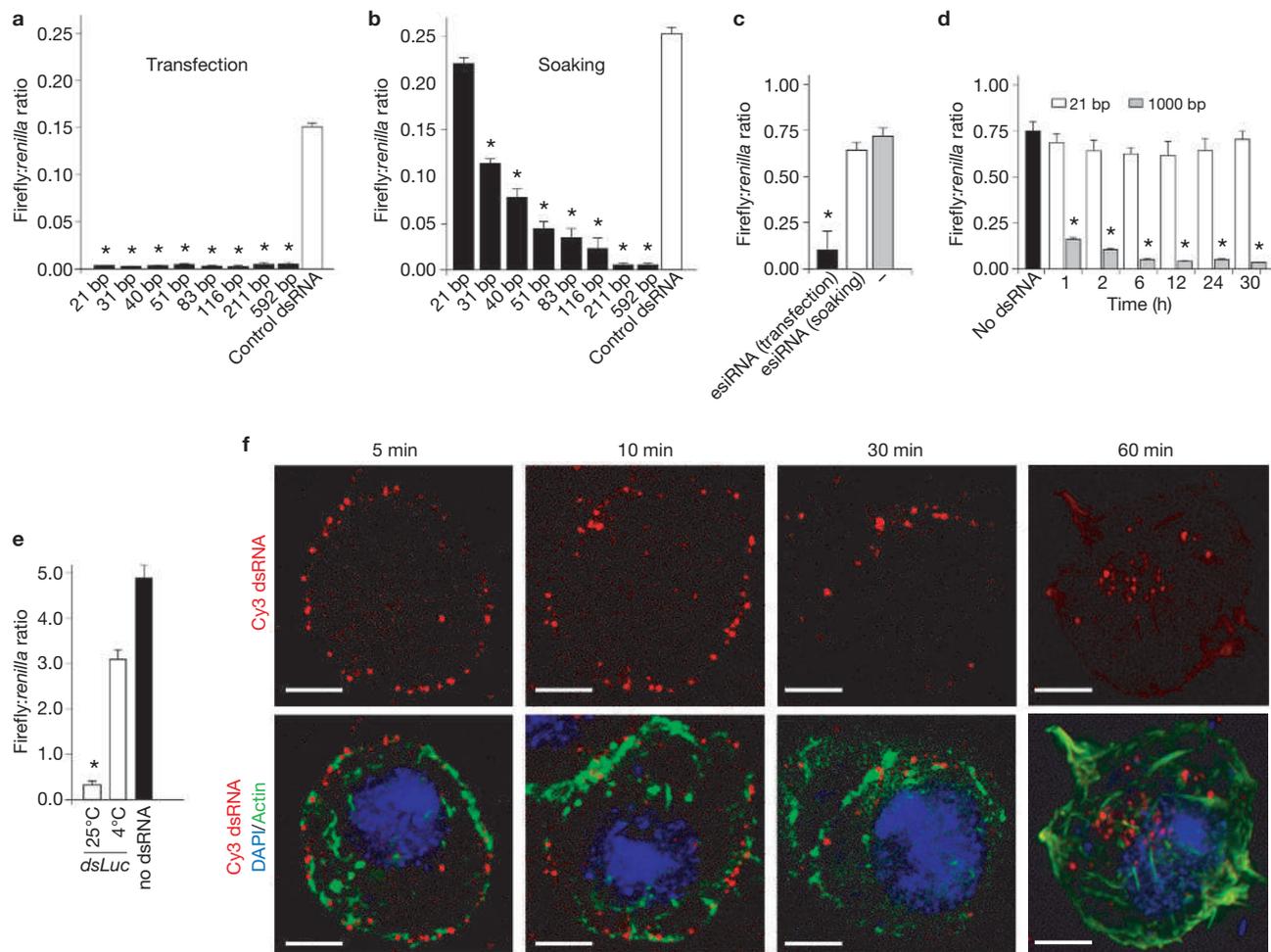
Figure 1 RNAi in *Drosophila* S2 cells is dependent on the length of the dsRNA. (**a, b**). Silencing of *luciferase* expression after exposure of S2 cells to dsRNA by transfection (**a**) or by adding dsRNA in the culture supernatant (soaking; **b**). S2 cells were cotransfected with expression plasmids encoding firefly and *Renilla luciferase*. Specific dsRNA targeting firefly *luciferase* was either transfected into the cell in conjunction with the expression plasmids or was added to the culture supernatant one day after transfection. Luciferase activity was monitored after 48 h and it is expressed as firefly:*Renilla* ratio. Luciferase activity after treatment with specific dsRNA of different sizes was compared with treatment with a non-specific dsRNA (control dsRNA). Results represent averages and s.d. from four independent experiments. The asterisk indicates $P<0.01$ with respect to untreated control (–). (**c**) Silencing of firefly *luciferase* expression after transfection or soaking pools of specific 21 bp siRNAs generated by cleavage of long dsRNA by recombinant Dicer (esiRNA) compared with luciferase activity in the absence of dsRNA (–). Results represent averages and s.d. from three independent experiments. The asterisk indicates $P<0.01$ with respect to untreated control (–). (**d**) Time course comparing silencing of

luciferase after soaking of S2 cells in dsRNA of 21 or 1000 bp. The experiment was performed as described in **a** and **b** but dsRNA was washed away after the indicated incubation times. Results represent averages and s.d. from three independent experiments. The asterisk indicates $P<0.01$ with respect to no dsRNA control. (**e**) Silencing of luciferase expression after soaking cells with specific dsRNA at 4 °C or 25 °C. S2 cells were transfected with expression plasmids encoding firefly and *Renilla luciferase* and exposed to dsRNA at 4 °C or 25 °C. After 1 h incubation, the cells were washed and further cultured at 25 °C for 48 h, and luciferase activity was monitored. Results represent averages and s.d. from three independent experiments. The asterisk indicates $P<0.01$ with respect to no dsRNA control. (**f**) Cellular localization of Cy3-labelled dsRNA over time. Shortly after incubation, dsRNA seemed to bind in a punctate pattern to the cell surface (5 min). Over the course of 60 min the dsRNA was internalized but remained in small punctate structures. Optical sections were deconvolved and flattened into a two-dimensional projection for presentation. The 60 min time point corresponds to an independent experiment. The scale bar in **f** represents 2 µm.

(Fig. 1a). In contrast, uptake of dsRNAs added to the medium was clearly length-dependent (Fig. 1b). It was possible that uptake of dsRNA could exhibit sequence specificity, as this could influence efficient uptake of short dsRNA to a greater extent than longer dsRNAs. Therefore, the RNase Dicer was used to derive a mixed pool of 21-mer siRNAs from a 1000 bp dsRNA fragment. Importantly, this diverse pool of short siRNAs (enzymatically generated siRNA; esiRNA) contained a mix of all the sequences used in Fig. 1a and b, yet failed to enter S2 cells, even though it effectively induced silencing after transfection (Fig. 1c).

The kinetics of long and short dsRNA uptake by S2 cells were then compared. Cells were pulsed by incubation with dsRNAs targeting firefly

luciferase for different times, washed extensively to remove free dsRNA and cultured for 48 h to allow subsequent transfer of the dsRNA into the RNAi machinery and cleavage of the target RNA. dsRNA of 1,000 bp rapidly became tightly associated with cells — a 1 h pulse already yielded significant firefly *luciferase* silencing compared with untreated control (Fig. 1d). Similar kinetics were observed using dsRNA of 200 bp (data not shown). In contrast, exposure of S2 cells to 21 bp siRNA did not result in any significant silencing, even after prolonged incubation times of up to 30 h (Fig. 1d).

Whether internalization of functional dsRNA by the endogenous S2 cell uptake machinery is temperature dependent was also examined
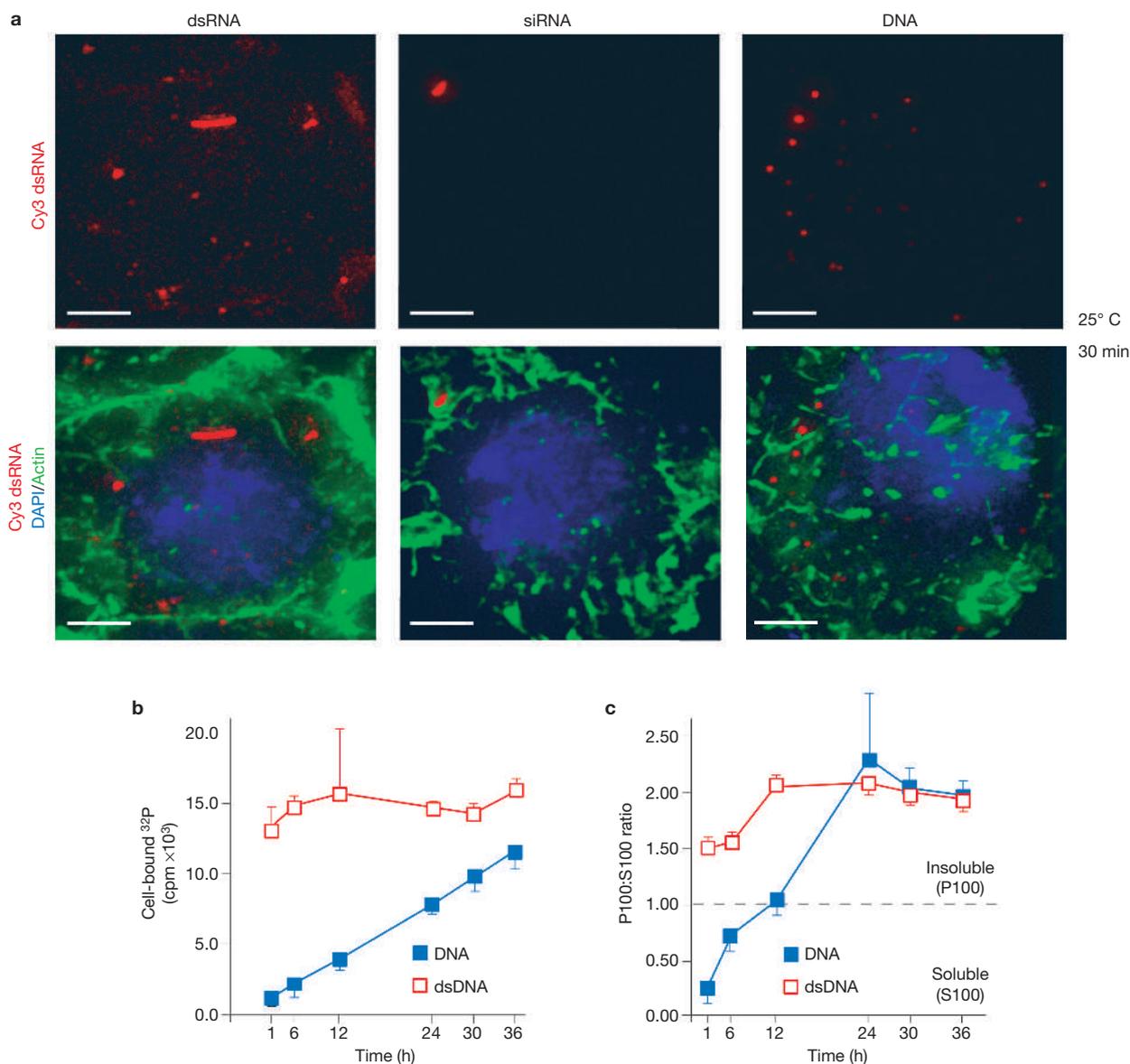
Figure 2 An active mechanism for uptake of long dsRNA in *Drosophila* S2 cells. (**a**) Subcellular localization of Cy3-labelled 500 bp dsRNA, siRNA and 500 bp DNA. (**b**) Association of radiolabelled dsRNA or DNA with S2 cells over time. S2 cells were incubated with radiolabelled dsRNA or DNA of the same size and sequence and cell-bound radioactivity was determined in a scintillation counter. Results represent averages and s.d. from two independent experiments. (**c**) Subcellular localization of radiolabelled dsRNA or DNA with S2 cells over time. S2 cells were incubated with dsRNA or DNA for the indicated times, lysed and fractionated by ultracentrifugation. The data are expressed as the ratio of radioactivity in pellet to supernatant. Results represent averages and s.d. from two independent experiments. The scale bar in **a** represents 2 μm.

(Fig. 1e). RNAi silencing of firefly *luciferase* was inefficient when cells were pulsed with dsRNA for 60 min at 4 °C instead of 25 °C (Fig. 1e). This temperature dependence indicates that natural uptake of dsRNA into *Drosophila* S2 cells is an active process.

The kinetics of uptake were also examined by fluorescence microscopy to determine the subcellular distribution of dsRNA during the early phases of uptake. Cells were incubated with cy3-labelled dsRNA followed by extensive washing and monitoring by fluorescence microscopy (Fig 1f). Importantly, the labelled Cy3 dsRNA was fully active in a functional assay (see Supplementary Information, Fig. S1b, c). Shortly after incubation (5 min), dsRNA seemed to bind in a punctate pattern to the cell surface (Fig. 1f). Over the course of 60 min at 25 °C, the dsRNA was internalized but remained in

punctate structures (Fig. 1f). Thus, S2 cells have an active mechanism for uptake and subcellular localization of long dsRNA.

The uptake pathway discriminates between dsRNA and DNA

Fluorescence microscopy revealed distinctions in the interaction of dsRNA, DNA or siRNAs with S2 cells. Long dsRNA bound to cells and was localized in large puncta in the cell interior (Fig. 2a). Low-level binding and no obvious internalization of siRNA was observed (Fig. 2a). DNA bound less efficiently than long dsRNA and, while seemingly internalized, it was localized to peripheral puncta that were smaller than those seen for dsRNA (Fig. 2a).

Consistent with the fluorescence microscopy analysis, the time course of uptake and localization of dsRNA and DNA was substantially
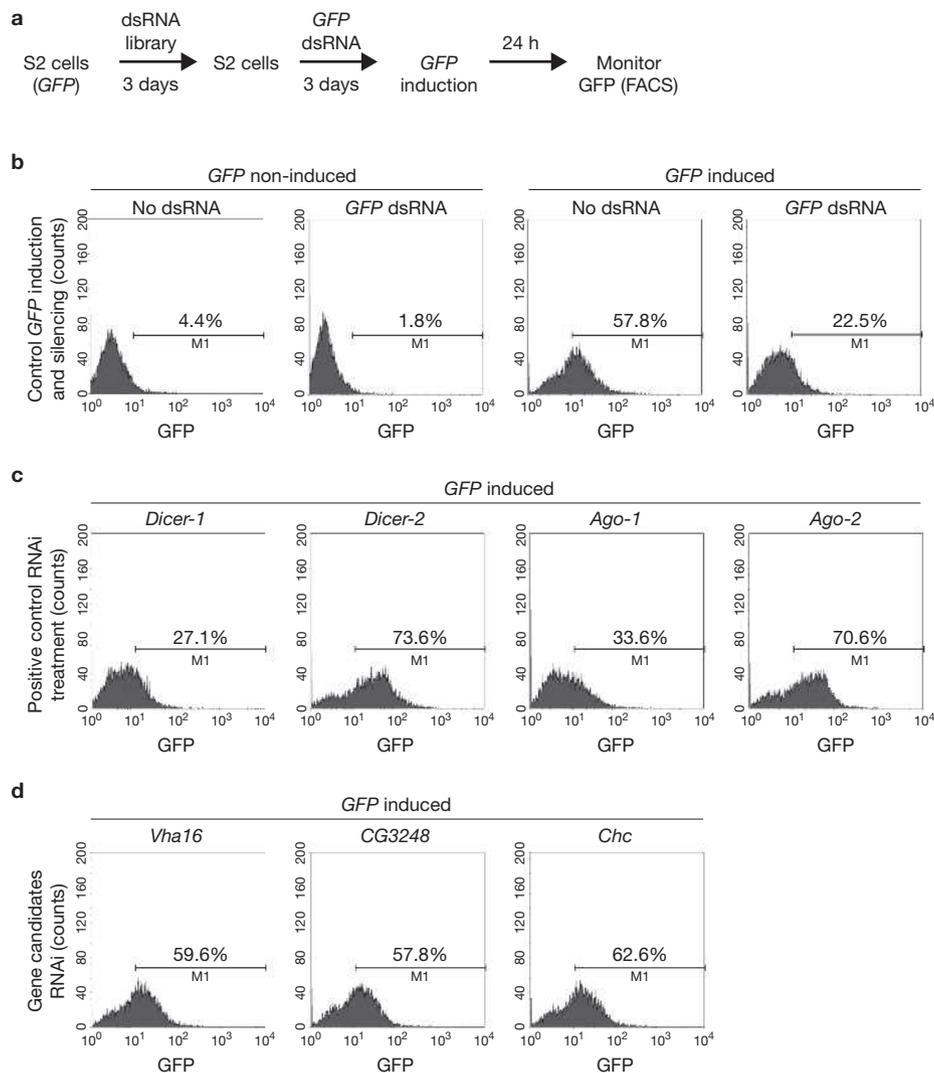
Figure 3 RNAi screen for genes involved in the RNAi pathway. (**a**) Schematic representation of the screening approach. S2 cells stably transfected with GFP under the inducible metallothionein promoter are treated with dsRNA from the RNAi library for three days. The cells are then split and refed with dsRNA from the RNAi library and with dsRNA targeting *GFP*. After a further three day incubation, *GFP* expression is induced by the addition of CuSO$_4$ to the culture supernatant and GFP expression is monitored. (**b**) Induction of *GFP* expression and RNAi in the reporter cell line. GFP expression is monitored on a FACS Calibur flow cytometer and analysed using Cellquest software. *GFP* expression is induced on addition of CuSO$_4$ in the culture medium. (**c**) Validation of the RNAi of RNAi approach. S2–GFP cells were pretreated with dsRNA targeting the RNAi-associated genes *Dicer-1*, *Dicer-2*, *Ago-1* and *Ago-2*, followed by RNAi of the *GFP* marker gene. (**d**) Examples of three genes identified in the RNAi screen. Knockdown of V-H-ATPase subunit (*Vha16*), *CG3248* and Clathrin heavy chain (*chc*) inhibits subsequent RNAi of *GFP*, as evident by a high GFP expression.

different. Incubation of S2 cells with ^{32}P-radiolabelled dsRNA or DNA of the same sequence and length, followed by measurement of cell-associated radioactivity (^{32}P), showed that 1000 bp dsRNA rapidly associated with the cells (Fig. 2b). This measurement is in good agreement with the rapid uptake of dsRNA observed using the RNAi functional assay (Fig. 1d). In contrast, DNA association with cells was less efficient and slower (Fig. 2b).

The subcellular distribution of dsRNA during uptake was also analysed using a biochemical approach and compared with the uptake of DNA. Cells were incubated with ^{32}P-labelled DNA or dsRNA for the indicated times, washed to remove unbound material, lysed and separated into soluble (S100) and insoluble (P100) fractions. Whereas the S100 fraction contained a marker for soluble cytoplasmic components (tubulin), P100 contained markers corresponding to membranous organelles, including the plasma membrane, Golgi, endosomes and lysosomes

(see Supplementary Information, Fig. S1d). The amount of ^{32}P-labelled DNA or dsRNA in each fraction was then estimated and expressed as the ratio of P100:S100 (Fig. 2c). Strikingly, as early as 1 h after incubation, dsRNA was enriched in the pellet fraction. In contrast, DNA was initially observed in the soluble fraction and was only found in the pellet fraction at later times.

These data show distinctions in the binding and uptake of short dsRNA, longer dsRNA and DNA with S2 cells and indicate that long dsRNA is rapidly bound on the cell surface and seems to accumulate in intracellular structures or bodies.

Genome-wide screen for genes involved in dsRNA uptake

To identify components required for RNAi silencing triggered by exogenously added dsRNA, we undertook a genome-wide functional screen. An 'RNAi of RNAi' approach[13,14] was used to downregulate cellular genes

Table 1 Identification of genes involved in RNAi function in *Drosophila* S2 cells

Group[a]	Gene ID	Gene name	Readout[b]			
			Relish–GFP	LAMP–GFP	dIAP1	Luciferase
Proton transport	CG3161	*Vha16*	+++	+++	++	+++
	CG17332	*VhaSFD*	++	+	+	++
Vesicle mediated transport	CG9012	*Clathrin hc*	+++	+++	++	+++
	CG7057	*AP-50*	++	++	++	++
	CG5915	*Rab7*	++	++	++	++
	CG6025	*Arf72A*	+	+	++	+++
Intracellular transport	CG54125	*ninaC*	+	+	++	++
	CG6177	*ldlCp*	+++	++	+	+
	CG3248		+++	++	++	++
	CG3911		+	++	+	++
	CG18028	*light*	+	+	+	+
Lipid metabolism	CG3495	*Gmer*	+	+	++	+++
	CG5373	*Pi3K59F*	+	+	++	+++
	CG12070	*Saposin-r*	++	+++	+++	+++
Proteolysis and peptidolysis	CG4572		+++	+++	+++	+++
	CG5053		+	+	++	++
	CG8184		+	+	++	++
	CG8773		+	+	++	++
Other	CG9659	*egghead*	+++	+	++	++
Unknown	CG5161		++	+	++	+
	CG5382		++	+	++	++
	CG5434		+++	+++	++	++
	CG8671		+	+	++	++
RNAi function	CG6493	*Dicer 2*	+++	++	+	++
	CG4792	*Dicer 1*	+	++	++	++
	CG7439	*Argonaute 2*	+++	+++	+++	+++
Control			–	–	–	–

[a]Genes were classified according to their biological function as annotated in Flybase (http://flybase.bio.indiana.edu/annot/). [b]Inhibition of RNAi function was assayed using four different secondary RNAi assays. Score: +++, expression of reporter gene equal to control in which expression of marker genes is not suppressed by RNAi; ++ and +, intermediate levels of expression of the reporter gene; –, expression of the reporter gene was silenced to the same level as control that only received marker gene RNAi treatment.

required for RNAi silencing of an inducible *GFP* reporter (Fig. 3a, b). We used a dsRNA library that targets the 7,216 *Drosophila* genes that have known homologues in *C. elegans* and mammals and corresponds to approximately 50% of the *Drosophila* genome[15]. Positive controls for RNAi-mediated downregulation of the *GFP* reporter included the core components of the RNAi machinery, *Dicer 2* and *Argonaute 2* (*Ago-2*, Fig. 3c). Fluorescence activated cell sorting (FACS) analysis indicated that targeting *Dicer-2* or *Ago-2* reduced the level of silencing and thus increased the GFP signal (Fig. 3c). In contrast, targeting their close homologues *Dicer-1* and *Ago-1*, which do not function in processing of long dsRNA but instead in the microRNA pathway, inhibited GFP silencing to a lesser extent (Fig. 3c). The initial screen identified 66 genes required for RNAi silencing (Fig. 3d and data not shown). Three secondary screens using different RNAi reporters yielded a subset of 23 genes required for RNAi silencing of all reporters (Fig. 3d and Table 1). Importantly, downregulation of the genes identified in this screen did not affect microRNA production or function (see Supplementary Information, Fig. S2). Thus, the genes identified in our screen are specifically involved in exogenous dsRNA uptake and processing.

Strikingly, components of the endocytic pathway were strongly represented in the screen, including genes for clathrin heavy chain and its adaptor AP-50, which mediate early endocytic uptake, as well as *rab7*, *Arf72A* (*ARF-like 1* orthologue), *light* (vacuolar protein sorting *Vsp41* orthologue) and *vacuolar H+-ATPase* (*V-H-ATPase*), involved in controlling endocytic vesicle trafficking and protein sorting (Table 1). The screen also identified members of the conserved oligomeric Golgi complex (COG) family: *ldlCp* and *CG3248*, (*Cog2* and *Cog3* orthologues respectively), the gene *CG3911* (transport protein particle –*TRAPP- component 3* orthologue) and genes involved in cyoskeleton organization and protein transport (*ninaC*). Therefore, it seems that exogenous dsRNA enter the RNAi pathway through the intracellular vesicle network. This conclusion is further supported by the identification of two genes involved in lipid metabolism and modification, *Pi3K* and *Saposin–r*. In addition to these relatively well-annotated genes, the screen also identified genes of unknown function. Taken together with the conclusions of our biochemical analysis, this genome-wide screen indicates that the pathway of dsRNA uptake relies on receptor-mediated endocytosis.

Pharmacological inhibitors of the dsRNA-uptake machinery

The role of the endocytic pathway in dsRNA entry was then explored using a pharmacological approach that tested the effect of inhibitors of cellular uptake mechanisms on RNAi silencing. Bafilomycin-A1
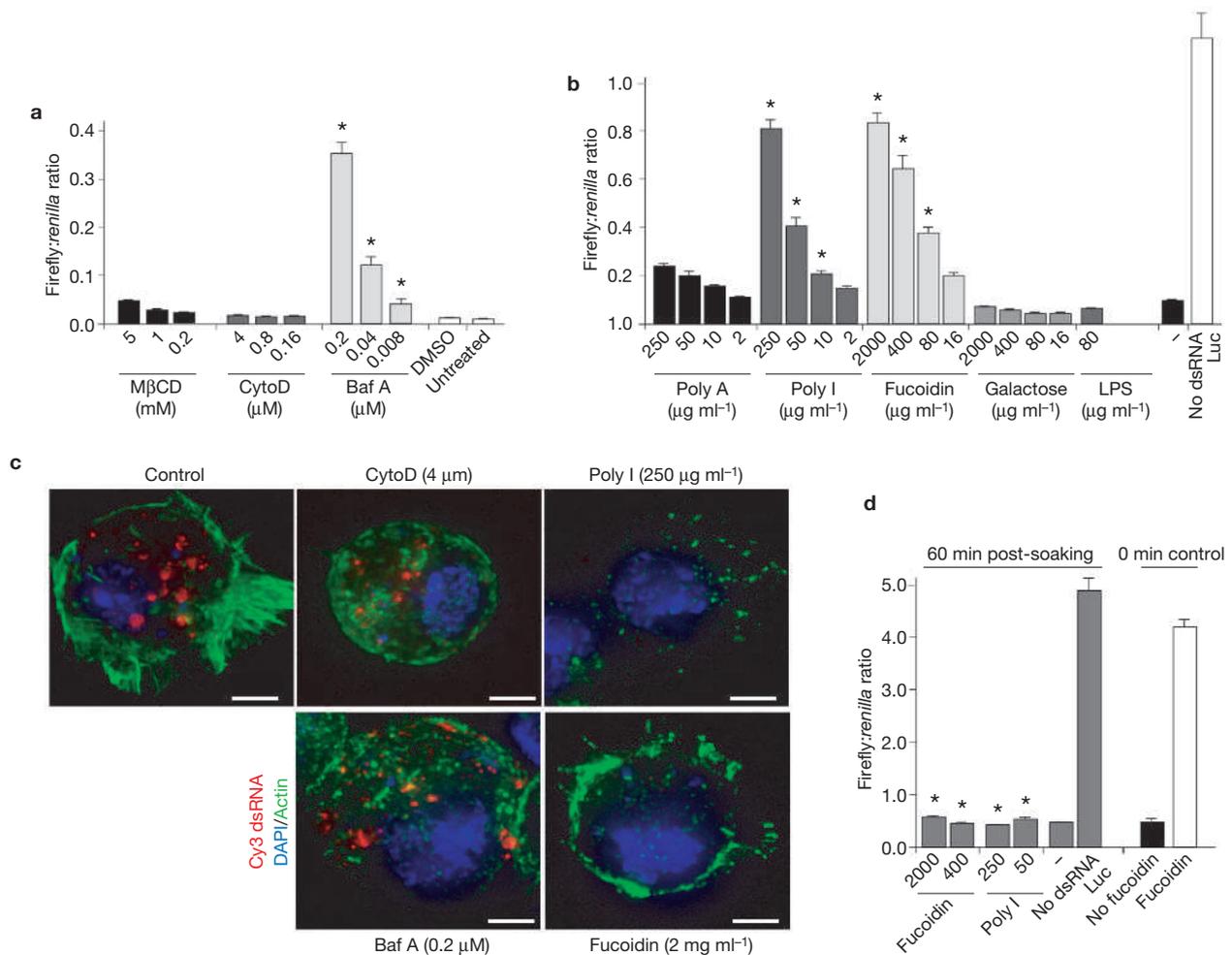
Figure 4 Endocytic uptake of dsRNA into *Drosophila* S2 cells is mediated by scavenger receptors. (**a**) Silencing of luciferase expression in the presence of inhibitors of the endocytic pathway. S2 cells were transfected with expression plasmids encoding firefly and *Renilla luciferase*. One day after transfection, the cells were exposed to different concentrations of methyl-β-cyclodextran (MβCD), Cytochalasin D (CytoD) or Bafilomycin A (Baf A) for 30 min and dsRNA was added in the presence of these inhibitors. Controls include incubations of dsRNA in the presence of the solvent dimethylsulfoxide (DMSO) and in the absence of inhibitor (untreated). Results represent averages and s.d. from three independent experiments. The asterisk indicates *P*<0.01 with respect to untreated control. (**b**) Silencing of *luciferase* expression in the presence of competitive inhibitors of scavenger receptors. The experiment was performed as described in **a**, using the inhibitors of scavenger receptors poly-inosinic acid (Poly I) and Fucoidin. Controls include incubation of S2 cells with dsRNA in the presence of compounds that are not known to inhibit scavenger receptors, poly-adenosinic acid, galactose and lipopolysaccharide (LPS) and in the absence of inhibitors (–). Results represent averages and s.d. from three independent experiments. The asterisk indicates *P*<0.01 with respect to untreated control (–). (**c**) Subcellular localization of Cy3-labelled dsRNA after incubation in the presence of inhibitors of the endocytic pathway and of scavenger receptors. (**d**) Inhibitors of scavenger receptors do not affect RNAi when added after incubation of S2 cells with dsRNA. S2 cells were transfected with plasmids encoding firefly and *Renilla luciferase*. Cells were then incubated with dsRNA for 1 h, washed and fucoidin or poly I was added to the culture. The bars labelled '0 min control' indicate incubations of Fucoidin in the presence of dsRNA and confirm the data in Fig. 4b. Results represent averages and s.d. from three independent experiments. No significant statistical difference was observed (asterisk), *P*>0.05, with respect to untreated control (–). The scale bar in **c** represents 2 μm.

(Baf A), a specific inhibitor of V-H-ATPase, strongly inhibited silencing of the reporter gene (Fig. 4a). In contrast, methyl-β-cyclodextran and cytochalasin-D, inhibitors of caveolae mediated endocytosis or phagocytosis[16,17], respectively, did not affect RNA silencing (Fig. 4a). Therefore, it seems that V-H-ATPase, a component of the endosomal–lysosomal acidification process[18], is required for dsRNA entry and RNA silencing in S2 cells, but not caveolae or phagocytosis. These conclusions are consistent with our finding that downregulation of subunits of V-H-ATPase inhibit silencing that is mediated by exogenous dsRNA (Table 1). In good agreement with the role of V-H-ATPase at early stages of the endocytic pathway[19–21], the exogenous dsRNA still accumulated in vesicles on treatment with Baf A (Fig. 4c).

## dsRNA uptake is blocked by inhibitors of pattern-recognition receptors

Although our biochemical experiments hinted at the existence of a surface receptor for entry of long dsRNAs, the screen did not identify any putative candidates for this function. This would be expected if these genes are not represented in the initial dsRNA library, which only targeted conserved genes. Alternatively, dsRNA uptake could be mediated by several related receptors with overlapping function. As dsRNA is a long polymer with a relatively regular structure, we reasoned that dsRNA recognition may be mediated by receptors that recognize repetitive patterns in biological macromolecules. There are two major classes of these so-called 'pattern-recognition receptors': the Toll receptors and the scavenger receptors[22,23].

A candidate approach was used to systematically examine the contribution of different classes of receptors to dsRNA uptake and silencing. RNAi-mediated downregulation was carried out for all annotated pattern-recognition receptors and the efficiency of the RNAi treatment was monitored by semi-quantitative RT–PCR (see Supplementary information, Fig. S3d, e). Downregulation of eight Toll receptors had no effect on dsRNA uptake or silencing (see Supplementary information, Fig. S3c). Similarly, individual downregulation of nineteen annotated genes coding for scavenger receptors in *Drosophila* did not result in significant inhibition of RNAi silencing (see Supplementary Information, Fig. S3a and Table S1), even under conditions where downregulation of scavenger receptor class C, type I (Sr-CI) dramatically reduced bacterial uptake (see Supplementary Information, Fig. S3b).

The putative role of pattern-recognition receptors in dsRNA uptake and silencing was then evaluated using a pharmacological approach. We examined whether macromolecules known to interact with scavenger receptors competed for binding of the dsRNA, and thus inhibited RNAi silencing. PolyI and fucoidin, well known ligands of the scavenger-receptor family[24], strongly inhibited both dsRNA binding and uptake, as assessed by fluorescent dsRNA (Fig. 4c), and dsRNA-initiated silencing (Fig. 4b). In contrast, chemically related molecules that interact with other receptors, but do not inhibit scavenger receptors (such as the polysaccharide LPS and the nucleic acid polyA, or the monosaccharide galactose), did not affect dsRNA-initiated silencing (Fig. 4b). Importantly, polyI and fucoidin inhibited silencing only if added early during dsRNA uptake (Fig. 4d), suggesting that binding and uptake of dsRNA is mediated by members of the scavenger-receptor family. Although RNAi downregulation of individual scavenger receptors did not result in inhibition of RNAi function, the strong inhibition observed by the pharmacological treatments may indicate that multiple scavenger receptors with overlapping functions participate in dsRNA uptake to induce RNAi. To explore this possibility, mixed dsRNAs targeting each known receptor were transfected into S2 cells. None of these mixtures produced a significant reduction in RNAi silencing (data not shown). Consistent with these results, a recent report showed that fluorescent dsRNA is internalized by Sr-CI and Eater receptor-mediated endocytosis in S2 cells[25]. However, this study was also unable to demonstrate that downregulation of scavenger receptors impairs RNAi function. In principle, it is possible that the simultaneous targeting of multiple genes by RNAi is not effective enough to impair the dsRNA binding to the remaining receptors. It is also possible that novel, unidentified members of this diverse family are responsible for this uptake function.

Conservation of entry pathways between *Drosophila* and *C. elegans*

Many components of the core RNAi machinery are highly conserved through evolution. However, organisms exhibit substantial differences in their ability to take up and spread dsRNA for silencing[26]. To determine whether the genes identified in the *Drosophila* 'RNAi of RNAi' screen also participate in RNAi function in other organisms, we studied the effect of knocking down *Drosophila* orthologues in the nematode *C. elegans*. Worms were fed *Escherichia coli* expressing dsRNAs targeting the *C. elegans* orthologues of the *Drosophila* genes required for dsRNA uptake. Two days later the progeny were challenged with a second dsRNA targeting *unc*-52, which is essential for muscle development in both embryos and larvae (Fig. 5). Knockdown of *unc*-52 causes severe defects in
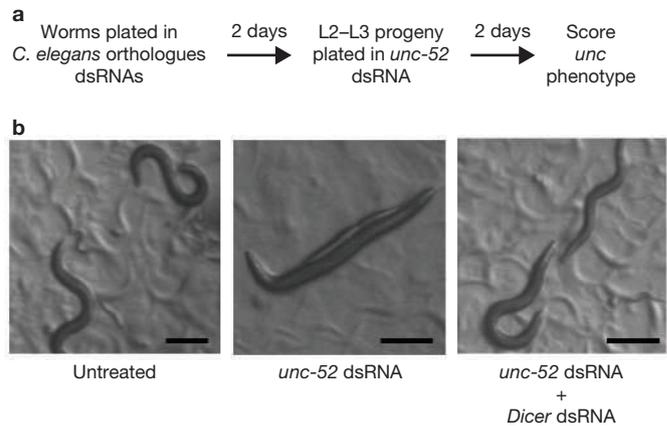
Figure 5 Orthologues of the *Drosophila* genes confer an RNAi phenotype in *C. elegans*. (**a**) Schematic representation of the experimental design. Worms were grown on bacteria expressing dsRNA targeting specific genes. L2–L3 progeny was subsequently plated on bacteria expressing dsRNA specific for *unc-52* and unc phenotype was monitored after 48 hours. (**b**) *C. elegans* grown on bacteria expressing dsRNA control targeting *Dicer* are incapable of processing *unc-52* dsRNA and do not display the *unc52* RNAi phenotype. The scale bars represent 0.2 mm.

myofilament assembly and leads to paralysis[27,28]; therefore, inhibition of RNAi silencing should alleviate the phenotype normally associated with RNAi knockdown of *unc-52*. As expected, knockdown of *Dicer* suppressed the phenotypic consequences of the treatment with *unc*-52 dsRNA (Fig. 5 and Table 2). Strikingly, worms treated with dsRNAs corresponding to several of the genes identified in the *Drosophila* screen inactivated the systemic spread of RNAi silencing in worms (Table 2, normal). Our experiments indicated that, similar to *Drosophila* S2 cells, several components of *C. elegans* intracellular vesicle transport (F22G12.5, C06G3.10, ZK1098.5 and ZK1098.5), as well as lipid modifying enzymes (R01H2.5 and Pi3K) are required for systemic RNAi. Notably, many of the core components of the endocytic pathway involved in dsRNA uptake in *Drosophila* were essential for viability of the worms and could not be tested (Table 2). In addition, the orthologues of several *Drosophila* genes with unknown function were also required for systemic RNAi in *C. elegans* (B0464.4, W05H7.3, Y45G12B.2 and C54H2.1). Thus, the basic machinery that mediates dsRNA upake in *Drosophila* is also required for systemic spread of the RNAi signal in *C. elegans,* suggesting that the dsRNA entry pathway is evolutionarily conserved and functionally relevant in intact organisms.

DISCUSSION
The phenomenon of RNAi silencing is widely conserved among all higher eukaryotes. Exploiting this process is becoming increasingly important as an experimental tool, as well as for therapeutic applications. Although most cells possess the basic RNAi core machinery, some cell types have the intriguing ability to naturally take up exogenous dsRNA and use it to initiate RNAi silencing[2,3,5–7]. Furthermore, some organisms, such as plants, *C. elegans* and planaria (*Girardia tigrina*) can transmit the RNA silencing signal from cell to cell, resulting in the systemic spread of the RNAi response[8,26,29,30]. It is currently believed that insects lack a pathway for the systemic spreading of RNAi. Nevertheless, injected dsRNA elicits cell non-autonomous RNAi in adult *Drosophila*, juvenile grasshopper, *Tribolium castaneum* (flour beetle) and *Anopheles gambiae*[9,31–33]. In plants, it seems that systemic spread relies on the plasmodesmal channel

Table 2 dsRNA uptake pathway is conserved among organisms: *Drosophila* orthologues affect RNAi in *C. elegans*

Group	*C. elegans* gene ID	*C. elegans* gene name	*Drosophila* orthologue	IPTG[a] 0.2 µM	2.0 µM
Proton transport	R10E11.2	*vha-2*	*Vha16*	Non-viable	Non-viable
	T14F9.1	*vha-15*	*VhaSFD*	Non-viable	Non-viable
Vesicle mediated transport	T20G5.1	*chc-1*	*Clathrin hc*	Non-viable	Non-viable
	R160.1	*dpy-23*	*AP-50*	Unc	Unc
	W03C9.3	*rab-7*	*Rab7*	Unc	Non-viable
	F54C9.10	*arl-1*	*Arf72A*	ND	Normal
Intracellular transport	F22G12.5		*ninaC.5*	Normal	Normal
	C06G3.10	*cgo-2*	*ldlCp*	Normal	Unc
	ZK1098.5		*CG3911*	Normal	Normal
	F32A6.3	*vps-41*	*light*	Normal	Normal
Lipid metabolism	R01H2.5		*Gmer*	Normal	Normal
	B0025.1	*vps-34*	*Pi3K*	Normal	Unc
Proteolysis and peptidolysis	F41C3.5		*CG4572*	Unc	Unc
	K05B2.2		*CG5053*	Unc	Unc
	T07F10.1		*CG8773*	Unc	Unc
Other	B0464.4	*bre-3*	*egghead*	Normal	Unc
Unknown	W05H7.3	*sedl-1*	*CG5161*	Normal	Normal
	Y45G12B.2		*CG5382*	Normal	Unc
	F08D12.1		*CG5434*	Unc	Non-viable
	C54H2.1	*sym-3*	*CG8671*	Normal	Normal
RNAi function	K12H4.8	*dcr-1*	*Dicer 2*	Normal	Normal
Control	Vector Control			Unc	Unc

[a]Expression of dsRNA in the bacteria used for the primary RNAi treatment was induced with two different concentrations of IPTG. ND, non-determined. Unc, *unc-52* RNAi phenotype.

system, which connects all the cells in the plant[34,35]. However, this system is absent from animal organisms. Despite the importance of RNAi processes, little is known about the machineries that mediate either dsRNA uptake or systemic spread of the RNAi signal in animal cells. A number of genetic screens using *C. elegans* have identified components required for systemic spread of an RNAi signal. Because systemic RNA silencing is a multistep process that requires uptake, amplification and spread of the silencing signal, the specific functions of these components within this complex process have not been precisely defined. Here, we sought to specifically identify the machinery that mediates uptake of exogenous dsRNA to induce an RNAi response using a less complex model system. As *Drosophila* S2 cells can efficiently take up exogenous dsRNA they provided with a well-defined system to identify the mechanism and components of dsRNA entry. Using biochemical, genomic and pharmacological approaches we found that dsRNA enters the RNAi pathway through an active and specific pathway that involves clathrin-mediated endocytosis. Furthermore, biochemical and pharmacological analyses implicate scavenger-like pattern-recognition receptors in dsRNA entry. We also examined whether *C. elegans* homologues of components of the *Drosophila* dsRNA entry pathway function in systemic spread of an ingested dsRNA signal. Whereas downregulation of core endocytosis components (such as clathrin and V-H-ATPase) was lethal in *C. elegans*, downregulation of several components of vesicular intracellular transport and lipid metabolism blocked systemic spread of the RNAi signal. It thus seems that RNAi spread is an active process that involves vesicle-mediated intracellular trafficking and depends on lipid modifications and cytoskeleton guidance. Based on these experiments, we hypothesize that the dsRNA entry

pathway we have identified in *Drosophila* is conserved in other animal cells. The severity of the phenotype observed for downregulation of the endocytic pathway may account for the inability to detect this pathway of entry in screens carried out in whole *C. elegans* animals.

The identification of components of the endocytic pathway required for dsRNA entry to initiate an RNAi response raises a number of interesting questions. Several lines of evidence, including the requirements of clathrin, ARF72A, V-H-ATPase and Rab 7 for exogenous dsRNA-initiated silencing (Table 1 and Fig. 4a), suggest that endocytic vesicles are critical in the entry pathway. However, the RNAi uptake pathway would need to deviate from standard endocytic uptake at some point if it is to deliver dsRNA to the cytoplasm. It is tempting to speculate that the RNAi signal may be directly translocated, perhaps through SID-1-like channels, from specialized entry vesicles to the RNAi machinery. Intriguingly, several components of the RNAi machinery, including dicer and ago-2, are membrane associated or have membrane-anchoring domains (Saleh, M.C., University of California San Francisco and Joachimiak, M., University of California Berkley; unpublished observations and ref. 36). Our observation that in cells defective for V-H-ATPase, dsRNA still accumulates in vesicles (Fig. 4c) but does not initiate an RNAi response (Table 1 and Fig. 4a) suggests that the V-H-ATPase activity controls progression of the dsRNA through the RNAi entry pathway. Future studies should determine the mechanisms by which dsRNA is loaded onto the RNAi apparatus. This, in turn, may explain why some cells are uniquely able to take up exogenous dsRNA to initiate an RNAi response.

The observation that members of the scavenger-receptor family act as receptors of dsRNA may provide insight into the physiological role of this

pathway, as these proteins have well-known roles in the ancestral innate immune response[24,37]. For example, scavenger receptors participate in the uptake of bacterial pathogens and have also been implicated in the uptake of chaperone–peptide complexes[38]. It is thought that the chaperone-bound peptide is translocated from vesicles to the cytosol to enter the antigen-presentation pathway in a process that bears some similarities to dsRNA uptake[39,40]. The pathway for dsRNA uptake may thus serve a protective role to prevent the spread of viral infections by uptake of viral replicative intermediate dsRNAs that are released on cell lysis.

RNAi has tremendous potential for specific and effective therapeutic applications but the main obstacle to achieving *in vivo* therapies by RNAi technologies is delivery. Our observations that the pathway of dsRNA entry utilizes components of the endocytic machinery may provide a starting point to develop novel strategies for RNAi delivery. The identification and exploitation of this natural RNAi entry pathway may provide more effective and non-toxic strategies of dsRNA delivery. □

METHODS

Cells, plasmids and reagents. *Drosophila* S2 cells (Invitrogen, Carlsbad, CA) were cultured at 25 °C in Schneider's *Drosophila* medium (GIBCO-Invitrogen, Carlsbad, CA), supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin. Stable S2 cell lines were cultured in the same medium, additionally supplemented with 300 μg ml⁻¹ hygromycin B. S2 cells stably expressing GFP–Relish protein have been previously described[15]. S2 Lamp–GFP cells were kindly provided by the laboratory of R. Vale at University of Calforria San Francisco. Firefly and *Renilla luciferase* sequences from the plasmids pGL3 and pRL-CMV (Promega, Madison, WI) were cloned into pMT/V5-HisB (Invitrogen) allowing copper-inducible expression from a metallothionein promoter. A *luciferase* construct that can be targeted by the endogenous miRNA, *miR2b,* was generated by inserting two copies of the mature *miR2b* sequence in sense and antisense orientation of the 3′UTR of pMT-GL3. Transfections were performed using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Firefly and *Renilla* luciferase expression was analysed using the Dual-Luciferase reporter assay system (Promega) and analysed on a Tecan Ultra-evolution plate reader. To evaluate the significance of the differences in firefly luciferase counts a student's *t*-test was used. Pharmacological inhibitors of the endocytic pathway and competitive inhibitors of scavenger receptors were purchased from Sigma Aldrich (St Louis, MO).

RNAi methods. dsRNA was generated by *in vitro* transcription using T7 RNA-polymerase and RNAi experiments were performed as previously described[15]. Synthetic siRNA targeting firefly *luciferase* mRNA was obtained from Dharmacon, (Lafayette, CO). A pool of esiRNA targeting firefly *luciferase* mRNA was generated by cleavage of 1000 bp dsRNA with recombinant human Dicer (Stratagene, La Jolla, CA), according to the manufacturers' instructions. The residual uncleaved dsRNA was removed from the esiRNA preparation using Microcon 100 microconcentrators (Millipore, Billerica, MA).

The *Drosophila* RNAi library has been previously described[15]. Genes involved in RNAi were identified using an RNAi of RNAi approach[13,14]. dsRNA from the RNAi library was used to knockdown specific *Drosophila* mRNAs (primary RNAi). RNAi function was assessed using secondary RNAi of a GFP–Relish fusion. For the genome-wide RNAi screen, approximately 2 μg of library dsRNA were aliquoted in 100 μl of S2 medium and 4 × 10⁴ S2 GFP–Relish cells were added in an additional 100 μl of medium to 96-well microplates. At day 4, the cells were split at the same approximate initial density and 2 μg of library dsRNA and 2 μg of dsRNA targeting the *GFP–Relish* mRNA were added into glass-bottomed 96-well microplates (BD Biosciences Pharmingen, San Diego, CA). At day 7 *GFP–Relish* expression was induced by the addition of 500 μM CuSO₄. At day 8, the cells were washed, fixed with 3.7% formaldehyde and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). GFP expression was evaluated visually under a Leica IMRB microscope. Positive candidates in the initial screen were confirmed using the same approach, but GFP expression was evaluated using a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson, Franklin Lakes, NJ).

To confirm the identity of the dsRNA in the RNAi library, the templates for *in vitro* transcription were cloned and sequenced in pCRII-Topo vectors (Invitrogen). These plasmids were used as templates for *in vitro* transcription and the assay was repeated with similar results (data not shown). Positive candidates were further confirmed with the same approach using three additional secondary RNAi assays: RNAi against a LAMP–GFP fusion in S2 cells stably expressing this fusion protein; RNAi against the anti-apoptotic factor dIAP1 in wild-type S2 cells using cell viability as a readout; and RNAi against firefly luciferase after transient transfection of wild-type S2 cells with firefly and *Renilla luciferase* expression vectors.

RNAi in *C. elegans*. All *C. elegans* experiments were performed with wild-type N2 worms at 20 °C. RNAi was induced by feeding the nematodes with bacteria expressing dsRNA. The RNAi constructs were obtained from the Ahringer RNAi library[41]. HT115 bacteria transformed with RNAi vectors expressing dsRNA of the genes of interest were grown at 37 °C in LB with 10 μg mL⁻¹ tetracycline and 50 μg mL⁻¹ carbenicillin, then seeded onto nematode growth media-carbenicillin plates and supplemented with two different concentrations of IPTG (0.2 and 2 μM). The presence of the insert of each clone of interest from the RNAi library was verified by PCR analysis with T7 primer.

Feeding of RNAi bacteria to worms was carried out as previously described[42,43]. Briefly, young adult worms were transferred to candidate RNAi bacteria in two different concentrations of IPTG. L2–L3 progeny were recovered and plated in *unc-52* RNAi bacteria plates with 2.0 μM IPTG. Two days later, the unc phenotype was scored.

Immunofluorescence microscopy. dsRNA and DNA were fluorescently labelled using the *Silencer* siRNA labelling kit Cy3 (Ambion, Austin, TX). Unincorporated dye was removed using HS200 gel filtration columns (Pharmacia, Piscataway, NJ). Labelling of dsRNA was verified by a decreased electrophoretic mobility on agarose gel of the labelled dsRNA as compared with unlabelled dsRNA (see Supplementary Information, Fig. S1b). S2 cells were incubated for the indicated times with labelded dsRNA, washed with PBS and deposited on Superfrost Plus Gold slides (Fisher Scientific, Pittsburg, PA) for immunofluorescence microscopy. Cells were fixed for 10 min in 4% formaldehyde (Sigma). Actin was visualized with oregon green 488-coupled phalloidin (Molecular Probes, Eugene, OR). Cells were mounted using Vectashield with DAPI (Vector, Burlingame, CA) as a nuclear counterstain. Images were captured on an Olympus IX70 microscope driven by DeltaVision software (Applied Precision, Issaquah, WA). Optical sections were deconvolved using the same software and flattened into a two-dimensional projection for presentation. All the images were then imported to and processed in Adobe Photoshop and Adobe Illustrator.

Incorporation of radiolabelled nucleic acids. Uniformly labelled dsRNA was generated by *in vitro* transcription in the presence of α-³²P-UTP. DNA was terminally labelled using polynucleotide kinase and γ-³²P-ATP. The same amount of counts of radiolabelled dsRNA or DNA were added to S2 cells after serum starvation for 1 h. At different times, the cells were washed with PBS and lysed in hypotonic buffer (10 mM HEPES at pH 7.3, 6 mM β-mercaptoethanol, complete protease inhibitor (Roche, Indianapolis, IN) and 0.5 units ml⁻¹ RNasin (Promega). After ultracentrifugation at 100.000*g* for 60 min, the amount of radioactivity in the soluble and insoluble fraction was measured in a scintillation counter (Beckman Coulter, Fullerton, CA). The pellet was resuspended in cold PBS and sonicated at low level for 5 sec.

The efficiency of separation of membrane bound and cytoplasmic fractions over pellet and supernatant was assessed by western blotting using antibodies directed against Syntaxin as marker for plasma membrane, Lava 1 (Golgi), Rab5 (early endosomes), cathepsin-L (lysosome) and tubulin (cytoplasm). Antibodies were kindly provided by the laboratory of G. Davis at University of California San Francisco.

Note: Supplementary Information is available on the Nature Cell Biology website.

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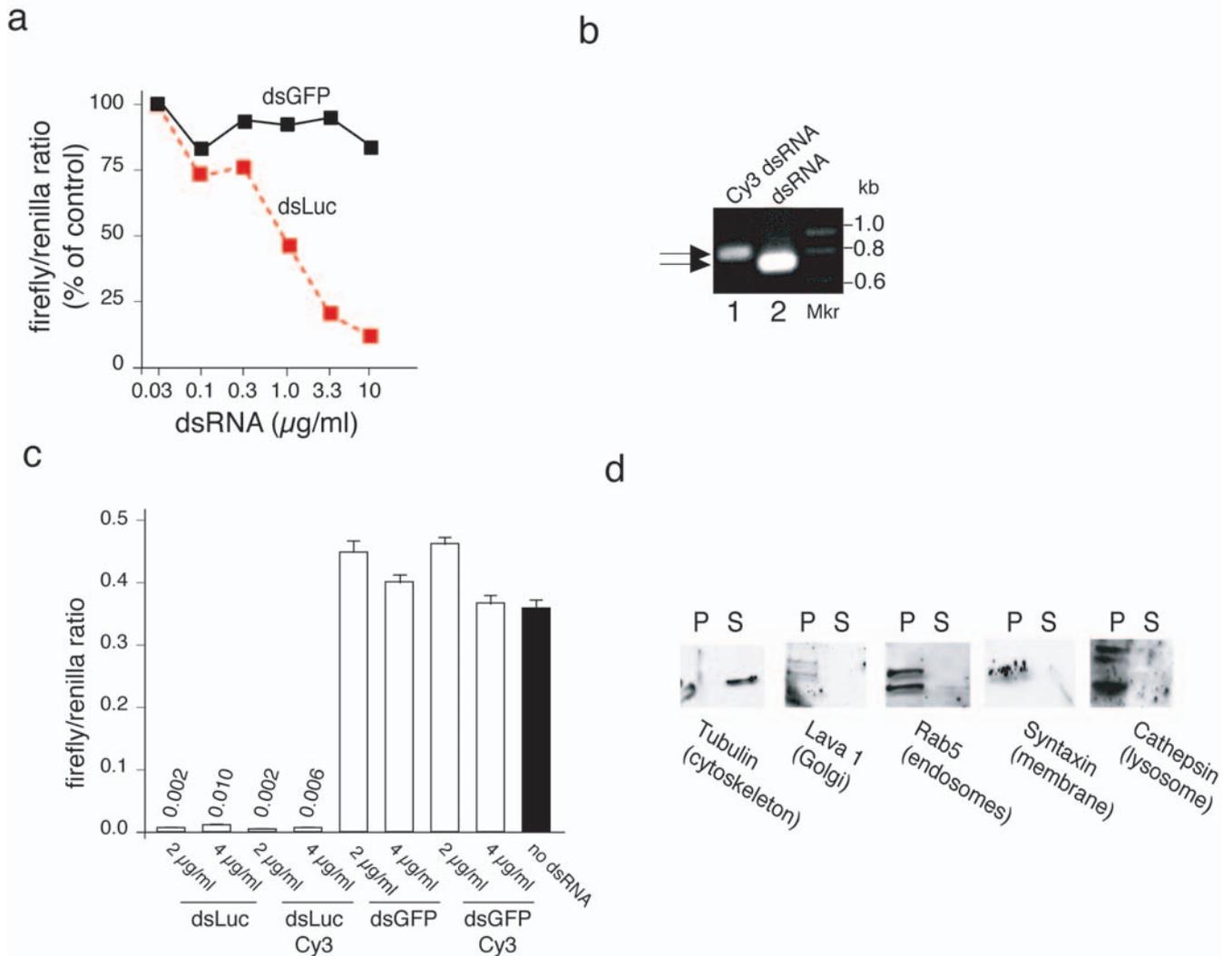
Figure S1 (a) Concentration dependent RNAi in *Drosophila* S2 cells. S2 cells were transfected with expression plasmids encoding firefly and *Renilla* luciferase. Different concentrations of specific dsRNA targeting firefly luciferase was added to the culture supernatant at one day after transfection. Luciferase activity was monitored 48 hours later. Firefly over *Renilla* luciferase ratios were expressed as percentage of control transfection without dsRNA treatment. **(b)** Cy3 labeled dsRNA is taken up by *Drosophila* S2 cells and functional in RNAi. Analysis of unlabeled and Cy3-labeled dsRNA on agarose gel. Reduced mobility of Cy3-labeled dsRNA indicates that all dsRNA molecules in the preparation are labeled. **(c)** Functionality of Cy3-labeled dsRNA. S2 cells were transfected with plasmids encoding firefly and *Renilla* luciferase. 24 hours after transfection, the cells were soaked in medium containing unlabeled or Cy3-labeled luciferase specific dsRNA with the indicated concentrations, or in non-specific dsRNA (targeting GFP). Cy3-labeled dsRNA is functional in RNAi, although it is less efficient than unlabeled dsRNA in suppressing luciferase activity. Average firefly/*Renilla* ratio is indicated on top of each bar. The asterisk indicate *P*<0.01 respect to no dsRNA control. **(d)** Characterization of pellet and supernatant fraction after ultracentrifugation of S2 cell lysates at 100.000xg. Western blot analysis of pellet and supernatant indicate an enrichment of membranes, endocytic vesicles and golgi complex in the pellet (P) and the presence of a cytoskeletal marker in supernatant (S) fraction.
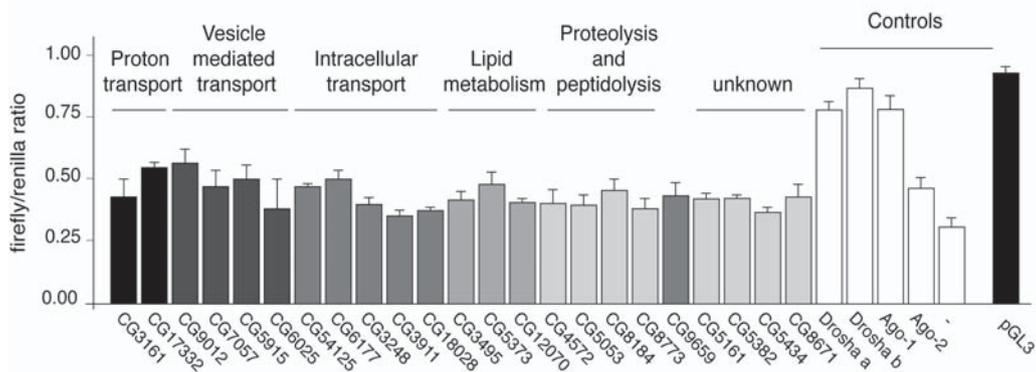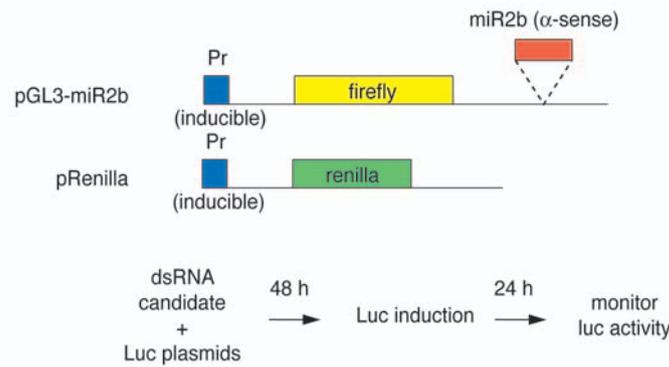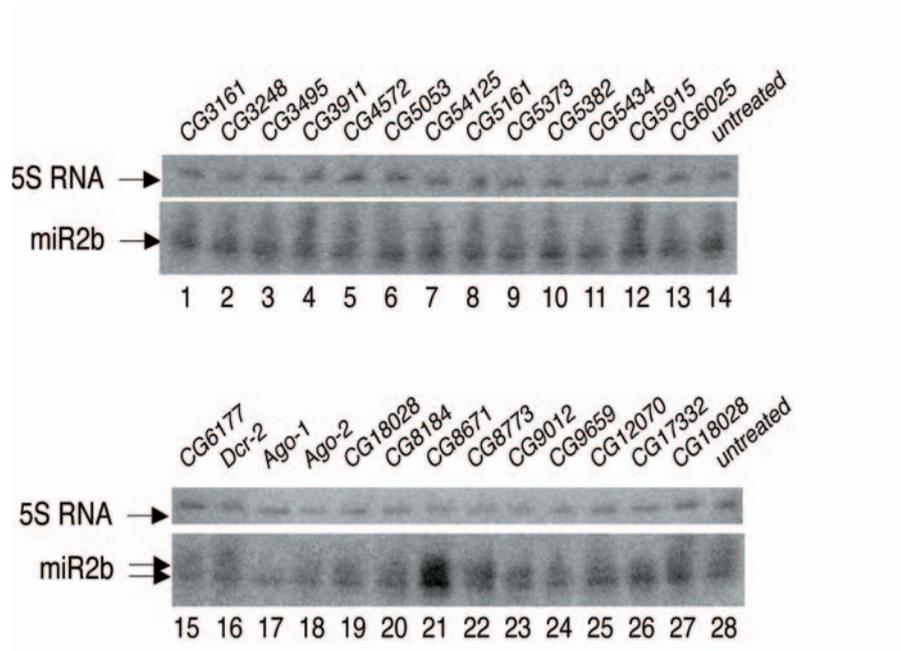
Figure S2 Genes identified in screen for novel components in the RNAi pathway do not affect miRNA biogenesis. **(a)** Detection of miR2b in cells treated with dsRNA targeting the indicated genes for 4 days. Mature miRNA and 5S rRNA are indicated by arrows. None of the candidate RNAi genes affect miRNA biogenesis. **(b)** Assay for miRNA-mediated RNA silencing. Schematic representation of genes encoding *Renilla* and firefly luciferase. The firefly luciferase mRNA contains two copies of a sequence that is perfectly complementary to mature miR2b inserted at the 3′UTR. S2 cells were co-transfected with dsRNA specific for candidate gene and the expression plasmids. Forty eight hours after transfection luciferase expression was induced by adding $CuSO_4$ to the culture supernatant and luciferase activity was monitored after another 24 hour incubation. Controls include treatment of cells with dsRNA targeting genes known to be involved in the miRNA pathway, *Drosha* (two different, non-overlapping dsRNA preparations) and *Argonaute 1*. The bar labeled pGL3 indicates expression of firefly luciferase from a control plasmid without miR2b complementary sequences. The asterisk indicate $P<0.01$ with respect to pGL3 control.

Fig. S3
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Fig. S3 (cont')
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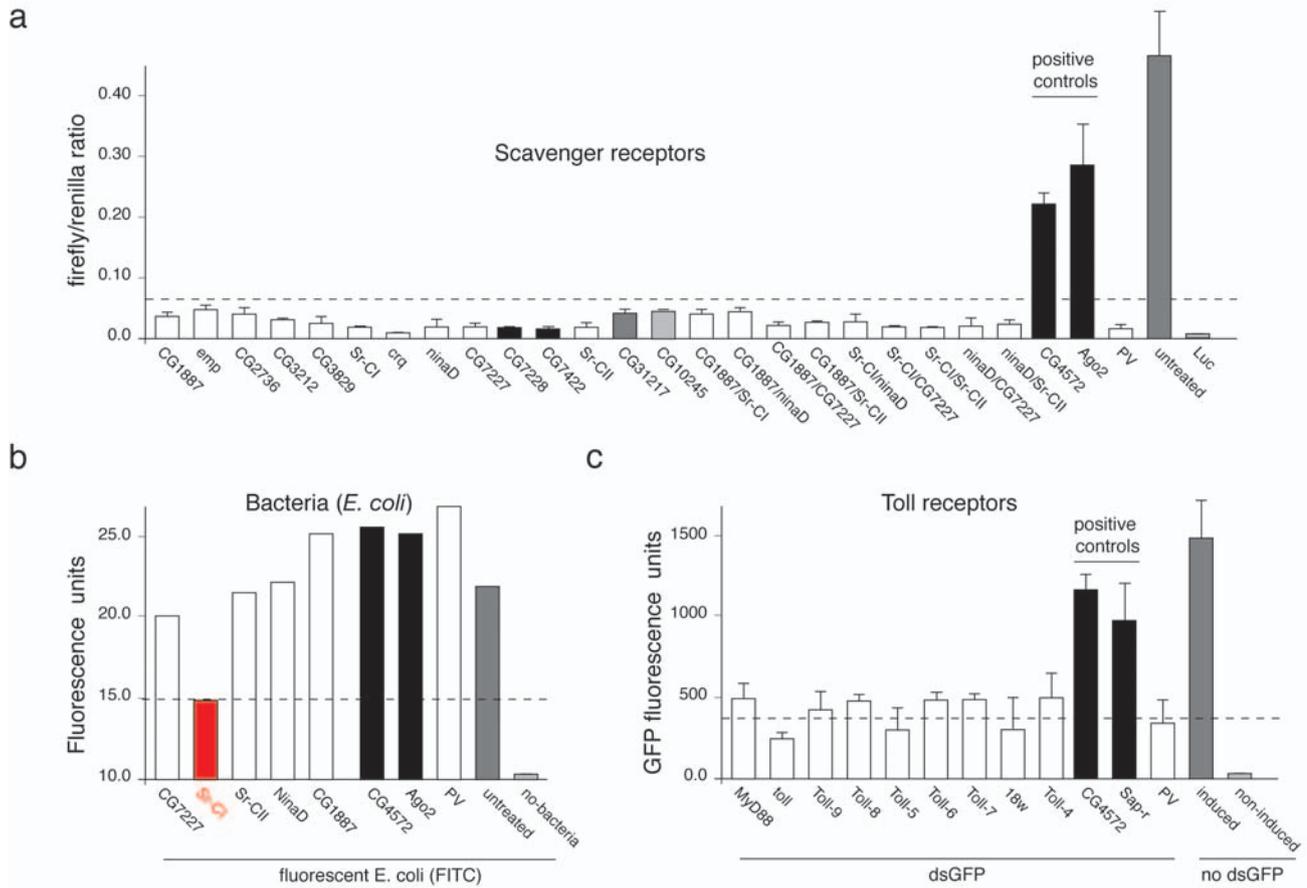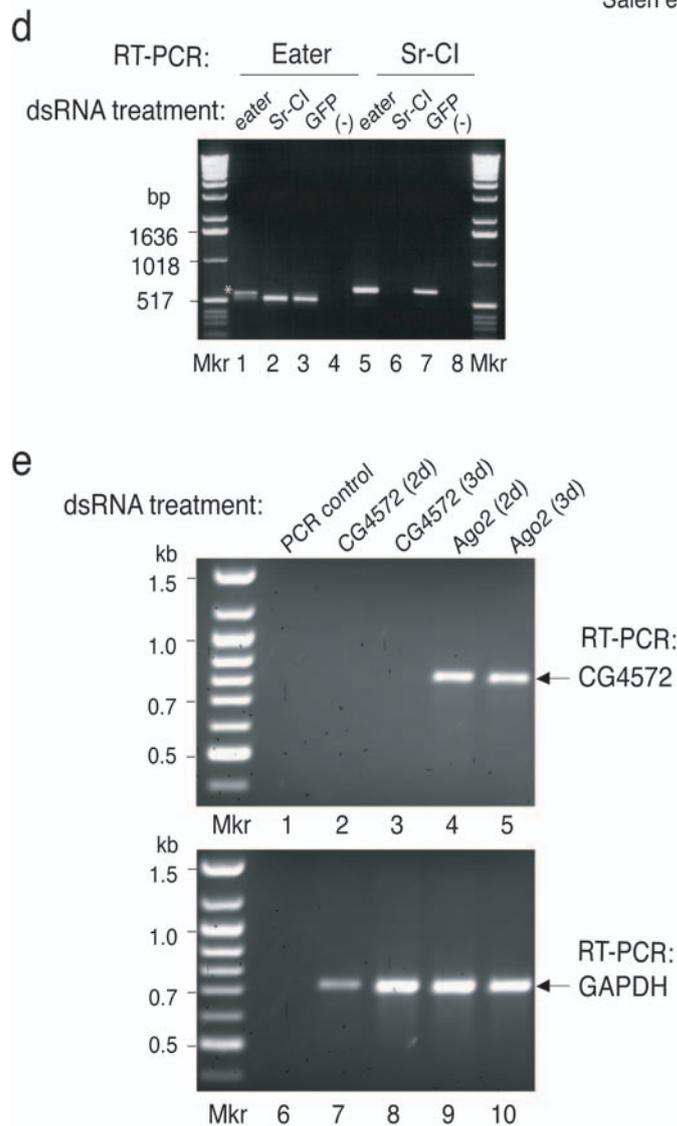
Figure S3 Knockdown of Pattern-Recognition receptors fails to inhibit RNA silencing **(a)** Knockdown of scanvenger receptors does not impair silencing of luciferase. S2 cells were cotransfected with dsRNA specific for different pattern recognition receptors and firefly and *Renilla* luciferase plasmids. After two days, cells were fed with dsRNA specific for firefly luciferase. Luciferase expression was induced by addition of CuSO4. Data are presented as the ratio between firefly and *Renilla* activity. Poliovirus (PV) specific dsRNA was used as a negative control. Positive controls include two candidates identified in our screen to be involved in RNAi. The asterisk indicate *P*<0.01 with respect to untreated control. **(b)** Knockdown of scavenger receptor *Sr-CI* impairs phagocytosis of bacteria in S2 cells. S2 cells were fed with dsRNA specific for scavenger receptors for three days. Cells were then serum starved for 90 min, incubated with FITC-labelled *E. coli* for 20 min, washed with PBS and fluorescence was measured by flow cytometry. As described previously (M. Ramet *et al.* Immunity 2001 15: 1027-1038), phagocytosis of *E. coli* was inhibited after Sr-CI knockdown. This experiment serves as a positive control for knockdown of Sr-CI in a functional assay. **(c)** Knockdown of Toll receptors fails to inhibit RNA silencing of luciferase expression. GFP:Relish S2 cells were treated with dsRNA specific for toll family members and three days later, cells were soaked in dsRNA targeting GFP. After induction of GFP expression by addition of CuSO4, GFP expression in cytoplasmic extract was monitored in

a Tecan Safire plate reader. Poliovirus (PV) specific dsRNA was used as a negative control. Positive controls include two candidates identified in our screen to be involved in RNAi. The asterisk indicate *P*<0.01 with respect to no dsGFP control. **(d)** Scavenger receptors mRNA levels are reduced after RNAi. S2 cells were treated with dsRNA specific for the scavenger receptors Eater, and Sr-CI or for GFP as a negative control. After three days, poly-A RNA was extracted, reverse transcribed and amplified using primers for Eater (lanes 1 to 4) or Sr-CI (lanes 5 to 8). Lane 4 and 8 represent negative controls for PCR in the absence of input cDNA. While Sr-CI mRNA is undetectable after dsRNA treatment (lane 6), Eater mRNA quantity was reduced by 70% (lane 1). The band indicated by an asterisk in lane 1 corresponds to the amplification of genomic DNA contamination in the RNA preparation as denoted by the slower mobility of the band since PCR primers are flanking a 55 bp intron and should yield a larger band than that derived from cDNA. **(e)** dsRNA treatment induce mRNA knockdown within 3 days of treatment. S2 cells were fed with dsRNA specific for CG4572 or Ago2. After 2 or 3 days of treatment, poly-A RNA was extracted, reverse transcribed and amplified using primers for CG4572 (lane 1-5) and for internal control GAPDH (lane 6-10). CG4572, a gene identified in our screen to be involved in RNAi, is undetectable after incubation of S2 cells with dsRNA for as little as two days.

Table S1 Scavenger receptor-like genes identified in Drosophila melanogaster		
Gene ID	Symbol	Gene name
CG4099	Sr-CI	Scavenger receptor class C, type I
CG8856	Sr-CII	Scavenger receptor class C, type II
CG31962	Sr-CIII	Scavenger receptor class C, type III
CG3212	Sr-CIV	Scavenger receptor class C, type IV
CG10345		
CG12789		
CG1887		
CG2736		
CG31217		
CG31741		
CG3829		
CG7422		
CG7000		
CG7227		
CG7228		
CG4280	crq	croquemort
CG2727	emp	epithelial membrane protein
CG31783	ninaD	neither inactivation nor afterpotential D
FBgn0082595	eater	