size of CdiB. Proteolytic fragments of CdiA were detected in the growth medium (*5*) but were not growth-inhibitory (fig. S4), indicating that the secreted forms of CdiA are inactive.

High amino acid sequence identity was found between CdiA/CdiB and predicted proteins from uropathogenic *E. coli* (UPEC), including strain 536 (*9*). Complementation analysis indicated that UPEC 536 and four additional UPEC strains contain genes that are functional homologs of *cdiB* (Fig. 3C) and *cdiA* (fig. S8). Bioinformatic analysis showed that *Yersinia pestis* (plague) and *Burkholderia pseudomallei* (melioidosis) also encode possible CdiAB homologs (fig. S7). Filamentous hemagglutinin from *Bordetella pertussis* (whooping cough) appeared more distantly related, sharing sequence identity to CdiA primarily in the N-terminal portion of the protein (fig. S7).

The *cdiAB* homologs in UPEC 536 are present within pathogenicity island II (*10*), but a *cdiI* homolog is not present, nor is it found in the sequenced genome of UPEC CFT073, which also contains a *cdiAB* homolog (*11*). This observation suggests that *cdiAB* expression in UPEC strains would inhibit their growth. Pathogenicity island II in UPEC 536 also contains a pyelonephritis-associated pili (*pap*) operon closely linked to *cdiAB*. Because Pap pili are expressed at the cell surface, we tested the possibility that pili expression might affect CDI because contact between CdiA and the target cell surface could be blocked. *E. coli* K-12 constitutively expressing P pili (*12*) or S pili (*13*) showed resistance to CDI, whereas cells expressing type 1 pili were 1000- to 10,000-fold more sensitive to growth inhibition (Fig. 4). Thus, resistance to CDI conferred by P and S pili involves specific interaction(s) and is not likely to be the result of nonspecific steric hindrance that blocks cell-to-cell contact.

Many UPEC strains contain *fim* (type 1 pili), *pap* (P pili), and *sfa* (S pili) operons (*10*, *11*). The expression of these pili types is normally subject to reversible off/on switching, generating diversity within bacterial populations by a differentiation mechanism (*14*, *15*). Such a mechanism might play a role in the temporal control of the differentiation observed for UPEC strains inside bladder cells, during which the bacteria progress through distinct developmental stages, including a quiescent growth state (*16*). *E. coli* K-12 cells inhibited by CDI appear to be nonviable because of their lack of growth on agar medium. However, CDI-inhibited cells appeared to be viable because they excluded propidium iodide (*5*), a standard criterion for distinguishing viable cells from nonviable cells (*17*). The identification of this sophisticated mechanism in *E. coli,* with possible homologs in a broad range of species, opens the door for exploration of the potential roles of CDI in controlling bacterial development and pathogenesis.

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Supporting Online Material

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Genome-Wide RNAi Screen for Host Factors Required for Intracellular Bacterial Infection

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Most studies of host-pathogen interactions have focused on pathogen-specific virulence determinants. Here, we report a genome-wide RNA interference screen to identify host factors required for intracellular bacterial pathogenesis. Using *Drosophila* cells and the cytosolic pathogen *Listeria monocytogenes*, we identified 305 double-stranded RNAs targeting a wide range of cellular functions that altered *L. monocytogenes* infection. Comparison to a similar screen with *Mycobacterium fortuitum*, a vacuolar pathogen, identified host factors that may play a general role in intracellular pathogenesis and factors that specifically affect access to the cytosol by *L. monocytogenes*.

During bacterial infections, macrophages play a critical role in eliminating engulfed pathogens. However, intracellular bacterial pathogens have evolved varying strategies to avoid elimination by host macrophages (*1*). One strategy used by pathogens, such as *Mycobacterium tuberculosis*, is to modify the phagosomal compartment to allow for vacuolar replication (*2*). Other bacterial pathogens, such as *Listeria monocytogenes*, escape the phagocytic vacuole to enter the host cell cytosol where replication occurs (*3*). Whereas numerous bacterial determinants that facilitate intracellular infection have been characterized from diverse bacterial species (*4*), less is known about the host factors that are exploited or subverted by intracellular bacterial pathogens. Here, we report the results of a genome-wide RNA interference (RNAi) screen conducted in *Drosophila* SL2 cells to identify host factors required for infection by *L. monocytogenes*, a cytosolic pathogen. In addition, we present the results of a comparison to a similar RNAi screen conducted with *Mycobacterium fortuitum*, a vacuolar pathogen (*5*).

Both *Drosophila* and cultured *Drosophila* cells are tractable models for analysis of *L. monocytogenes* pathogenesis (*6*, *7*). We tested the ability of macrophage-like *Drosophila* SL2 cells to support intracellular infection by *L. monocytogenes*. DH-L1039, a green fluorescent protein (GFP)–expressing *L. monocytogenes* strain derived from wild-type 10403S, replicated within *Drosophila* SL2 cells (fig. S1, A and B). In contrast, GFP fluorescence of DH-L1137, a variant lacking the pore-forming cytolysin listeriolysin O (LLO), was punctate in appearance and growth was inefficient in SL2 cells (fig. S1, A and B), consistent with LLO-negative bacteria that remained trapped within phagocytic vacuoles (*3*, *8*). Next, we developed a microscopy-based, high-throughput RNAi screen to identify host factors required for intracellular infection by *L. monocytogenes* (Fig.

1A) (*9*). We confirmed the feasibility of the approach by analyzing the impact of β-COPI double-stranded RNA (dsRNA) treatment on *L. monocytogenes* infection. Interfering with β-COPI expression by RNAi results in a strong defect in *Escherichia coli* phagocytosis (*10*). The impact of blocking *L. monocytogenes* entry by β-COPI dsRNA treatment was clearly detectable by fluorescence microscopy (fig. S1C). For the screen, we used a library of ~21,300 dsRNAs (*11*) that target >95% of annotated genes in the *Drosophila* genome (*12*), and we identified several phenotypes (Fig. 1, B to F): (i) Decreased GFP fluorescence ("down" phenotype). In some instances only a few host cells were infected, but in those cells the infection was as robust as observed in control wells, consistent with a defect in entry (Fig. 1C). In other cases, the percentage of host cells displaying GFP fluorescence appeared to be similar to control wells, yet the intensity of GFP fluorescence per cell was decreased, consistent with a defect in intracellular replication (Fig. 1D). Combinations of these two phenotypes were also observed. (ii) Punctate GFP fluorescence ("spots" phenotype). This phenotype was similar to the phenotype observed during infection by DH-L1137 (fig. S1A) and is consistent with a defect in vacuole escape. However, bacteria in dsRNA-treated wells appeared to be capable of increased replication within the localized clusters (Fig. 1E). (iii) Increased GFP fluorescence ("up" phenotype). The intensity of GFP fluorescence per infected SL2 cell was more robust, consistent with an increase in intracellular replication rate or increased bacterial uptake (Fig. 1F).

From the primary RNAi screen, we identified 358 *Drosophila* genes that potentially affect *L. monocytogenes* infection, including genes coding for ribosome components (61 genes) and proteasome components (25 genes). With the exception of the dsRNAs targeting ribosome and proteasome components [supporting online material (SOM) text], the candidate dsRNAs identified in the primary RNAi screen were retested at least six times and ~70% were confirmed as affecting *L. monocytogenes* infection. Of these dsRNAs, less than 13% were shown to have effects on host cell viability in a previous screen with the use of the same dsRNA library (*11*), suggesting a specific effect on intracellular bacterial infection rather than nonspecific effects on the host cells. The putative host factors required for *L. monocytogenes* infection spanned a wide range of cellular functions (tables S1 and S2). Many of the identified targets resulting in a down phenotype are predicted to be involved in vesicular trafficking (19%), signal transduction (10%), and cytoskeletal organization (10%). Knockdown of many of these targets resulted in a phenotype consistent with an entry defect, including Syx5 (vesicular trafficking) (Fig. 1C), Cdc42 (signal transduction), and Arp2/3 complex members (cytoskeleton). Other targets in these categories had phenotypes consistent with defects in steps beyond entry, such as Tor (target of rapamycin) (signal transduction) (Fig. 1D) and Rab2 (vesicular trafficking) (SOM text). Furthermore, dsRNAs targeting 48 *Drosophila* genes caused an up phenotype (Fig. 2A and table S1). The gene products of these targets are predominantly predicted to be involved in cell cycle (40%) and RNA processing (13%).

To determine whether the identified host factors were specific to *L. monocytogenes* infection or more generally required for intracellular pathogenesis, we compared the results with a similar RNAi-based screen that tested *M. fortuitum* (*5*, *9*). We focused our comparative analysis on candidates that caused decreased infection (Fig. 2, B and C). The majority of dsRNAs that decreased infection by both pathogens target factors that are predicted to be involved in vesicular trafficking or cytoskeleton components (table S3 and Fig. 2C). Many of these may be required for nonspecific uptake of both pathogens, because they were also found to affect phagocytosis of *E. coli* (*5*). However, some host factors appeared to be specific for entry. For example, a knockdown of CG7228 (Pes), a member of the CD36 family of scavenger receptors, reduced entry of both *L. monocytogenes* (fig. S2) and *M. fortuitum*, but had no effect on uptake of *E. coli* or *Staphylococcus aureus* (*5*). In addition, several candidates affecting both pathogens appeared to have roles other than entry (SOM text).

We also identified dsRNAs that decreased infection by one pathogen but not the other. For example, dsRNA targeting CG6121 decreased intracellular infection by *M. fortuitum*, but did not interfere with *L. monocytogenes* infection (Fig. 3). CG6121 (dTip60) is a member of a complex with histone acetyltransfer-

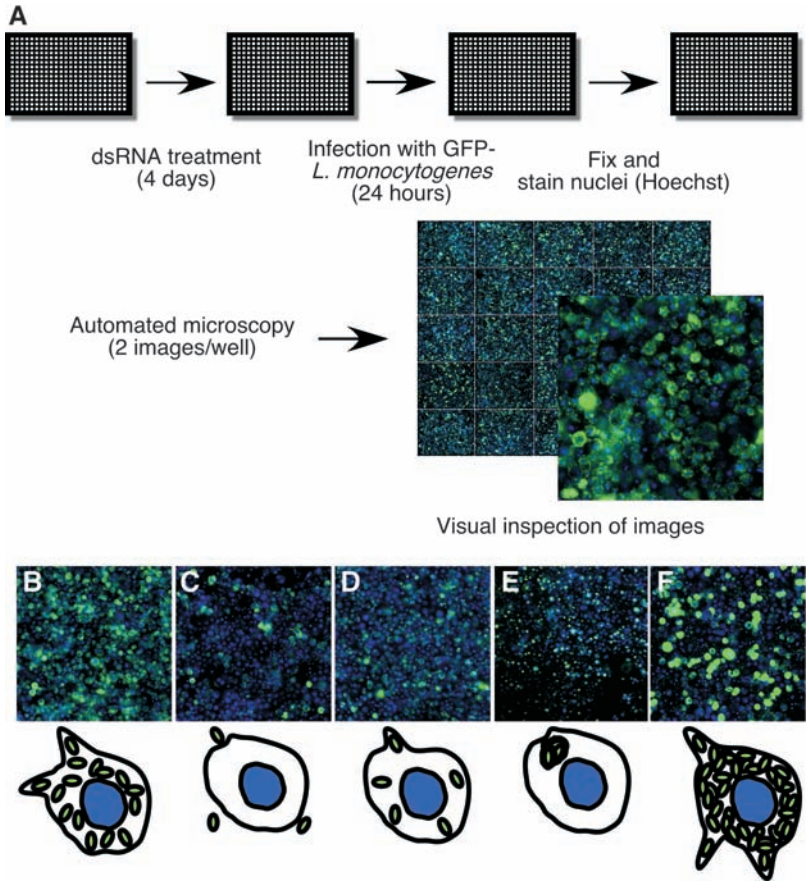

Fig. 1. RNAi screening procedure and phenotypes observed. (**A**) Screen design. [(B) to (F)] Candidates representative of the observed phenotypes. (**B**) No dsRNA (control). (**C**) Syx5: down phenotype in which a decreased percentage of cells expressed GFP. (**D**) Tor: down phenotype in which each cell displayed decreased GFP expression. (**E**) CG5691: spots phenotype, in which GFP expression was clustered within the cell. (**F**) CG3605: up phenotype, in which each cell displayed increased GFP expression. Below each image is a schematic depiction of the corresponding phenotype.

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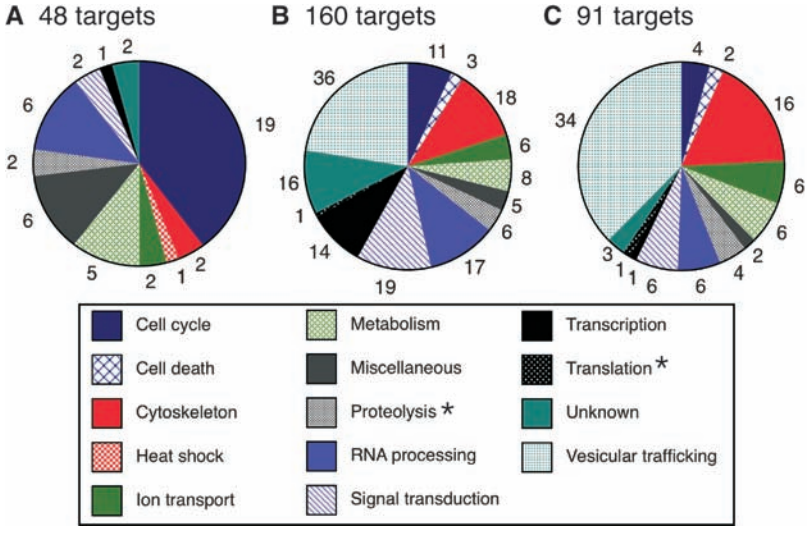

Fig. 2. Functional categories of dsRNA targets identified in *L. monocytogenes* and *M. fortuitum* RNAi screens. [(A) to (C)] Functional categories identified. The numbers surrounding the charts indicate the number of target genes within each functional category. Stars (*) indicate targets other than proteasome or ribosome subunits that are categorized as proteolysis or translation. (**A**) dsRNAs resulting in an up phenotype for *L. monocytogenes*. (**B**) dsRNAs resulting in a down phenotype for *L. monocytogenes*. (**C**) dsRNAs resulting in a down phenotype for both *L. monocytogenes* and *M. fortuitum*.

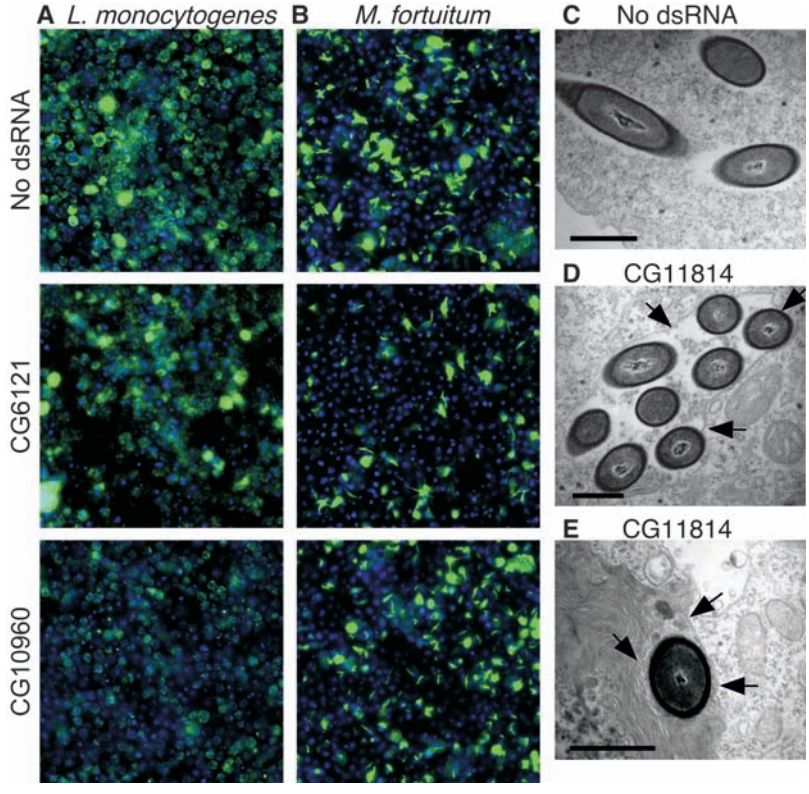

Fig. 3. Representative phenotypes for dsRNAs that uniquely affect *L. monocytogenes* or *M. fortuitum* infection. (**A** and **B**) SL2 cells were untreated (top), treated with CG6121 dsRNA (middle), or treated with CG10960 dsRNA (bottom) and infected with *L. monocytogenes* (A) or *M. fortuitum* (B) expressing GFP (green) (*5*). At 24 hours postinfection (*L. monocytogenes*) or 48 hours postinfection (*M. fortuitum*) cells were fixed and stained with Hoechst dye (blue). Fluorescent images (20×) are representative of infections performed at least six times for each pathogen. (**C** to **E**) Electron micrographs of (C) untreated or [(D) and (E)] CG11814 dsRNA-treated SL2 cells infected with wild-type *L. monocytogenes*. Arrows indicate membranes surrounding bacteria in the CG11814 dsRNA-treated cells. Scale bars, 0.5 μm.

ase activity (*13*). Two other members of the *Drosophila* Tip60 complex, dom and E(Pc), were also identified as affecting *M. fortuitum* infection. Human immunodeficiency virus Tat protein interaction with Tip60 in mammalian cells alters expression of host cell genes (*14*). Thus, it is possible that *M. fortuitum* similarly targets the Tip60 complex to modify the expression of specific host factors. In all, 17 dsRNAs that decreased infection by *M. fortuitum* did not affect *L. monocytogenes* infection. Six dsRNAs that decreased infection by *M. fortuitum* caused an up phenotype for *L. monocytogenes* (table S3). A total of 59 dsRNAs, including those targeting a putative glucose transporter, CG10960, decreased infection by *L. monocytogenes* but did not affect *M. fortuitum* (table S3 and Fig. 3). Ten dsRNAs that caused a down phenotype for *L. monocytogenes* appeared to increase *M. fortuitum* intracellular growth (table S3). Furthermore, all 11 dsRNAs resulting in the spots phenotype for *L. monocytogenes* (table S1) did not detectably affect *M. fortuitum* infection. As confirmed by electron microscopy, the spots phenotype corresponded to a vacuolar escape defect. In untreated SL2 cells, *L. monocytogenes* were found free in the cytosol (Fig. 3C), whereas treatment with CG11814 dsRNA, which caused a spots phenotype, resulted in membrane-bound compartments harboring multiple bacteria (Fig. 3D). CG11814 is predicted to have roles in lysosomal transport. Spots vacuoles appeared to be more permissive for growth of *L. monocytogenes*. Indeed, LLO-negative bacteria replicated two times more in SL2 cells treated with CG11814 dsRNA than in untreated SL2 cells (fig. S3). Of membrane-bound compartments containing one or more bacteria in CG11814 dsRNA-treated cells, 52% were surrounded by multilamellar structures (Fig. 3E) compared with 8% in untreated control cells. Similar structures surround mutants of another cytosolic pathogen, *Shigella flexneri* (*15*). These multilamellar structures result from an attempt of the host cell to control intracellular infection by a process related to autophagy (*15*). Thus, autophagy may represent a defense mechanism that limits *L. monocytogenes* infection (SOM text). Because infection of *Drosophila* cells by intracellular bacterial pathogens is similar to infection of mammalian host cells, it is likely that many homologous mammalian host factors will be conserved in their requirement for intracellular infection.

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Drosophila RNAi Screen Reveals CD36 Family Member Required for Mycobacterial Infection

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Certain pathogens, such as *Mycobacterium tuberculosis*, survive within the hostile intracellular environment of a macrophage. To identify host factors required for mycobacterial entry and survival within macrophages, we performed a genome-wide RNA interference screen in *Drosophila* macrophage-like cells, using *Mycobacterium fortuitum*. We identified factors required for general phagocytosis, as well as those needed specifically for mycobacterial infection. One specific factor, Peste (Pes), is a CD36 family member required for uptake of mycobacteria, but not *Escherichia coli* or *Staphylococcus aureus*. Moreover, mammalian class B scavenger receptors (SRs) conferred uptake of bacteria into nonphagocytic cells, with SR-BI and SR-BII uniquely mediating uptake of *M. fortuitum*, which suggests a conserved role for class B SRs in pattern recognition and innate immunity.

About one-third of the world's population is infected by *M. tuberculosis*, which is responsible for more deaths yearly than any other bacterial pathogen. In addition, other pathogenic mycobacteria, including *M. fortuitum*, are capable of causing infection in humans (*1*). Although macrophages play a central role in host defense, recognizing and destroying pathogens, pathogenic mycobacteria are able to survive within this hostile environment. Mycobacteria can escape phagosome-lysosome fusion (*2*) and grow in a variety of evolutionarily divergent phagocytic cells, including mammalian macrophages, fish monocytes (*3*), fly hemocytes (*4*), and amoeba (*5*). Thus, mycobacteria appear to target evolutionarily conserved molecules for intracellular survival and growth. Although several factors involved in phagosome maturation arrest have been studied (*6*), there has been no systematic, genetic approach for identifying host factors required for mycobacterial survival. Here we describe a model of infection using *Drosophila* S2 cells, a macrophage-like cell line (*7*–*9*) that is readily amenable to RNA interference (RNAi). This allowed us to conduct a systematic functional genomic screen to identify host factors required for uptake and growth of mycobacteria.

M. fortuitum has several properties that make it a useful model mycobacterium. Like *M. tuberculosis*, it restricts interferon-γ (IFN-γ)–induced nitric oxide production and limits phagosome fusion with lysosomes (*10*), suggesting it has virulence properties in common with other mycobacteria. In addition, *M. fortuitum* infects *Diptera* in nature (*11*), so flies may have innate mechanisms to combat infection. Practically, *M. fortuitum* grows relatively rapidly at 25°C, the temperature at which S2 cells grow, thus facilitating development of a robust assay of intracellular growth. To detect intracellular growth, we tested constructs in which green fluorescent protein (GFP) expression is under control of the *map24* and *map49* promoters that are induced when the fish pathogen *Mycobacterium marinum* infects macrophages (*12*). We found that these promoters could also be used to efficiently detect intracellular growth of *M. fortuitum* (fig. S1). By 24 hours after infection of S2 cells, expression of *map24* and *map49* was induced (figs. S1 and S2; Fig. 1A).

In mammalian cells, recruitment of the Arp2/3 complex is required for phagocytosis (*13*), whereas expression of a dominant-negative version of Rab5 causes internalized *Mycobacterium avium* to be delivered to the lysosome (*14*). Thus, we reasoned that double-stranded RNAs (dsRNAs) targeting

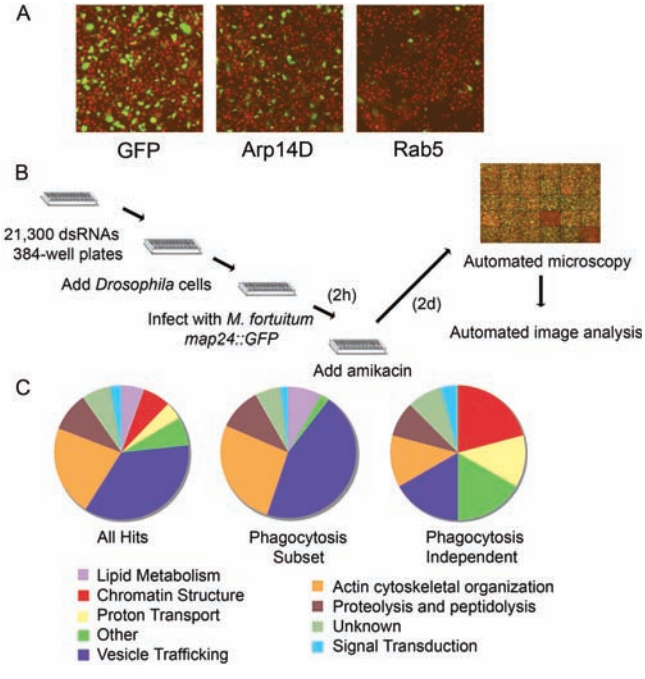

Fig. 1. Host factors required for *M. fortuitum* infection identified by RNAi. After treatment with dsRNAs, S2 cells were infected with *M. fortuitum map24::GFP*. (**A**) dsRNA targeting Arp14D or Rab5 decreased infection as compared to controls treated with dsRNA targeting GFP. (**B**) This assay was used as the basis of a genomewide screen (*15*). A composite image of 35 wells is shown. (**C**) Pie charts based upon GeneOntology (GO) index biological function show the categories of host factors that reproducibly decreased infection after 3 days of dsRNA treatment, as well as the subsets that are required for phagocytosis and those that are apparently phagocytosis independent.

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