

Review

Illuminating the host – How RNAi screens shed light on host-pathogen interactions

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Over millions of years pathogens have coevolved with their respective hosts utilizing host cell functions for survival and replication. Despite remarkable progress in developing antibiotics and vaccination strategies in the last century, infectious diseases still remain a severe threat to human health. Meanwhile, genomic research offers a new era of data-generating platforms that will dramatically enhance our knowledge of pathogens and the diseases they cause. Improvements in gene knockdown studies by RNA interference (RNAi) combined with recent developments in instrumentation and image analysis enable the use of high-throughput screening approaches to elucidate host gene functions exploited by pathogens. Although only a few RNAi-based screens focusing on host genes have been reported so far, these studies have already uncovered hundreds of genes not previously known to be involved in pathogen infection. This review describes recent progress in RNAi screening approaches, highlighting both the limitations and the tremendous potential of RNAi-based screens for the identification of essential host cell factors during infection.

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1 Introduction

Pathogen infections represent a major health risk causing millions of deaths each year. By exploiting host cellular machineries, pathogens have evolved to master successful host cell infection and cell-to-cell spread. Most of the host cell proteins utilized by pathogens, however, have not been identified yet. After sequencing the human genome [1] a new era in investigating biological processes and gene regulations has begun, enabling the elucidation of cel-

lular gene functions involved in pathogen infections. RNA interference (RNAi) has been widely used to study loss-of-function phenotypes by specifically down-modulating gene expression. RNAi was first described in plants as post-transcriptional gene silencing [2], but also observed in fungi and worms [3, 4] and later identified in trypanosomes [5] and in the nematode *Caenorhabditis elegans* [6], when the introduction of long double-stranded RNA (dsRNA) led to the degradation of homologous mRNA. Further interest in RNAi was raised by the observation that gene silencing also occurred in mammalian cells [7]. RNAi has been identified as an evolutionarily conserved process in which long dsRNAs are cleaved into short RNA duplexes of 21–23 nucleotides in length, termed small interfering RNAs (siRNAs), a process mediated by the RNase III-like enzyme Dicer. Processed siRNAs are directed to the RNA-induced silencing complex (RISC) where one strand of the siRNA serves as a guide sequence for the recognition of the homologous target mRNA, resulting in mRNA cleavage (for further information

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Abbreviations: ER, endoplasmic reticulum; HCV, hepatitis C virus; HIV, human immunodeficiency virus; mRNA, messenger RNA; RNAi, RNA interference; siRNA, small interfering RNA; VSV-G, vesicular stomatitis virus G-protein; WNV, West Nile virus

see [8]). The observation that RNAi can be initiated by the introduction of chemically synthesized siRNAs revolutionized biomedical research and therapy. In recent years researchers started to investigate the regulation of gene expression using siRNAs and explored the potential therapeutic use of RNAi. For a systematic analysis of gene functions in cells at a genome-wide scale, platforms for high-throughput screening approaches had to be developed. So far, several genome-wide siRNA-based screens have been performed, but only a few studies investigating gene functions during pathogen infections are known to date.

2 RNAi screens to identify host genes involved in parasitic infections

So far, reports on the use of RNAi to identify host factors that influence parasitic infections are restricted to a small number of studies, in which specific groups of genes were targeted. These focused on infection by *Plasmodium*, the causative agent of malaria, a parasite whose complex life cycle involves two obligate hosts: the invertebrate host and the vertebrate host (reviewed in [9, 10]).

2.1 The invertebrate host

Female *Anopheles* mosquitoes are the vectors of malaria transmission and *Plasmodium*'s invertebrate host, where the sexual replication of the parasite occurs. Although no large-scale RNAi screens of *Anopheles* have been reported, a number of RNAi-based studies aimed at identifying factors from this host that are important during *Plasmodium* infection have been carried out and are worth mentioning in the context of this review. Gene silencing through direct injection of dsRNA into adult *Anopheles* mosquitoes was first reported by Blandin *et al.* [11]. This technique was employed to evaluate the role of the anti-microbial peptide defensin in the anti-bacterial and anti-parasitic responses in *A. gambiae*. Later on it was successfully applied to identify factors that regulate development of the rodent malaria parasite *P. berghei* in the mosquito host. RNAi depletion of a complement-like thioester-containing protein, TEP1, and a leucine-rich repeat protein, LRIM1, significantly increased the number of developing oocysts even in fully susceptible mosquitoes, whereas knockdown of two genes encoding C-type lectins induced strong melanization response to *P. berghei* [12, 13]. Melanization is a unique immune response system in Arthropods that involves the production and deposition of melanin pigments on invading

pathogens and parasites [14]. dsRNA-mediated gene silencing was later used to assess the roles of a selected set of 21 genes belonging to the *CLIPA* and *CLIPB* subfamilies of genes encoding serine proteases, potentially involved in innate immunity, in the melanization of *P. berghei* ookinetes [15]. The authors showed that, whereas most CLIPBs promote melanization, several CLIPAs seem to play contrasting roles in this process. More recently, Brandt *et al.* [16] used *Drosophila* as a surrogate mosquito to identify host factors required for the growth of the avian malaria parasite *P. gallinaceum*. Five *A. gambiae* homologs of the 18 *D. melanogaster* loss-of-function mutants showing impaired parasite growth were then targeted by injection of appropriate dsRNA molecules. Subsequent assessment of *P. berghei* growth in *A. gambiae* mosquitoes showed that knockdown of the expression of four of these five genes led to altered parasite growth phenotypes. It should, however, be noted that while knockdown of two of these genes led to a decrease in parasite load in the mosquito, that of the other two had the opposite effect. Nevertheless, these studies highlight the potential of RNAi to identify and define the roles of genes from mosquito vectors of infection, which may constitute valid intervention targets against the spread of disease. Importantly, injection of dsRNA into *A. gambiae* mosquitoes has also been employed to compare the mosquito's melanization response to *P. falciparum*, the deadliest of all human malaria parasite species, to the one mounted against the rodent parasite *P. berghei* [17, 18]. These studies suggested that both universal and *Plasmodium* species-specific responses occur in the mosquito [17], and that the human malaria parasite may have evolved specific immune evasion strategies [18].

The relatively small number of RNAi-targeted genes in these studies reveals the difficulties of employing this *in vivo* RNAi strategy to large numbers of target genes. In this context, an informed pre-selection of the genes to be assessed by RNAi, either through bioinformatics- [15] or microarray-based analysis [17] or by carrying out a previous forward genetic screen [16] should be considered.

2.2 The vertebrate host

Following injection into their mammalian host, *Plasmodium* parasites undergo an outstanding asexual replication phase inside liver cells, which results in the release of newly formed parasites into the blood stream, where they infect erythrocytes and cause the symptoms of disease. Given their anucleated nature, red blood cells are not easily amenable to RNAi studies. However, *in vitro*

RNAi was recently used for the first time to identify vertebrate host factors that influence *Plasmodium* infection of liver cells [19, 20]. Both these studies employed siRNAs to modulate gene expression levels and used high-content, high-throughput fluorescence microscopy to assess the effect of gene knockdown on infection of the human hepatoma cell line, Huh7, by *P. berghei* parasites (Fig. 1). These medium-to-large-scale RNAi screens focused on two groups of genes whose implication on infection was previously suggested or was likely in view of available data. In Rodrigues *et al.* [19], the authors followed up a reported link between hepatocyte invasion by *Plasmodium* and apolipoprotein clearance in the liver [21, 22] and used RNAi to establish whether or not this reflected the involvement of a host lipoprotein receptor in the infection process. A total of 53 genes expressed in the liver and annotated as having validated or putative roles in lipoprotein assembly, binding, or uptake were targeted, from which the scavenger receptor class B type I (SRBI) emerged as the strongest modulator of infection. In the same report, the authors show evidence that SRBI is not only implicated in the invasion of host cells by the parasite, but also in the latter's intracellular development process that follows. The establishment of the importance of SRBI during infection confirms the validity and relevance of the RNAi approach used. Prudêncio *et al.* [20] took an unbiased approach to investigate the role of host signaling pathways during infection by *Plasmodium*. RNAi was used to selectively silence

the expression of 727 genes encoding proteins with known or putative kinase activity, as well as kinase-interacting proteins, thereby covering the entire annotated kinome [23]. This three-step screen employed increasingly stringent selection criteria, and resulted in a list of five "hits" whose influence on infection was consistently observed. One of the genes identified, *MET*, is, in fact, the first host signaling molecule to have previously been implicated in *Plasmodium* liver infection [24, 25]. One other protein identified in this screen, the atypical PKC ζ , was shown by the authors to influence the early stages of *Plasmodium* infection of hepatocytes, once again lending support to the RNAi-based strategy employed to assess the role of an informed selection of genes on malaria liver infection [20]. Importantly, both these reports made use of a novel method to silence genes in the living animal by employing siRNA molecules formulated in liposomal nanoparticles to confirm the relevance of selected target genes in the context of infection of a rodent model of malaria [19, 20]. This novel class of lipid-like delivery molecules, termed lipidoids, can be formulated with siRNAs to facilitate high levels of specific silencing of endogenous gene transcripts in mice, rats and nonhuman primates [26]. Mammalian *in vivo* RNAi thus emerges as a tool of outstanding potential to evaluate the importance of carefully selected genes in a physiological context of malaria infection.

The process of liver cell infection by *Plasmodium* comprises two consecutive steps: invasion of

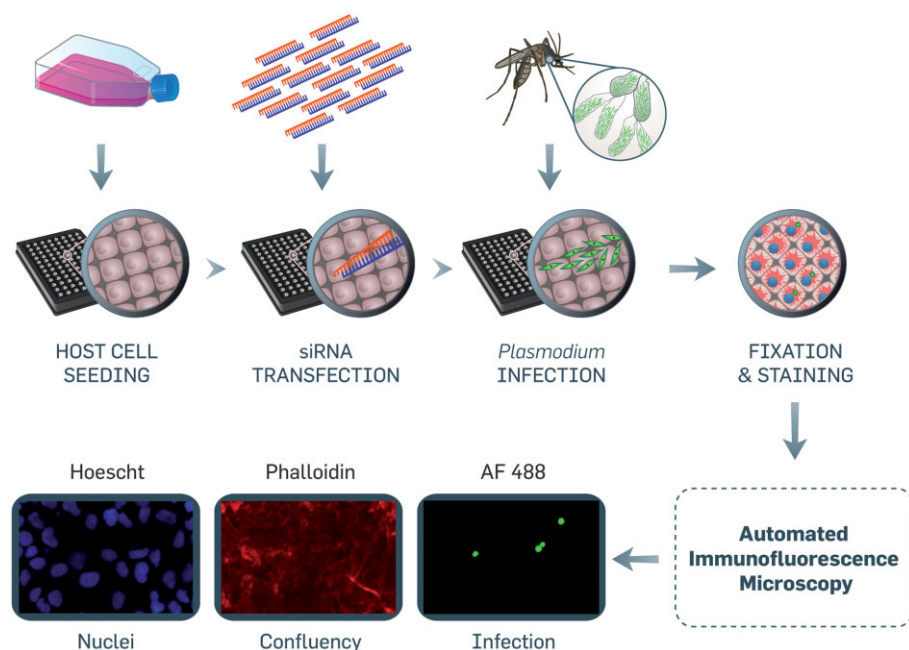


Figure 1. Experimental setup for high-throughput, high-content, RNAi-based screening of host factors involved in *Plasmodium* liver infection. Host cells are transfected with siRNAs 24 h after seeding and infected 48 h later with *Plasmodium* sporozoites freshly extracted from the salivary glands of female *Anopheles* mosquitoes. At 24 h after infection, cells are fixed and stained for parasite (green), actin (red) and nuclei (blue) and imaged by immunofluorescence microscopy. Infection is quantified and normalized following automated image acquisition and analysis.

the cells by the parasites and intracellular development of the latter [10]. Since the screening approach outlined in Fig. 1 contains information not only about parasite number but also about their size, cell invasion and parasite development can be independently assessed for each of the conditions tested with this setup. Moreover, in addition to allowing the normalization of infection levels, actin and nuclei staining of the host cells provide information about whether the RNAi treatment affected cell proliferation/morphology [19, 20]. High-content image-based RNAi screening thus constitutes a powerful technique to identify and study mammalian host factors that play a role during hepatocyte infection by *Plasmodium*. Nevertheless, other approaches may be envisaged to assess infection following RNAi-mediated knockdown of gene expression, such as flow cytometry-based assays [27]. In the case of malaria hepatic infection, flow cytometry presents the advantage that it allows the specific monitoring of the early events of cell invasion by the parasite by analyzing cells at early time

points after infection, which cannot be achieved by a high-throughput, automated analysis of image data. However, unlike microscopy, flow cytometry depends on the availability of a transgenic fluorescent pathogen, which may, in some cases, constitute a limitation. Finally, the desired readout content should also be taken into consideration. Through a selection of appropriate stainings, flow cytometry may provide simultaneous data about various cellular features. However, if information about cellular or sub-cellular morphology is required this may be better achieved by an image-based approach.

3 RNAi screens to identify host factors involved in bacterial infections

Bacterial infections have been the focus of a significant number of RNAi-based screens targeting host genes (Table 1). The amenability of *Drosophila* cells to RNAi [28, 29] has made them important tools in several large-scale screens to identify host factors

Table 1. RNAi-based screens to identify host factors involved in bacterial infections

Bacteria	Screen design			Outcome		Reference
	Host cells	Library	Readout	Main cellular processes identified ^{a)}	Validation in mammalian cells/confirmation of knockdown	
<i>Escherichia coli</i>	<i>Drosophila</i>	1000	FACS	VT, CO, CSR	No/Yes	30
<i>Staphylococcus aureus</i>	S2	random				
<i>Listeria monocytogenes</i>	<i>Drosophila</i>	21 300	Microscopy	VT, ST, CO, CC	No/No	31
	S2	genome-wide				
<i>Mycobacterium fortuitum</i>	<i>Drosophila</i>	21 300	Microscopy	VT, CO, LM, ST	Yes/No	32
	S2	genome-wide				
<i>Listeria monocytogenes</i>	<i>Drosophila</i>	7216	Microscopy	VT, ST	No/No	33
	S2	phylogenetically conserved				
<i>Legionella pneumophila</i>	<i>Drosophila</i>	73	Microscopy	VT	Yes/Yes	38
	S2	membrane trafficking				
<i>Brucella melitensis</i>	<i>Drosophila</i>	240	Microscopy	VT, ST	Yes/Yes	39
<i>Brucella abortus</i>	S2	ER + random				
<i>Chlamydia caviae</i>	<i>Drosophila</i>	16 000	Microscopy	Tr, VT, ST	Yes/Yes	41
	S2	(not specified)				
<i>Chlamydia trachomatis</i>	<i>Drosophila</i>	7261	Microscopy	CO, CC, ST, VT	Yes/Yes	42
	S2	phylogenetically conserved				
<i>Pseudomonas aeruginosa</i>	<i>Drosophila</i>	80	CFU counts	CO	Yes/Yes	44
	S2	actin cytoskeleton				
<i>Salmonella typhimurium</i>	Human	779	Microscopy	ST	Yes/Yes	46
	MCF7	kinome				

a) VT, vesicle transport; CO, cytoskeletal organization; CSR, cell surface receptor; ST, signal transduction; CC, cell cycle; LM, lipid metabolism; Tr, transcription.

that influence bacterial pathogenesis. *D. melanogaster* macrophage-like Schneider 2 (S2 or SL2) cells were used for RNAi screening for the first time in a flow cytometry-based study that employed 1000 random dsRNAs. The authors identified 34 *Drosophila* gene products involved in phagocytosis of FITC-labeled *Escherichia coli* and *Staphylococcus aureus* [30]. Besides proteins that participate in, among other processes, vesicle transport and actin cytoskeleton regulation, a cell surface receptor, the peptidoglycan recognition protein LC (PGRP-LC), was shown to be involved in phagocytosis of Gram-negative but not Gram-positive bacteria [30]. Later, two seminal image-based screens took a genome-wide RNAi approach to study infection of *Drosophila* cells by GFP-expressing bacteria of the obligatory intracellular species *Listeria monocytogenes* [31] and *Mycobacterium fortuitum* [32]. In both of these reports approximately 21 300 dsRNAs targeting >95% of the annotated *Drosophila* genome were used, and the resulting infection phenotypes were evaluated by a high-throughput automated microscopy-based analysis of bacterial GFP readouts. Another fluorescence microscopy-based RNAi study screened 7216 genes to identify host pathways that control compartmentalization of *L. monocytogenes* [33]. These studies identified a large number of *Drosophila* genes whose knockdown had an effect on bacterial infection. Their corresponding gene products span a wide array of cellular functions, including vesicular trafficking, cytoskeletal organization and lipid metabolism. Whereas several of the host factors identified, such as Rab5 (Fig. 2), were shown to influence infection by both *L. monocytogenes* and *M. fortuitum*, some dsRNAs decreased infection of only one of the two pathogens [31]. Interestingly, Philips *et al.* [32] showed that uptake of not only *M. fortuitum* but also of *E. coli* and *S. aureus* is strongly influenced by SR-BI, a molecule that has also been implicated in infection by hepatitis C virus (HCV) [34], as well as other bacteria like *Salmonella typhimurium* and *L. monocytogenes* [35], and *Plasmodium* parasites [19].

Bacterial pathogens often replicate inside an intracellular vacuolar compartment, whose formation involves recruitment and trafficking of membrane material between the endoplasmic reticulum (ER) and Golgi. The maturation of such a replicative compartment with ER-like properties is known to involve the bacterial Type 4 secretion system and fusion with membranes that contain ER-resident proteins, but it is thought that bacteria also exploit host pathways for intracellular replication [36, 37]. However, the molecular mechanisms employed by bacteria to secure this replicative niche remain

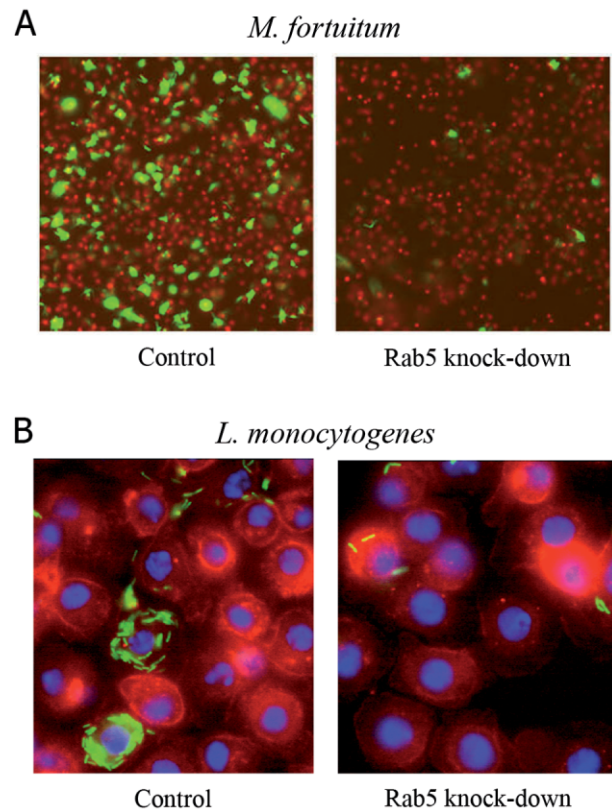


Figure 2. Effect of dsRNA-mediated knockdown of *Drosophila* Rab5 expression on infection by *M. fortuitum* (A) and *L. monocytogenes* (B). Bacteria are shown in green, actin in red and nuclei in blue. Adapted with permission from Philips *et al.* [32] (A) and Agaisse *et al.* [31] (B).

poorly understood. Two recent RNAi screens specifically addressed this issue by knocking down the expression of genes involved in secretory pathways, endocytic transport and ER-associated processes and monitoring the subsequent outcome of infection by *Legionella pneumophila* [38] and *Bruceella* spp. [39]. These screens employed 73 and 240 dsRNAs, respectively, and infection was assessed by fluorescence microscopy following immunostaining of the bacteria. The results obtained with *L. pneumophila* indicated that the replication vacuole of this bacterium is fed by distinct pathways of the secretory system. The observation that combinations of knockdowns of various ER-Golgi transport proteins were necessary to impair *L. pneumophila* replication is in agreement with the notion that multiple and redundant host secretory pathways may be exploited during infection by this pathogen [37, 40]. In contrast, the single down-modulation of the expression of Cdc48/p97, involved in ER-associated ubiquitination and targeting of unfolded proteins to the proteasome, caused defective replication of the bacteria. Importantly, this result was

confirmed by RNAi-mediated knockdown of expression of human Cdc48/p97 in HEK 293 cells, indicating that this complex is required for intracellular replication in mammalian cells [38]. The *Brucella* study identified 52 host factors whose expression knockdown influences infection by this bacterium [39]. Fourteen of these factors were shown by various RNAi screens to also mediate infection by other bacterial pathogens [31–33, 38, 41, 42]. Among the genes whose involvement in infection had not been previously reported, the authors concentrated their efforts on IRE1. This signaling molecule is involved in the regulation of the host cell's unfolded protein response, which, in turn, is associated with enhanced expression of genes encoding proteins that participate in ER-associated degradation [43]. The authors showed that *Brucella* replication in IRE1 $\alpha^{-/-}$ mouse embryonic fibroblasts (MEFs) is significantly lower than in wild-type MEFs, thereby confirming the importance of this host factor in *Brucella* replication in mammalian cells [39].

Chlamydia species are obligate intracellular bacteria that cause a wide range of infections in humans. Two large scale RNAi screens were carried out to identify host factors that influence infection of *Drosophila* S2 cells by *C. caviae* [41] and *C. trachomatis* [42]. Both screens were based on high-throughput microscopy assessments of infection levels following staining with a *Chlamydia*-specific FITC-conjugated antibody. It is interesting to notice that, despite the similarities of the systems and experimental approaches employed, while Derré *et al.* [41] targeted approximately 16 000 genes and identified 54 factors whose depletion leads to a decrease in infection, Elwell *et al.* [42] screened about 7200 genes and report an effect on infection for 266 of them. Rather than reflecting actual differences between the two infection and screening setups, this about tenfold difference in "hit rates" likely reflects the distinct criteria chosen by the authors for candidate gene selection. Both authors selected groups of genes for further confirmation of their role in infection. Derré *et al.* [41] highlighted the importance of the multiprotein Tim-Tom complex (involved in the mitochondrial import of nuclear-encoded proteins) in the *Chlamydia* infection process and the specificity of Tom40 and Tom22 on the infection of mammalian cells by *C. caviae* but not by *Listeria* or *C. trachomatis*. The report by Elwell *et al.* [42] gives particular emphasis to signaling molecules for which a decrease in expression influenced bacterial uptake by the host cells, most notably those belonging to the PDGFR and Abl kinase signal transduction pathways, which are frequently involved in Rac-dependent cytoskeletal re-

arrangements. A sister report from the same laboratory focused on about 80 *Drosophila* genes that regulate the actin cytoskeleton and assessed the effects of the RNAi-mediated knockdown of their expression in terms of the ability of *Pseudomonas aeruginosa* to enter *Drosophila* S2 cells [44], as monitored by the previously described method of counting colony-forming units following the eukaryotic cell lysis [45]. The authors found that down-modulation of the expression of several components of the Abl signaling pathway, including Abl, its target Crk, and the p21-activated kinase, Pak1, regulates host cell entry by *Ps. aeruginosa*.

Signaling molecules were the focus of another study that aimed at identifying host kinases that could be used as targets against the intracellular growth of *S. typhimurium* and *Mycobacterium* spp. [46]. The authors used a combined image-based RNAi and kinase inhibitor screening strategy to elucidate signaling pathways that influence infection by these intracellular pathogens. An siRNA library was used to target 779 genes covering the entire kinome and kinase-associated or kinase-regulatory proteins, and infection of the human breast cancer cell line MCF7 by GFP-expressing *S. typhimurium* was monitored by high-throughput automated microscopy. A reproducible decrease in *S. typhimurium* growth was observed following down-modulation of ten of the targeted kinases, which were found to cluster around the AKT1 signaling network. Interestingly, none of these were identified by the genome-wide screens that focused on infection of *Drosophila* macrophage-like cells by *L. monocytogenes* or *M. fortuitum* [31, 32]. While this may be the result of pathogen-specific host responses, it may also reflect differences arising from the type of host cells employed in these studies, an aspect that should always be carefully considered when undertaking an RNAi-based screening approach. If the amenability of *Drosophila* cells to RNAi-mediated gene expression knockdown and the availability of dsRNA libraries for this system make it an attractive model to identify and study host factors that influence bacterial infection, the use of a mammalian-derived cell line may provide useful information in a more relevant context of infection.

4 RNAi screens to identify host factors that influence viral infections

Viruses differ from many other pathogens in that they fully depend on the host cell machinery for their replication. In the past, classical virology focused on the virus itself, ignoring cellular functions

and complex biological processes in the cell as an essential part of the viral life cycle. Recently, screening approaches have become increasingly important for investigating host cell functions exploited by viruses for their replication. To date only a few RNAi screens, on a small number of viruses, have been reported, including studies on human immunodeficiency virus (HIV-1), influenza virus, HCV and West Nile virus (WNV), the causative agent of the mosquito-borne West Nile fever.

In a hallmark study Pelkmans and coworkers [47] used vesicular stomatitis virus (VSV) and simian virus 40 (SV40) as probes in an siRNA-based high-throughput screening approach to explore the functions of human kinases in different endocytic entry pathways. VSV enters cells *via* clathrin-mediated endocytosis into early and late endosomes, while SV40 is transported to the ER upon caveolae-mediated endocytosis *via* caveosomes. A total of 210 kinases involved in endocytosis have been uncovered, several of which were not previously known to play a direct mechanistic role in endocytosis. Interestingly, high selectivity for one of the two entry routes was observed and some kinases were shown to have a positive effect on one endocytic route at the expense of the other, suggesting that clathrin- and caveolae-mediated endocytosis are strongly coordinated. Furthermore, many of the kinases shown to play an essential role in endocytosis also function in various signaling pathways, as in the case of p38MAPK, arguing for a controlled interplay between signaling and endocytosis [47].

In a recent genome-wide RNAi study, 305 host proteins were identified as influencing infection by WNV [48]. Here, 283 proteins facilitated WNV infection (host susceptibility factors), while 22 host proteins were found to reduce WNV infection (host resistance factors). Functional clustering of the host genes revealed a wide variety of cellular processes and pathways required for successful infection [48]. The assay was performed by infection of gene-silenced HeLa cells with WNV, followed by a microscopy-based quantification of infected cells 24 h later. The RNAi screen confirmed 11 host factors that had already been previously characterized, like vATPase, endosomal transport regulators and IRF3 [49–55], but also identified 294 novel interaction partners of WNV. Among the host susceptibility factors, RNA-binding proteins, ubiquitination-related proteins such as the ubiquitin ligase CBL1, transcription factors, C-type lectins and protocadherins like PCDHB5 were detected, while the monocarboxylic acid transporter MCT4 was identified as a strong host resistance factor [48]. The study also revealed that silencing of 36% of the

host factors exploited by WNV also reduced infection by Dengue Virus, a related flavivirus, suggesting both overlapping and unique interaction networks with their host.

Using a genome-wide screen in *Drosophila*, more than 100 previously unrecognized host proteins required by influenza virus were identified, demonstrating the feasibility and value of using *Drosophila* RNAi screening to elucidate novel host factors [56]. Since *Drosophila* cells do not express the cognate receptor for human influenza virus, cells were infected with VSV glycoprotein (VSV-G)-pseudotyped influenza virus particles. Having screened over 13 000 genes, the human homologues of *ATP6V0D1*, *COX6A1* and *NXF1* as well as mitochondrial electron transporter complexes III and IV were shown to have key functions in the replication of H5N1 and H1N1 influenza A virus.

Many RNA viruses, like HCV or rhinovirus, utilize internal ribosomal entry sites (IRES) for translation. To elucidate host factors required for IRES-dependent translation and viral replication, Cherry *et al.* [57] performed a genome-wide RNAi screen in *Drosophila* cells infected with *Drosophila* C virus (DCV). The authors reported the identification of 66 ribosomal proteins that specifically inhibited DCV replication, when they were depleted, without any effect on non-IRES-containing RNA viruses. This study suggests another direction for antiviral drug discovery by modulating the host cell's ribosome activity [57].

Recently, three genome-wide RNAi screens on host genes required for the replication of HIV-1 were reported (Table 2). The first screen to be published was performed in cultured human HeLa cells expressing CD4 as the essential receptor for HIV-1 entry [58]. The goal was to identify host proteins involved in early events of infection such as virus entry, uncoating, reverse transcription, integration and viral gene expression as well as factors needed for late stages in infection such as viral assembly and release. Considering more than 21 000 genes, Brass *et al.* [58] identified 273 host factors required for HIV-1 replication. Preliminary characterization of three of the genes revealed previously unknown functions: Rab6, which is involved in the retrograde transport from endosomes to the Golgi and from the Golgi to the ER, was shown to be important for virus entry. Transportin-3 (TNPO3), important for the import of multiple proteins into the nucleus, revealed a function after reverse transcription but prior to integration and the depletion of the mediator complex Med28 specifically affected the reverse transcription of HIV-1 genes. Of the 273 genes, 237 had not previously been identified as important for HIV-1 infection. In

Table 2. RNAi-based screens to identify host factors involved in HIV-1 replication

Virus	Host cells	Number of investigated genes	Time of siRNA treatment	Infection time	Readout	Number of identified host factors	Reference
HIV-1 (IIIB)	HeLa derived TZM cells (CD4 ⁺ , β-gal reporter)	21 121 genome-wide	72 h	Part 1: 48 h Part 2: 24 h	Part 1: p24 stain Part 2: β-gal activity	273	58
VSV-G pseudotyped HIV-1 luc vector	293T	19 628 genome-wide	48 h	24 h	Luciferase activity	295	59
HIV-1 (HXB2)	HeLa P4/R5 cells (CD4 ⁺ , β-gal reporter)	19 707 genome-wide	24 h	48 h and 96 h	β-gal activity	232	61

a second study by König *et al.* [59], the investigators performed a genome-wide RNAi screen by infecting human 293T cells with replication-defective pseudotyped HIV-1 particles bearing the VSV-G instead of HIV-1 envelope proteins. The internalization of VSV-G pseudotyped particles is mediated by the cellular endocytic machinery and it has been shown recently that wildtype HIV-1 also fuses within endosomes [60]. However, it remains to be elucidated if VSV-G pseudotyped viral particles require the same host factors for infection as wild-type virions. By applying a diverse setup of analytical methods at a genome-wide scale, including yeast-two-hybrid analyses, König and coworkers [59] compiled a list of more than 2400 potential host genes required by HIV-1. Finally, the number of selected host factors was narrowed down by a series of filters removing genes that affected the replication of control viruses or genes known to be associated with cell death. Following this selection process, 295 genes were scored as cellular host factors important for HIV-1 replication, including cytoskeletal proteins (most likely involved in the intracellular transport of the viral reverse transcription complex), protein complexes that regulate ubiquitin conjugation (possibly required for virus uncoating), nucleic acid binding proteins (presumably interacting with the viral reverse transcriptase), factors involved in RNA splicing and, surprisingly, proteins related to DNA-damage response. Remarkably, in this study 55 factors were mapped to specific steps in the HIV-1 life cycle [59]. The genome-wide RNAi screen on HIV-1 carried out by Zhou *et al.* [61] was set up to determine host factors involved in all stages of virus infection from virus entry to release and viral cell-to-cell spread. In total, 390 genes were identified and confirmed as playing an important role in the HIV-1 replication cycle. After screening *in silico* against databases containing information on expression levels of over 11 000 genes in diverse tissues (including activated T cells and macrophages as the two predominant cellular targets of HIV-1) only 232

genes remained to be considered as host factors for HIV-1. As with the other screens, all identified genes could be grouped into different functional classes including virus entry, mitochondrial functions or energy metabolism. In addition, the mediator complex and the NF-κB signaling pathway were identified as being involved in the pathogenesis of HIV-1. Although each of these three screens identified about 300 cellular host factors exploited by HIV-1, a comparison between the identified host genes revealed only three validated genes in common, *i.e.*, Med 6 and Med 7, two subunits of the mediator complex, as well as RELA, a binding partner of NF-κB. The severe discrepancy between the results obtained is intriguing, but may be explained by the different conditions of the three screens, such as using different cell lines, different siRNA libraries and different virus strains. Moreover, siRNA treatment times were different and infection periods varied, suggesting that each screen detected a subset of genes involved in a specific stage of infection. The fact that many host factors uncovered by the three genome-wide screens have been independently identified in the past, like the nuclear import factor TNPO3/Transportin SR2 [62, 63], cyclinT1 [64] or factors involved in the NF-κB signaling pathway [65], indicates that all three screening approaches reveal gene lists of enormous interest for future exploration.

Among other viruses, HCV remains a severe threat to human health. HCV infects 3% of the world's population establishing a chronic infection in 70–80% of exposed humans, leading to liver failure or hepatocellular carcinoma. Thus, identifying cellular cofactors of the HCV life cycle might lead to novel targets for anti-HCV therapies. Using a genome-wide siRNA library, Tai *et al.* [66] recently identified 96 human genes that support HCV replication in a Huh7/Rep-Feo replicon cell line [67, 68] measuring luciferase activity 72 h after siRNA treatment. Among these genes, 27 were found to be involved in lipid metabolism, membrane biogenesis, sorting and trafficking, supporting previous ob-

servations that HCV, like other positive-strand RNA viruses, replicates on altered host membranes, also described as “membranous webs” [69, 70]. Furthermore, 6 genes were identified as being associated with the cellular RNA machinery, including *SERBP1*, *HNRPAB* and *NOP5/NOP58*. A high-ranking hit in the study by Tai *et al.* was the phosphatidylinositol 4-kinase PI4KA, whose function in mammalian cells has not been well defined to date. Previous studies, however, already revealed a direct function of PI4KA in HCV replication [71]. PI4KA appears to be an attractive target for anti-HCV therapeutics as *PI4KA* silencing is well tolerated in cultured cells [66]. In addition, the COPI vesicle coat complex and hepcidin, a peptide hormone regulating iron homeostasis [71], were shown to affect HCV replication. Previous RNAi studies have identified several host cell factors involved in HCV replication by applying limited siRNA libraries targeting about 4000 [72], 510 [73] and 62 genes [74], respectively. There was little overlap between the results of these three screens and the study by Tai *et al.* [66]. Remarkably, only 1 gene out of 11 top ranked hits from the three prior screens could be reconfirmed by Tai and coworkers when re-tested [66]. The reasons for the discrepancies might be the same as discussed for HIV-1 above. Importantly, studies based on a HCV replicon model only allow identification of host cofactors involved in replication. Thus, in future, fully infectious, cell-culture-adapted HCV strains will have to be used to explore other aspects of the HCV life cycle, such as entry, uncoating and assembly.

5 Conclusion

Host factors have been emerging as promising targets for intervention strategies against infections by various pathogens [75, 76]. In this context, RNAi-based screening approaches, combined with bioinformatic analysis methods, have become powerful tools to study overall gene functions, and have been successfully applied to identify genes involved in pathogen replication. Although the functions of several host genes during infection have already been elucidated, the search for host factors that play a role during pathogen invasion and multiplication is far from complete.

The identities of host cell factors identified by RNAi-based screens seem to depend strongly on the assay conditions and readouts chosen, suggesting that further genes will be identified by additional screens with different experimental setups. Without any doubt, all pathogen-related RNAi screens performed so far are of great interest,

shedding light on a large number of previously unknown gene functions and identifying novel host cellular pathways utilized by pathogens. However, the cells used in these studies were often chosen for experimental convenience and do not always represent physiological targets for the pathogens under study, making it advisable to confirm the importance of the genes identified in a physiologically more relevant system [77, 78]. Moreover, genes whose functions are redundant with those of other genes might not be detected, as siRNA-mediated knockdown will only deplete a single gene product at a time. This indicates the need for future combinatorial approaches employing several siRNAs targeting different genes at the same time. Furthermore, to prevent the detection of false-positive hits due to off-target effects, several siRNAs targeting different regions of a given mRNA must yield similar phenotypes, which also has to be shown to result from successful gene knockdown [79]. On the other hand, false-negative results might also become an obstacle if siRNAs are not efficient enough in silencing their target mRNAs or if the gene product is so stable that a loss-of-function phenotype cannot be monitored even though the respective siRNA is fully functional. Thus, the efficacy of the siRNAs used has to be confirmed either at the mRNA level (by using quantitative RT-PCR to determine gene expression levels) or at the protein level (by employing Western blotting, in the case that the corresponding antibody is available) [80]. Furthermore, the validation of hit information by alternative screening techniques and/or targeted assays such as the use of blocking antibodies and inhibitors should always be sought.

A close scrutiny of the reported RNAi screens to identify host factors involved in pathogen infections reveals that the selection criteria for “hit” definition vary considerably between different studies. This, in conjunction with overall inconsistencies concerning the question of how to validate potential hits, makes the gene lists obtained by different screening approaches hard to compare. Thus, selection criteria need to be standardized, at least for screens focused on similar pathogens and employing similar experimental setups. Nevertheless, siRNA screens offer large sets of promising data, allowing the exploration of the life cycle of a pathogen as well as the definition of targets for novel therapeutic approaches. As more data will be generated in the future by further RNAi screening-based studies, it will become increasingly necessary to establish widely accessible databases containing information about all host genes implicated in pathogen replication.

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