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Drosophila RNAi Screen Reveals CD36 Family Member Required for Mycobacterial Infection

Jennifer A. Philips,[1]* Eric J. Rubin,[3] Norbert Perrimon[1,2]*

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

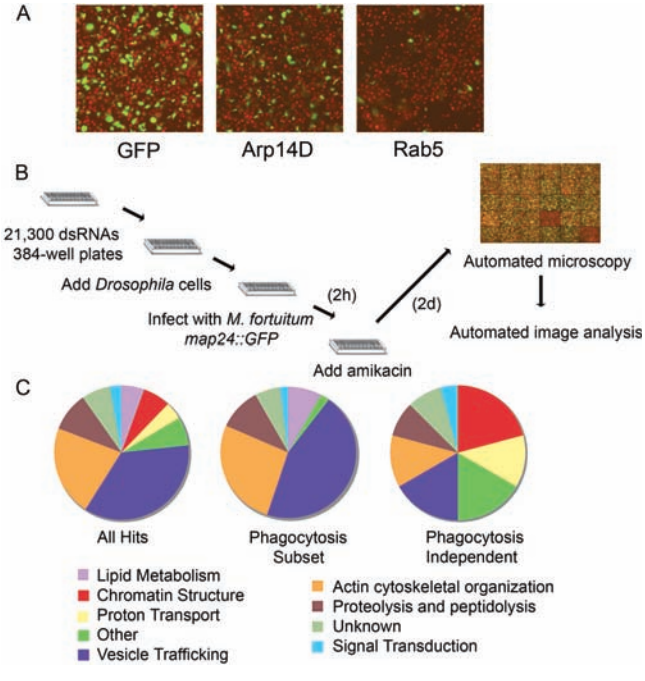

Fig. 1. Host factors required for *M. fortuitum* infection identified by RNAi. After treatment with dsRNAs, S2 cells were infected with *M. fortuitum map24::GFP*. (**A**) dsRNA targeting Arp14D or Rab5 decreased infection as compared to controls treated with dsRNA targeting GFP. (**B**) This assay was 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.

[1]Department of Genetics, [2]Howard Hughes Medical Institute, Harvard Medical School, 77 Louis Pasteur Avenue, Boston, MA 02115, USA. [3]Department of Immunology and Infectious Disease, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA.

*To whom correspondence should be addressed. E-mail: jphilips@partners.org (J.A.P.), perrimon@receptor.med.harvard.edu (N.P.)

Arp14D and Rab5 would decrease infection in *Drosophila* cells. Indeed, we found that these dsRNAs blocked intracellular growth of *M. fortuitum* (Fig. 1A). Using this assay, we performed a genomewide RNAi screen in which each unique dsRNA was tested for its ability to disrupt infection on the basis of visual inspection and automated image analysis (*15*) (Fig. 1B). We found six out of seven Arp2/3 components, five out of seven COPI components, and all six alleles of actin, suggesting a false-negative rate of less than 20%. Upon repeat testing of the candidates identified in the primary screen, 86 dsRNAs decreased infection by 2 SDs ($P < 0.05$; tables S1 and S3). These dsRNAs target genes involved in basic cellular processes, the largest categories of which are predicted to have a role in vesicle trafficking and actin cytoskeletal organization (Fig. 1C).

Because GFP expression requires internalization of bacteria, we expected to find dsRNAs that block general phagocytosis. Indeed, 54 dsRNAs caused a significant decrease in phagocytosis, as measured by the uptake of fluorescent *Escherichia coli* ($P < 0.01$; table S2 and fig. S3). About two-thirds of these dsRNAs target factors involved in the actin cytoskeleton and vesicle trafficking (Fig. 1C). Nearly all of the actin cytoskeletal components that affected *M. fortuitum* infection appeared to be generally required for phagocytosis, including Cdc42, the Arp2/3 complex, actin capping proteins, and cofilin, all molecules previously implicated in phagocytosis (*13*). Most vesicle trafficking genes also affected phagocytosis, with exceptions such as CG1515 (predicted to have SNAP receptor activity) and Rab2.

However, the requirement for many genes for infection could not be explained by their role in phagocytosis. For example, dsRNA that targets *chickadee* (*chic*), which encodes a Profilin, caused a small increase in phagocytosis (118% of GFP-treated well; $P < 0.05$), similar to what has been reported in hemocytes heterozygous for *chic* ($chic^{01320}/+$) (*9*). Hence, Chic is unlikely to act at the level of uptake and may play a unique role in mycobacterial infection. Some dsRNAs, such as those that target Rab5 and Rac2, caused a mild defect in phagocytosis relative to their severe defect in the *M. fortuitum* infection, indicating that they may have additional roles later in infection. Finally, some categories of host factors were not required for bacterial uptake in general but were nonetheless needed for infection with *M. fortuitum*. These include components of the vacuolar adenosine triphosphatase and chromatin factors, along with many that do not fit into distinct categories (Fig. 1C).

We further characterized one host factor that appeared to be specifically required for mycobacterial uptake. The dsRNA that targeted CG7228, a member of the CD36 family of scavenger receptors (SRs), blocked infection by *M. fortuitum* (Fig. 2A), but appeared to be dispensable for phagocytosis in general. dSR-CI, a class C scavenger receptor, makes a small contribution toward the uptake of *E. coli* and *Staphylococcus aureus* in S2 cells (*8*), but we could not detect a similar contribution from CG7228 (Fig. 2B). However, CG7228 was required for uptake of *Mycobacterium smegmatis*, as were more general phagocytosis factors (Fig. 2C). In addition, CG7228 is required for uptake of *Listeria monocytogenes* (*16*). That CG7228 is required for infection with *M. fortuitum* and for uptake of *M. smegmatis* and *L. monocytogenes*, but makes no contribution toward the uptake of *E. coli* or *S. aureus*, suggests that it functions in pattern recognition, detecting some component of *Mycobacteria* and *Listeria*. On the basis of its role in bacterial infection, we have named it *peste* (*pes*).

To determine whether Pes was sufficient to confer uptake of mycobacteria, we expressed it in human embryonic kidney 293 (HEK293) cells that are refractory to infection with *M. fortuitum*. HEK293 cells transfected with Pes could be infected with *M. fortuitum* (Fig. 3A), showing that the *Drosophila* SR can mediate uptake of mycobacteria in human cells. Pes also caused a small increase in the uptake of *E. coli* and *S. aureus* when transfected in HEK293 cells (Fig. 3, B and C), although it did not seem to be required for their uptake in S2 cells. This apparent discrepancy may be explained by genetic redundancy provided by PGRP-Lc (*7*), dSR-CI (*8*), and potentially other SRs that mediate uptake of *E. coli* and *S. aureus* in S2 cells.

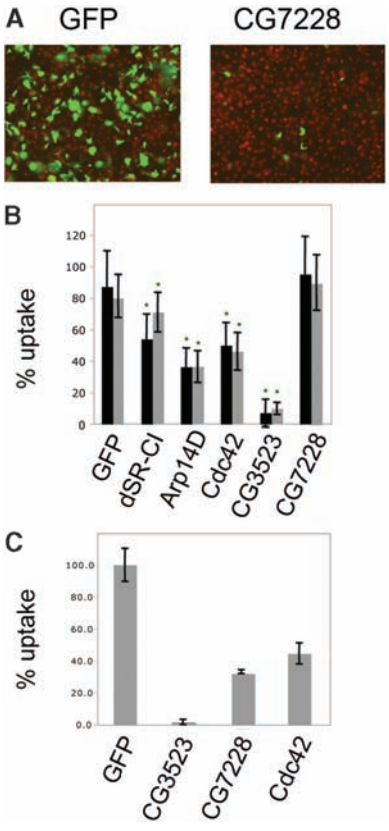

Fig. 2. CG7228 is required in S2 cells for infection by *M. fortuitum* and uptake of *M. smegmatis* but not *E. coli* or *S. aureus*. (**A**) dsRNA against CG7228 blocked infection by *M. fortuitum* as compared to controls treated with dsRNA against GFP. (**B**) Uptake of fluorescein isothiocyanate (FITC)–conjugated *E. coli* (black bars) and *S. aureus* (gray bars) is blocked by dsRNA toward dSR-CI, Arp14D, Cdc42, and CG3523, but not CG7228. Data show the percent uptake as compared to that in untreated cells (fig. S3). The mean ± SD of six experiments is shown in which each dsRNA was tested in triplicate (*$P < 0.05$ compared to GFP-treated wells, Students *t* test.) (**C**) Antibiotic protection experiments demonstrate the role of CG7228, CG3523, and Cdc42 in the uptake of *M. smegmatis*. Data are normalized to GFP; the mean ± SD is shown from a representative experiment performed in triplicate.

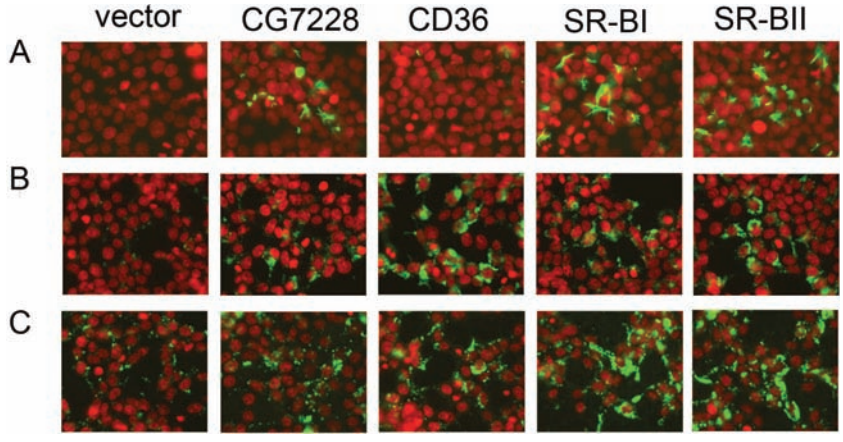

Fig. 3. Class B SRs mediate uptake of *M. fortuitum*, *E. coli*, and *S. aureus*. HEK293 cells transfected with vector (pcDNA3.1) or expression constructs for CG7228 (Pes), CD36, SR-BI, or SR-BII were (**A**) infected with *M. fortuitum map24::GFP* or (**B**) incubated with FITC–*E. coli* or (**C**) FITC–*S. aureus*. In (A) green represents GFP expression from the *map24* promoter, whereas in (B) and (C) green shows the FITC signal from internalized bacteria. Nuclei are stained red. Magnification, ×40.

Whereas in *Drosophila* there are more than 10 class B SRs, in humans and mice there are four—CD36, SR-BI, its splice isoform SR-BII, and LIMP-II. We tested the ability of these molecules to mediate infection with *M. fortuitum* and found that human SR-BI and SR-BII (*17*) resulted in an even greater level of infection than Pes when transfected in HEK293 cells. In contrast, expression of murine CD36 caused little, if any, increase in infection with *M. fortuitum* (Fig. 3A). However, cells transfected with CD36 were capable of taking up *E. coli*, and to a lesser extent *S. aureus*. SR-BI and SR-BII seemed more indiscriminate in terms of their bacterial recognition, as they were able to mediate uptake of all bacteria tested (Fig. 3, B and C). Whereas SR-BI and SR-BII have been extensively studied for their role in cholesterol transport in liver and steroidogenic tissue, their role in macrophages is less clear (*18*). We suggest that these receptors may be important in host defense against a broad range of bacteria, including *M. fortuitum*, *E. coli*, and *S. aureus*, and could play a role in the nonopsonic uptake of *M. tuberculosis*. Consistent with this idea, fucoidin, a nonspecific inhibitor that blocks both class A SRs and SR-BI (*19*), interferes with binding and uptake of *M. tuberculosis* in monocyte-derived macrophages (*20*). CD36 may play a partially overlapping role in recognizing bacteria, but is unable to mediate uptake of *M. fortuitum*. Because CD36 augments Toll-like receptor 2 (TLR2) signaling in response to a subset of TLR2 ligands (*21*), we speculate that CD36, SR-BI, and SR-BII serve partially overlapping roles in both bacterial uptake and TLR signaling, which may represent an evolutionarily conserved strategy in host defense, linking phagocytosis to antimicrobial signaling.

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Effects of Telomerase and Telomere Length on Epidermal Stem Cell Behavior

Ignacio Flores, María L. Cayuela, María A. Blasco*

A key process in organ homeostasis is the mobilization of stem cells out of their niches. We show through analysis of mouse models that telomere length, as well as the catalytic component of telomerase, Tert, are critical determinants in the mobilization of epidermal stem cells. Telomere shortening inhibited mobilization of stem cells out of their niche, impaired hair growth, and resulted in suppression of stem cell proliferative capacity in vitro. In contrast, Tert overexpression in the absence of changes in telomere length promoted stem cell mobilization, hair growth, and stem cell proliferation in vitro. The effects of telomeres and telomerase on stem cell biology anticipate their role in cancer and aging.

Tumor formation and aging are associated with alterations in the number or functional competence of tissue stem cells (*1*–*3*). Both processes have also been linked to alterations at the telomere (*4*–*7*), the nucleoprotein structure that caps chromosome ends (*8*, *9*), and to changes in the activity of telomerase, the reverse transcriptase that elongates telomeres (*10*, *11*). The catalytic subunit of telomerase (Tert) is expressed in the stem cell compartment of several adult tissues (*12*), although telomerase levels in these tissues are not sufficient to prevent progressive telomere shortening with age (*10*). Reduced telomerase activity due to mutations in telomerase components in the human diseases dyskeratosis congenita and aplastic anemia (*10*) leads to accelerated telomere shortening and premature loss of tissue regeneration, which suggests that telomerase levels in the adult organism are rate limiting and influence organ homeostasis. Further evidence for a role of telomerase and telomere length in organ homeostasis comes from the study of telomerase-deficient mice (*Terc*$^{-/-}$ mice), which show premature aging and a decreased proliferative potential of adult stem cell populations (*13*–*15*).

To investigate the role of telomerase and telomere length on stem cell biology, we used mouse models with altered telomerase activity (*16*). We compared epidermal stem cell number in different generations of telomerase-deficient mice, which have telomeres ranging from slightly reduced in length (first generation, G1 *Terc*$^{-/-}$ mice) to critically short (third generation, G3 *Terc*$^{-/-}$ mice) (*17*, *18*). Because telomerase activity per se is not required for cell proliferation when telomeres are long, the study of G1 and G3 *Terc*$^{-/-}$ mice allowed us to assess independently the impact of telomerase deficiency and telomere length on epidermal stem cells. To visualize epidermal stem cells, we used a labeling technique previously shown to mark self-renewing and multipotent epidermal cells, the so-called "label-retaining cells" (LRCs) (*16*, *19*) (Fig. 1A). Confocal microscopy revealed that LRCs are enriched at the bulge area of the hair follicle in *Terc*$^{+/+}$ (wild-type) mice, which corresponds to the niche of epithelial stem cells (Fig. 1B, fig. S1A, and

Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Centre (CNIO), Melchor Fernández Almagro 3, Madrid E-28029, Spain.

*To whom correspondence should be addressed. E-mail: mblasco@cnio.es