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Knockdown Screens to Knockout HIV-1

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Abstract

Three recent screens use siRNAs to identify host genes that are critical for HIV-1 replication. These screens have uncovered hundreds of human genes not previously known to be commandeered by the virus during infection. Although some caveats remain, this screening approach opens up a new landscape of viral-host interactions for future exploration.

With their short life cycle and high mutation frequency, viruses can adapt quickly to make maximal use of any host proteins that might promote their replication. Viruses are likely to exploit critical cellular functions and so may serve as a means to characterize the host cell machinery. Indeed, viruses have historically led to the discovery or characterization of the cellular machinery for many processes including membrane fusion, DNA replication at specific origins, RNA capping and splicing, and internal ribosomal entry during mRNA translation. Recent advances in genomics and RNA interference methods have led several laboratories to conduct whole-genome surveys to identify the entire set of cellular genes that can affect virus replication—the cellular or host "virome." Three such surveys, all targeting host genes needed for the replication of HIV-1, have recently been reported (Brass et al., 2008; König et al., 2008; Zhou et al., 2008). The results of these studies are surprisingly different, suggesting that the devil may be in the details.

The first screen (Brass et al., 2008), reported in *Science*, was performed in cultured human HeLa cells engineered to express CD4, the essential receptor for HIV-1 entry, and a β-galactosidase reporter gene responsive to tat, the major viral transactivator protein. Pools of short-interfering RNAs (siRNAs), four per gene, were used to transfect the HeLa cells 72 hr before they were infected with the replication-competent IIIB isolate of HIV-1. Forty-eight hours after virus infection, the cells were fixed and scored for the presence of the HIV-1 capsid protein p24 to monitor the efficiency of early events of infection, namely virus entry, reverse transcription, integration, and viral gene expression. The culture supernatants, which contain any viruses that were released, were removed and used to infect fresh cells, thus allowing Brass et al. to monitor the yield of infectious virions from the siRNA-treated cells. This yield was determined by the level of tat-activated β-galactosidase reporter gene expression in the cells 48 hr after infection. Of the more than 21,000 siRNA pools (and thus genes) tested, Brass et al. identified 273 genes whose depletion inhibited either p24 production or β-galactosidase activation by more than 2 standard deviations from the mean values (a ~2- to 3-fold greater inhibition relative to the controls). Remarkably, 237 of the 273 genes were not known to be involved in HIV-1 replication. The functions of these genes span virtually all aspects of cell physiology, and, in principle, they define the host proteins most important for HIV-1 replication. They encode components of the nuclear pore complex

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(NPC) and the Mediator complex required for RNA polymerase II-driven transcription, as well as proteins required for transport to the Golgi, protein glycosylation, and autophagy.

Brass et al. also performed preliminary characterizations of three of the genes identified in the siRNA screen, each involved in a distinct cellular process. Depletion of Rab6, a regulator of retrograde protein transport to the Golgi, blocked early stages of virus infection without affecting expression of the viral receptors CD4 and CXCR4 or tat protein function in host cells. Rab6 depletion seemed to specifically inhibit membrane fusion mediated by the envelope protein of HIV-1 and the CD4 virus receptor, implicating Rab6 as a new player in this process. Indeed, Rab6 depletion did not block infection by pseudotyped virions that utilize vesicular stomatitis virus G (VSV-G) envelope proteins instead of those of HIV-1. Like Rab6, depletion of Transportin 3-SR2 (TNPO3), a nuclear import factor for serine/arginine-rich (SR) substrates, also potently blocked early stages of infection. Unlike Rab6, TNPO3 depletion blocked infection after virus reverse transcription but before virus integration, strongly suggesting that it plays a role in nuclear targeting of the virus. Depletion of the third gene examined, the Mediator transcription activation complex component Med28, did not affect provirus formation. Med28 depletion specifically blocked HIV-1 gene expression but not gene expression of the closely related murine leukemia viruses (MuLVs), suggesting a special need for the Mediator complex in HIV-1 genome transcription.

In the second study by König et al. (2008) published in a recent issue of *Cell*, the authors used SV40 T antigen- and adenovirus E1A-transformed cultured human 293T cells instead of HeLa cells. To test for susceptibility to infection, a luciferase reporter gene was delivered to the cells using a replication-defective pseudotyped HIV-1 virus utilizing VSV-G envelope proteins instead of HIV envelope proteins. Thus, the screen only tested for genes involved in early events of infection; HIV-1 envelope-mediated entry and all late events were not scored. For comparison, luciferase reporter genes delivered by Moloney murine leukemia virus and adeno-associated virus were also tested. In the screen, cells were transfected with siRNAs targeting 20,000 genes, with about six siRNAs targeted to each gene. Forty-eight hours after siRNA treatment, the cells were infected with HIV-1 and were assayed for luciferase expression 24 hr later. König et al. scored as positive hits all genes whose depletion resulted in a greater than 45% inhibition of luciferase expression, indicating a decrease in virus infectivity. In addition, they examined by yeast two-hybrid protein-protein interactions other genes whose products were linked to these positive hits. In all, König et al. composed a list of more than 2400 candidate host cell genes involved in HIV-1 infectivity. These genes were then put through an elaborate series of filters that removed those candidates that also affected infection by the control viruses or killed the host cells when depleted. Genes that had shared functions with other hits, or were present in yeast two-hybrid host-virus interaction databases, as well as those that were coexpressed with CD4 and coreceptors, were ranked higher as candidates. Using this filtering and ranking process, König et al. pared down the list to ~800 genes. This list was further narrowed down to 295 genes whose depletion by siRNA inhibited HIV-1 infection by more than 45% in the original screen. The final set of genes consisted of a densely connected network that could be subdivided by function into a handful of groups.

König et al. partially characterized the genes they identified to determine the steps of the viral life cycle affected by their depletion. Some of these genes blocked infection before reverse transcription, whereas others altered the kinetics of DNA synthesis. In these classes, many of the genes encoded cytoskeletal proteins (likely to be involved in the intracellular movement of the viral reverse transcription complex), nucleic acid-binding proteins (plausibly interacting with reverse transcriptase), components of the ubiquitination/proteasome pathway (possibly required for virus particle uncoating), and, surprisingly,

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components of the DNA-damage response. Depletion of other genes including the nuclear pore complex component Nup153, the nuclear transport factor RANBP2, and TNPO3 blocked nuclear import as assayed by the appearance of circular viral DNAs. Still other genes specifically affected DNA integration of the HIV-1 genome when depleted.

A third screen, reported by Zhou et al. (2008) in *Cell Host & Microbe*, was similar to the Brass et al. screen in many respects. This screen was also performed in HeLa cells expressing CD4 and harboring a β-galactosidase reporter gene responsive to HIV transcription. The cells were transfected with pools of siRNAs directed against about 20,000 genes (~3 siRNAs targeted each gene) and were infected with the HXB2 isolate of replication-competent HIV-1 24 hr later. Unlike the two other screens, Zhou et al. examined β-galactosidase activity in the cells at 48 hr post-infection (to detect the first round of virus entry and *tat* transactivator expression) and again at 96 hr post-infection (to detect additional rounds of virus spreading into neighboring cells). Zhou et al. identified and confirmed 931 genes as hits. The siRNAs against these genes were then tested for their ability to inhibit the release of infectious virus by applying the culture supernatant to fresh cells and measuring activation of β-galactosidase expression 48 hr later. Depletion of 224 of the 931 genes inhibited the release of infectious virus, and these genes were also expressed in T cells, the normal target of HIV-1. Eighty-four of these genes directly inhibited tat-mediated activation of the β-galactosidase reporter.

As with the other screens, Zhou et al. grouped the 224 hits from their screen into several functional classes. These included genes likely to be involved in virus entry, tat-mediated transcription (such as components of the Mediator complex), and the NF-κB signaling pathway. Interestingly, genes involved in mitochondrial function (such as those required for oxidative phosphorylation) and genes involved in energy metabolism (including the Akt kinase, AMP-activated kinase, and components of the adipocytokine signaling pathway) were also identified. For three genes that were characterized based on the potential for their gene products to be inhibited by drugs, Zhou et al. determined that their depletion blocked virus infection before completion of reverse transcription.

At first glance, the results of these three screens seem to be in remarkable agreement: In each case, about 200–300 cellular genes with wide-ranging functions were identified and were found to play essential roles at some stage of HIV-1 infection. These extremely exciting findings expand the number of host cell factors involved in HIV-1 infection at least 5-fold. In principle, given that all genes of the human genome have been queried by these screens, all of the cellular machinery involved in virus replication down to the exact host genes may now be in hand. Indeed, virtually every step in the viral life cycle seems to involve multiple host proteins. Although the total number of host genes required may seem surprisingly high, this is not unexpected as similar studies of transposable elements in budding yeast have demonstrated that hundreds of genes may be involved in the retrotransposition of Ty elements, a process similar to retrovirus infection (Aye et al., 2004; Griffith et al., 2003; Irwin et al., 2005; Scholes et al., 2001).

Closer comparisons of the three screens, however, reveal a disconcerting fact: Although all three screens had the same goal, they did not recover the same sets of genes. Indeed, there was almost no overlap between some pairs of sets. Between the gene sets identified in the screens of Brass et al. and König et al. (consisting of about 273 and 295 genes, respectively), there were only 13 genes in common. Clearly, despite their common aim, these screens uncovered wildly different hits. Some of this discrepancy can be explained by the different conditions of the three screens (Table 1). Brass et al. used HeLa cells and replication-competent HIV-1, whereas König et al. used 293T cells and a pseudotyped HIV-1 vector. The conditions for siRNA treatment and virus infection also varied. Brass et al. treated the

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cells with siRNAs for 72 hr before infection and scored viral infectivity 48 hr post-infection. In contrast, König et al. treated their 293T cells with siRNAs for 48 hr and scored the readout 24 hr after infection. These differences suggest that the screens may have selectively identified subsets of genes involved in different stages of infection. For example, Brass et al. may have selectively recovered genes that are involved in virus entry via the CD4 receptor, are required for late stages of the virus life cycle, or encode relatively stable proteins that required longer times to decay after siRNA treatment. In contrast, König et al.'s screen may have been biased to recover genes that act only in the early steps of infection or encode proteins with short half-lives. Some of the explanation for the differing results may come from the secondary filtering algorithms imposed on the initial hits to pare them down to genes already implicated in HIV-1 biology. The different filters for "virus relevance" used by each screen may impose profound biases, and their ultimate value may prove wildly variable.

Comparison of the results from the Brass et al. and Zhou et al. screens is also puzzling. Between the two gene sets (about 273 in Brass et al. and 224 in Zhou et al.), again, there were only a few—15 in this case—genes in common. Here the conditions of the two screens were very similar. Both were performed in HeLa-derived cells, and both used replication-competent HIV-1. However, the two screens used different isolates of HIV-1 (the IIIB swarm versus the HXB2 clone) with unpredictable effects on the final results. In addition, there were differences in siRNA treatment times (72 hr versus 24 hr) and the primary readout of virus infection (p24 levels versus a tat-dependent reporter), possibly biasing the proteins identified in each of the two screens according to protein half-life or stage of viral infection. Here, also, the secondary filters imposed on the screens may have further contributed to the differing results. It is possible that many of the nonoverlapping genes identified by all three screens are actually false positives that are irrelevant to HIV-1 pathogenesis. Clearly, more work is necessary to critically validate all of the hits. Also, the optimal conditions for this type of siRNA screen still need to be determined. Regardless, whatever the explanation for the limited overlap among these gene sets, the fact that the results of these screens are of enormous interest is indisputable. For example, the nuclear import factor TNPO3/Transportin-SR2 uncovered by the screens has been independently validated to play a role in the nuclear import of the HIV-1 preintegration complex, PIC (Christ et al., 2008; Luban, 2008). TNPO3 binds directly to the viral integrase, and depletion of TNPO3 in the host cell blocks entry of PIC into the nucleus as determined by microscopy. Curiously, TNPO3 seems also to be involved in the uptake of truncated tRNAs into the nucleus, and it may transpire that HIV-1 is hijacking this poorly characterized system to gain entry to the nucleus (Zaitseva et al., 2006). The identification of other components of the nuclear pore complex, notably Nup153, suggests that they are also involved in this process. In addition, recovery of Mediator complex components strongly suggests that these proteins are essential for HIV-1 transcription. It is also of interest that genes connected to the NF-κB signaling pathway were pinpointed in the screens. Although not unexpected given that NF-κB-binding sites in the viral promoter are important for viral gene transcription, the identification of components of this pathway is reassuring. Other intriguing leads include genes required for ubiquitination, SUMOylation, DNA repair, and vesicular transport. Perhaps most exciting among the many hits are genes encoding proteins that are involved in mitochondrial function and energy production. This indicates that virus infection and the formation of viral progeny drains the resources of host cells, implying that inhibition of energy sources could limit virus replication.

Despite the many genes identified in the three screens, it is evident that the hunt for host factors is not yet over. The identities of the hits recovered seem to depend strongly on the conditions and readouts of the screens. Thus, new screens may identify even more sets of host genes. Indeed, it is likely that even these three large-scale screens have missed some

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important players. On balance, how do we evaluate these siRNA-based screens in comparison with more conventional genetic screens? Clearly, these new methods provide tremendous power. In principle, all genes of the genome are being tested, and the effect on the gene product—depletion—is likely to be simple and easy to interpret. Importantly, this method of depleting gene products is perfectly suited to screening in diploid cells, as both alleles of a gene are simultaneously suppressed. There are, however, some fundamental problems inherent in siRNA screens. Most obviously, the cells used in all of these screens were chosen for experimental convenience and are not the natural biological targets of the virus. Thus, all genes identified in the screens will need to be confirmed in the more physiologically relevant system of primary T cells. Moreover, any genes that are required for infection only in these cells and not in the cells used in the screens will simply be missed. Similarly, unannotated genes and microRNAs will also not be tested by the screen. Furthermore, as siRNAs will deplete only a single gene product at a time, the screen may not find those genes whose functions are redundant with other genes. Aside from missing certain categories of genes, siRNAs often have off-target effects on unexpected genes and could thus result in false positive hits. There may also be false negatives in cases when siRNAs may not have sufficient activity against mRNAs. Alternatively, false negatives can arise when a gene product is so stable that it decreases too slowly for the loss-of-function phenotype to be assayed even though its mRNA is efficiently depleted. For example, the key host factor LEDGF (lens epithelium-derived growth factor), which is known to partner with the HIV-1 integrase to establish proviral DNA, was not identified in any of the three screens. However, it is known that defects in viral infection are difficult to detect in LEDGF knockdown cells, possibly due to low levels of the protein remaining even after efficient mRNA depletion (Llano et al., 2004; Maertens et al., 2003). The secondary filters imposed on the screens also contribute to their limitations. The filters will discard those genes whose depletion is lethal or toxic to the host cell, even if they play essential roles in promoting virus infection. However, this is a limitation that is shared with most mutational studies and could possibly be overcome by the use of a conditionally active siRNA library. Lastly, the siRNA screens are likely to identify only those genes whose depletion confers recessive, loss-of-function, or hypomorphic effects. Unlike a conventional mutagenesis screen, the full range of mutations, such as overexpression or dominant mutations, are not likely to be found. In spite of these many limitations, siRNA screens still offer huge promise when used as a primary genetic screen. Indeed, they give the molecular biologists among us much to chew on.

No doubt, such siRNA screens can also be used to elucidate host factors required for infection by other isolates of HIV and SIV, as well as nonretroviruses. Indeed, similar screens have been performed in human cells to identify host genes important for infection by West Nile virus (Krishnan et al., 2008) and in cells of the fruit fly to study cellular factors important for the replication of influenza virus (Hao et al., 2008). Like the HIV-1 screens, these surveys identified hundreds of genes. Satisfyingly, the genes identified were involved in processes similar to those found in the HIV-1 screens, such as protein trafficking, ubiquitination, and transcription. The functions of the human homologs of the *Drosophila* genes, which were tested for the effects of their depletion on influenza virus pathogenesis, were particularly striking. These experiments confirmed the importance of genes involved in energy metabolism and in the export of RNAs from the nucleus to the cytoplasm. The broad overlap of critical host cell functions required for viral replication across such diverse virus families raises the prospect of developing antiviral interventions that could simultaneously act on many of these viruses, a holy grail of antiviral therapy.

The use of these cellular genes as targets for antiviral drugs is already becoming a reality. The key hope is that targeting host cell gene products that do not have the mutagenic potential of viral genes will minimize the acquisition of drug resistance and will provide

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long-lasting blocks to virus replication. Indeed, some of the most promising anti-HIV-1 drugs already approved or in clinical trials are targeted not against a viral protein but against CCR5, the cellular coreceptor for virus entry. These siRNA screens have provided dozens, if not hundreds, of new cellular targets for slowing or stopping the replication of HIV-1 and other lethal viruses. Such efforts should be spurred by the assembly of a database containing host genes implicated in HIV replication, which is currently underway (F.D. Bushman, personal communication).

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Table 1

Comparison of the Three siRNA Knockout Screens

	Host Cell Line	Time of siRNA Treatment	Virus Challenge	Time of Scoring Post-infection	Readout	# of Filtered Hits
Brass et al.	HeLa ($CD4^+$, β-gal reporter)	72 hr	Live HIV-1 (III B)	48 hr; 48 hr in new cells	p24 (CA); reporter activation	273
König et al.	293T	48 hr	HIV-1 luc vector, VSV-G pseudotyped	24 hr	Luc reporter	295
Zhou et al.	HeLa ($CD4^+$, β-gal reporter)	24 hr	Live HIV-1 (HXB2 isolate)	48 hr; 96 hr	β-gal reporter activation	224