CHAPTER ONE

Functional Genomic Strategies for Elucidating Human–Virus Interactions: Will CRISPR Knockout RNAi and Haploid Cells?

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Abstract

Over the last several years a wealth of transformative human–virus interaction discoveries have been produced using loss-of-function functional genomics. These insights have greatly expanded our understanding of how human pathogenic viruses exploit our cells to replicate. Two technologies have been at the forefront of this genetic revolution, RNA interference (RNAi) and random retroviral insertional mutagenesis using haploid cell lines (haploid cell screening), with the former technology largely predominating. Now the cutting edge gene editing of the CRISPR/Cas9 system has also been harnessed for large-scale functional genomics and is poised to possibly displace these earlier methods. Here we compare and contrast these three screening approaches for elucidating host–virus interactions, outline their key strengths and weaknesses including a comparison of an arrayed multiple orthologous RNAi reagent screen to a pooled CRISPR/Cas9 human rhinovirus 14–human cell interaction screen, and recount some notable insights made possible by each. We conclude with a brief perspective on what might lie ahead for the fast evolving field of human–virus functional genomics.

Advances in Virus Research, Volume 94
ISSN 0065-3527
http://dx.doi.org/10.1016/bs.aivir.2015.11.001

1. INTRODUCTION

The burden imposed upon the health of the world's population by just three of the major pathogenic viruses is staggering, with nearly 300 million people chronically infected by either HIV-1 (36 million) or HBV (250 million), and another 5–6 million severe infections by influenza A virus (IAV) occurring transiently each year (Ortblad, Lozano, & Murray, 2013; Schweitzer, Horn, Mikolajczyk, Krause, & Ott, 2015) (http://www.who.int/immunization/topics/influenza/en/). Collectively these three viruses cause the deaths of over 2.5 million people annually. These infections arise because viruses must find and exploit the host's cellular resources and machinery to produce their progeny. Elucidating human pathogenic viral dependencies has been a longstanding pursuit of health science researchers whose goal is to use this knowledge to treat and cure infections. For decades, mammalian *in vitro* tissue culture systems have proved tremendously useful for studying host–virus interactions. Over this same period, loss-of-function genetic screening produced an impressive number of discoveries and illuminated gene and pathway function in multiple model systems. While loss-of-function genetic screening proved extremely valuable in model systems, such technologies did not exist for mammalian cells until the discovery and implementation of RNA interference (RNAi) (Fire et al., 1998). The initial technologic revolution of RNAi, and later the development of haploid cell screening, resulted in a wave of discoveries that shed new light on many vital human viral requirements (Brass et al., 2008; Hao et al., 2008; Krishnan et al., 2008; Randall et al., 2007; Sessions et al., 2009). The ascendance of CRISPR/Cas9 technologies, which can dramatically alter gene expression, has heralded a new era in mammalian *in vitro* genetic screening (Shalem, Sanjana, & Zhang, 2015). This review will discuss the available functional genomics strategies, highlight their strengths and weaknesses including a comparison of matched MORR RNAi and CRISRP/Cas9 screens, and provide some future perspectives on the use of mammalian *in vitro* genetics to elucidate human host–virus interactions.

2. HOST–VIRUS GENETIC SCREENS

The numbers of host–virus functional genomic screens using these technologies, particularly RNAi, have been increasing rapidly attesting to their innovative discovery power, generalizability and remarkable ease of use (Table 1). *Drosophila* cell *in vitro* RNAi screens were the first to detect novel host factor interactions for several human pathogens with the practical focus being on arboviruses, although an elegant approach using a recombinant virus also made it possible to screen for IAV dependency factors in this system (Arkov, Rosenbaum, Christiansen, Jonsson, & Munchow, 2008; Cherry et al., 2005; Hao et al., 2008). RNAi screens using human cells have now been done for the majority of major human pathogenic viruses (Table 1); these efforts have largely used arrayed siRNA libraries combined with high-throughput imaging or plate reader-based assays as readouts for viral replication. Collectively these works have identified multiple previously unappreciated dependencies for each virus, as well as host cell defense mechanisms. Recent publications covering viruses that have been functionally interrogated by multiple independent groups including HIV-1, IAV, and HCV have been discussed elsewhere in detail (Bushman et al., 2009; Hao et al., 2013; Stertz & Shaw, 2011; Zhu et al., 2014). In this work, we focus on the functional genomic screening technologies and provide a resource noting many of the published host–virus screens along with some of their key attributes.

3. RNAi GENETIC SCREENING TECHNOLOGIES AND APPROACHES

Nearing a decade ago the Nobel Prize winning discovery of RNAi in *C. elegans* and its mercurial extension into mammalian systems provided virologists and geneticists alike with a powerful new tool for detecting viral dependencies (Elbashir et al., 2001; Fire et al., 1998; Grishok & Mello, 2002). Academia and industry both quickly embraced RNAi and paired it with the contemporaneous completion of the genetic annotation of the entire human genome to create multiple large-scale libraries for functional genomic screening (Paddison et al., 2004; Root, Hacohen, Hahn, Lander, & Sabatini, 2006; Silva et al., 2005). Because the RNA-induced silencing complex (RISC) machinery's expression is ubiquitous, virtually all mammalian cell lines can carry out RNAi, permitting host–virus screens to be carried out with any tropic cell line and virus pairing (Elbashir et al., 2001). Two major types of RNAi libraries, pooled and arrayed, have been constructed and dictate the two methods of screening discussed below.

Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions

	Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Haploid cells	Carette et al. (2009)	Influenza virus (PR/8/34; H1N1)	Haploid human suspension cells KBM-7	Pooled	Haploid cell Insertional mutagenesis with lentiviral exon trap	N/A	2–3 weeks	Survival	Yes	Multiple independent integrations	No	N/A	CMAS; SLC35A2	Entry	RT-PCR; immunofluorescence; complementation with cDNAs
	Carette et al. (2011)	rVSV-GP-Ebola virus	Haploid human adherent cells (HAP1)	Pooled	Haploid cell Insertional mutagenesis with lentiviral exon trap	N/A	Unknown	Survival	Yes	Multiple independent integrations	No	N/A	NPC1, HOPS complex	Entry, viral fusion in lysosomal compartment	Complementation with cDNAs; test against related viruses; small-molecule U1866A and imipramine; immunofluorescence/ electron microscopy viral entry assays; primary cell lines
	Jae et al. (2013)	rVSV-GP-Lassa virus	HAP1	Pooled	Haploid cell Insertional mutagenesis with lentiviral exon trap	Gene-Trap	Unknown	Survival	Yes	Multiple independent integrations	No	N/A	TMEM5; B3GALNT2; B3GNT1; SLC35A1; SGK196	Entry, presentation of laminin-binding carbohydrate	Null alleles TALENs; rescue cDNAs; analysis of know polymorphisms; flow cytometry; RT-PCR; clinical comparison
	Kleinfelter et al. (2015)	rVSV-Andes virus-GP	HAP1	Pooled	Haploid cell Insertional mutagenesis with lentiviral exon trap	N/A	8 days	Survival	Yes	Multiple independent integrations	No	N/A	S1P; S2P; SREBF2; SCAP; LSS; SQLE; ACAT2	Entry	S1P CRISPR/Cas9 gene editing in U2OS; complementation with cDNA; small-molecule inhibitor

siRNA															
Haploid cell and siRNA	Petersen et al. (2014)	rVSV-Andes virus, either recombinant or pseudoparticles expressing Renilla luciferase	HAP1	Pooled	Haploid cell Insertional mutagenesis with lentiviral exon trap	N/A	3 weeks	Survival	Yes	Multiple independent integrations	No	N/A	SCAP; S1P; S2P; SREBF2	Entry	Functionally deficient cells S1P, S2P, or SCAP null CHO and SREBP2 KD HEK293T; TALEN-mediated gene disruption; small-molecule PF-429242 and mevastatin
			HEK29	Arrayed	Ambion druggable genome library (9102 genes) (4 siRNAs/gene) (2 siRNAs/well)	72 h	24 h	Renilla luciferase expression	Yes 210 dsRNAs; 112 genes reconfirmed	In both pools: *Z* score for infection <−1.5 ($p<0.009$); viability <−2			SREBF2	Entry	3 additional unique siRNAs screened with ANDV and VSV-G pseudoparticles; validated by 1 siRNA repeating finding two times. 105 candidate genes—33 validated—9 specific for ANDV
	Brass et al. (2008)	HIV-1-IIIB	TZM-bl	Arrayed	Dharmacon siARRAY siRNA library (21,121 siRNA pools)	72 h	48 h	% Infectivity (anti-HIV-1 p24)	Yes	Decreased Infectivity by ≥2 SDs; viability not decreased by >2 SDs	No	N/A	RAB6A	Fusion	Subcellular localization; gene ontology (GO) biological processes analysis; Expression Genomic Institute of the Novartis Research Fund (GNF); individual shRNAs; individual siRNAs; infection with VSV-g; other cell lines Jurkat; qPCR
													TNPO3	Cytosolic post-RT–pre integration	
													MED28	Transcription	
	Hao et al. (2008)	Influenza A virus Flu-VSV-G-GFP	DL1	Arrayed	Ambion *Drosophila* RNAi library (13,071 genes)	48 h	24 h	Renilla luciferase activity	Yes	Inhibition >2.4 SDs; Viability reduction Z score >-3	Yes	Increase >3 SDs; viability reduction Z score >−3	COX6A1	PB2/PB1-F2-mediated functions	RT-PCR; reagent redundancy; test human homologues, knockdown in HEK293 cells; individual siRNAs; small-molecule inhibitors; related viruses: WSN, H5N1 Influenza A/Indonesia/7/05, VSV, VACV
									176 candidate genes—110 confirmed		123 candidate genes—11 genes confirmed		ATP6V0D1	Fusion	
													NXF1	RNA export pathway	

Continued

Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Krishnan et al. (2008)	West Nile virus WNV strain 2471	HeLa	Arrayed	Dharmacon siARRAY siRNA library (21,121 siRNA pools)	72 h	24 h	% Infectivity (viral E-proteins)	Yes	Infection reduction of $>$twofold	No	NA	CBLL1	Entry	Individual siRNAs, small-molecule: MG132, cyclohexamide; colocalization; enrichment analysis using Panther; gene expression—microarray; protein interaction network
	Dengue virus DENV New Guinea C strain					30 h		283 candidates				MCT4	Replication phase	
Tai et al. (2009)	Hepatitis C virus Subgenomic genotype 1b replicon	Huh7/Rep-Feo	Arrayed	Dharmacon siARRAY human genome siRNA library (21,094 genes)	72 h	N/A	Viral replication (luciferase)	Yes	Replicon expression decreases by >2 SDs	Yes	Increased replicon expression with threshold of $q<0.10$	PI1KA	Replication complex formation, generation of HCV nonstructural protein-associated membranes	Gene ontology; clustered; literature review; other cell line: OR6 replicon cell line, UHCVcon57.3; protein expression; Western blot; small-molecule Wortmannin, brefeldin A; reagent redundancy; shRNAs; localization studies; virus: HCV-JFH1
								236 pools—186 replicated—96 confirmed		13 pools		COPI-Coatomer	Early	
												Hepcidin	Cellular translation	
Li et al. (2009)	Hepatitis C virus JFH-1	Huh 7.5.1	Arrayed	Dharmacon siARRAY siRNA library; human genome (19,470 genes)	72 h	48 h	% Infectivity (HCV Core Antibody 6G7)	Yes	Infectivity $<50\%$ plate mean; cell number $>50\%$ of plate mean	Yes	Infectivity $>150\%$ pf plate mean; cell number $>50\%$ plate mean	RAB9p40	Needed for both HCV and HIV	Individual siRNAs, enrichment analyses for molecular function and biological process according to Panther classification; network analyses interactome screens + HPRD; RT-PCR
								407 candidate pools		114 candidate pools				

Sessions et al. (2009)	Dengue virus DENV-S2	Dipteran cells	Arrayed	Genome-wide RNAi library DRSC 2.0 (22,632 dsRNAs)	48 h	72 h	Expression of envelope protein	Yes 218 candidate dsRNAs—rescreen 179 dsRNA—identified 118 dsRNA = 116 genes—111 novel	Inhibited infection ≥1.5-fold with $p < 0.05$	No	N/A	FLJ20254; TAZ; EXDL2; CNOT2	RNA accumulation	Gene ontology; *in vivo* mosquito *Ae. aegypti*; validation of human homologue siRNAs in Huh-7 cells; other viruses: YFV 17D vaccine strain, Coxsackie B3 (strain 20; CB3); RT-qPCR
Brass et al. (2009)	Influenza A virus A/Puerto Rico/8/34	U2OS	Arrayed	Dharmacon siARRAY siRNA library; human genome (17,877 genes)	72 h	12 h	% Infectivity (anti-HA antibody)	Yes 312 pools	<55% infectivity; viability >40%	Yes 22 pools	>200% infectivity; viability >40%	IFITM3	Early	Rescreened candidates; (GO) enrichment analysis; other cell lines primary lung fibroblasts, HeLa, A549, ChEFs, MDCKs; other viruses: HIV, PR8, H3N2 A/Udorn/72, A/Brisbane/59/07 H1N1, A/Uruguay/716/07 H3N2, A/Aichi/2/68 H3N2, MLV, VSV-G; pseudoparticles MLV with the following envelopes: H1, H3, H5, H7, MACH, MLVRescue construct; overexpression; Western blot; immunofluorescence
Shapira et al. (2009)	Influenza A virus IAV PR8	HBECs	Arrayed	Dharmacon SMARTpool	72 h	48 h	Viral particle production (reinfection); IFN production	Yes	Change >twofold less replication compared to median	Yes	Change >twofold more replication compared to median	WNT/p53 pathway	NS1 related	Pathway analysis; clustering of expression data; functional annotations; yeast 2 hybrid

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Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Kolokoltsov, Saeed, Freiberg, Holbrook, and Davey (2009)	EBOV GP (Zaire)—pLENTI6-fluc	HEK293	Arrayed	Kinase and phosphorylase subset of Ambion druggable genome (720 genes)	48 h	36 h	Luciferase expression	Yes	Decrease $\geq 3\times$ standard deviation	Yes	Increase $\geq 3\times$ standard deviation	PI3K	Membrane turnover	Verified in Vero cells; redundant siRNA activity analysis; Ingwnuity pathways knowledge base network analysis; small molecule: inhibitor drugs, KN-93, KN-92, LY294002
												CAMK2	Transcription	
Konig et al. (2010)	Influenza A virus Recombinant A/WSN/33	A549	Arrayed	QIAGEN genome-wide (19,628 genes)	48 h	12, 24, 36 h	Luciferase activity	Yes	2 siRNAs Luciferase reduction $\geq$ 35%	No	N/A	COPI coat complex	Entry	Reagent redundancy; viability; enrichment analysis; protein interactions; WT virus, clustering; pseudoparticles; GO analysis; STRING analysis; other virus IAV A/Hamburg/04/2009, A/Vietnam/1203/2004; lifecycle assays; localization assay
Karlas et al. (2010)	Influenza A virus IAV A/WSN/33	A549/293T	Arrayed	QIAGEN	48 h	24 h	Nuclear protein staining/ luciferase	Yes	Robust Z score <-2	No	N/A	CLK1	Splicing viral mRNA	Reagent redundancy; viability assay; replication analysis; gene enrichment; network analysis; Western blot; lifecycle assay; RT-qPCR; small molecule: TG003; *in vivo* assay

Smith et al. (2010)	Human Papillomavirus Stable expressing HPV18LCR-Luc	C33A/BE2/18LCR Clone 4	Arrayed	Dharmacon human genome library (21,121 SMARTpools)	72 h	N/A	Luciferase activity	No	N/A	Yes	Z score ≥ 2	SMCX EP400 Brd4	E2-dependent transcriptional repression	Quantitative In-Cell Western; reagent redundancy; individual siRNAs; multiple different cell lines; protein interaction network; GO analysis; transient DNA transfections; immunoprecipitation; RT-qPCR
Moser, Jones, Thompson, Coyne, and Cherry (2010)	Poxvirus	DL1	Arrayed	Mini library *Drosophila* kinase and phosphate genes (440 genes)	72 h	48 h	% Infectivity (anti-B-gal antibody)	Yes 8 genes—7 validated	Robust Z score of <-2	No	N/A	AMPK	Entry	Secondary dsRNAs; RT-PCR; mammalian cells—MEFs (null), U2OS; VSV control virus; Northern blot for virus; AMPK inhibitor Compound C; dextran uptake
Panda et al. (2011)	Vesicular Stomatitis virus VSV-eGFP	HeLa	Arrayed	QIAGEN genome-wide siRNA library version 1 (22,909 genes)	52 h	18 h	Green fluorescence protein (GFP) intensity	Yes 233 genes	>5 SDs from mean	No	N/A	COPI; ARF1; GBF1	Viral gene expression	RT-qPCR; cell viability; clustering/enrichment analysis; reagent redundancy; other viruses: HPIV3, LCMV; lifecycle assay
Coyne et al. (2011)	Coxsackievirus B CVB Poliovirus PV	HBMECs	Arrayed	Ambion druggable genome library (5492 genes)	72 h	14 h	% Infectivity (viral VP1 antigen)	Yes CVB 144; PV 155; 38% confirmation; 46 validation overlap	Robust Z score <-2; viability <30% in cell number	Yes CVB 31; PV 65; 38% confirmation; 17 validated overlap	Robust Z score >2; viability <30% in cell number	Akt1/Akt2 MAP3K4; MAPK1 TLR8/IRK1 ADCYs	Akt/MAPK signaling Viral detection cAMP mediated CREB-dependent transcription	3 unique siRNAs; pathway enrichment; protein network analysis; microarray analysis; small-molecule Akt1/Akt2 inhibitor SH-6, TOR inhibitor rapamycin, ERK1/2 inhibitor FR180204; dominant negative mutant

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Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Hussain, Leong, Ng, and Chu (2011)	HEV71	RD cells	Arrayed	Dharmacon human genome siRNA endocytic and membrane trafficking genes subset library (119 genes)	48 h	12 h	Primary anti-HEV17 antibody	Yes	Viral antigen + cells <50% of control	No	N/A	AP2A1; CLTC; CLTCL1	Clathrin-mediated endocytosis	Dominant negative mutants; deconvolution of siRNAs; reagent redundancy; dosage-dependent KD; immunofluorescence entry assay; transmission electron microscopy entry assay; small molecule: Chlorpromazine, cytochalasin B, filipin, nystatin, methyl-B-cyclodextrin, EIPA
												MAP4K2; PAK1; PIK3CG; PIK3C2G; ROCK1	Signal transduction at viral entry	
Liu et al. (2011)	HIV-1$^{89.6R}$	HeLa-CD4	Arrayed	QIAGEN human whole genome siRNA Set V4.0 (19,121 genes)	72 h	48 h	% Infectivity (GFP expression)	No	N/A	Yes	GFP + Foci >3 SDs from mean	PAF1 complex	Innate defense	Network pathway analysis (IPA); individual siRNAs; WT viral strains NL4-3, 89.6wt; mRNA levels; Western blot; cell lines MDMs, CD4+ T cells; qPCR
	HIV-1$^{8.2N}$									192 candidates—114 validated		SETDB1	Preintegration	
Espeseth et al. (2011)	HXB2 HIV	HeLa P4/R5	Arrayed	siRNA DNA repair factor library	24 h	48 h	β-galactoside activity	Yes	Inhibition >40%	No	N/A	Base-excision repair pathway	Integration	cDNA rescue; lifecycle assays; qPCR; flow cytometry; GO annotation; cell line: murine embryonic fibroblasts (MEFs)
								41 siRNA pools						
Le Sommer, Barrows, Bradrick, Pearson, and Garcia-Blanco (2012)	Yellow Fever virus YF-17D	Huh-7	Arrayed	QIAGEN human genome library (22,909 genes)	51 h	42 h	% Infectivity (4G2 antibody)	Yes	Decrease % infection twofold	No	N/A	GRK2	Entry	Individual siRNAs; comparison to WNV + DENV screens; Western blot; other cell lines: MEFs; other virus: DENV-NGC, HCV-JFH1; qRT-PCR; lifecycle assays
								395 hits—98 candidates					Genome amplification	

Dziuba et al. (2012)	HIV-1 strain LAV	CD4+/CCR5+/CXCR4+ TZM-bl	Arrayed	Dharmacon siRNA SMARTpool custom library of trapped genes	48 h	48 h	HIV-1 p24 capsid production	Yes	50% inhibition	No	N/A	GTF2E1	Tat-dependent gene transcription	Rescue experiment; infectivity of surviving clones; Western blot; individual siRNA; RT-PCR; ELISA; other viral strains: SF162, ADA, 89.6 HIV-1; pathway analysis
												DHX8	Release of spliced mRNA	
												UBA3	Modification of HIV-1 proteins	
												KALRN; HAP1	Protein trafficking	
Arita, Wakita, and Shimizu (2012)	PV pseudovirus	HEK293	Arrayed	Thermo Scientific human membrane trafficking gene library	96 h	7 hr	Luciferase activity	Yes	Strongest novel hit	No	N/A	VCP	Viral RNA replication	Rescue KD with mutant protein; immunofluorescence microscopy; immunoprecipitation; Western blot; two-hybrid assay; PLA; PV mutant resistant to KD
Mercer et al. (2012)	Vaccinia virus VACV-EGFP	HeLa	Arrayed	QIAGEN druggable genome (7000 genes)	72 h	8 h	% Infectivity (GFP)	Yes	Median absolute deviation <−1.5	No	N/A	Proteasome subunits	Late viral gene expression	Reagent redundancy; functional annotation clusters; protein interaction analysis; immunofluorescence; lifecycle assay; small molecules: MG132, UBEI-41, cytosine arabinoside; Western blot
												Cullin 3	vDNA replication	
Ward et al. (2012)	Influenza A virus IAV A/WSN/33	HBEC30-KT	Arrayed	Dharmacon library (21,125 genes)	48 h	48 h	Luciferase assay	Yes 182 candidates	3 SDs below mean	Yes 53 candidates	3 SDs above mean	CDC2; CHEK1	Viral production	Network analysis; comparison to other screens; literature review; plaque assay; small molecule: SB218078, 3-IPEHPC; Western blot; immunofluorescence; other cell line: A549

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Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Ooi, Stiles, Liu, Taylor, and Kielian (2013)	Sindbis virus SINV-Luc	U2OS	Arrayed	Ambion Silencer human genome siRNA library V3 (21,687 genes)	48 h	24 h	Luciferase intensity	Yes	Robust Z score < -3	Yes	Robust Z score > 2	FUZ	Viral uptake	Individual siRNAs; individual shRNAs; multicycle infectivity assay; other cell lines: HeLa, primary endothelial cells; other viruses: SFV, CHIKV, VSV, DENV; immunofluorescence lifecycle assays; fusion assay; endocytic pathway assay; quantigene analysis of mRNA
								400 genes		59 genes		TSPAN9	Viral fusion	
Sivan et al. (2013)	Vaccinia virus VACV IHD-J/ GFP	HeLa	Arrayed	Ambion Silencer Select human genome siRNA library (21,500 genes)	48 h	18 h	% Infectivity (GFP + cells)	Yes	<-1.5 median absolute deviation; $<50\%$ reduction in cell number	Yes	<-1.5 median absolute deviation; $<50\%$ reduction in cell number	NUP62	Conversion of immature virion to mature virion	Gene network analysis (IPA); gene ontology (GO); common seed analysis; individual siRNAs; rescue experiment; Western blot; lifecycle evaluation; viral gene expression; TEM
				Dharmacon siGENOME SMARTpool siRNA (18,120 genes)				576 genes		530 genes				
Fusco et al. (2013)	Hepatitis C virus HCV-JFH1	Huh7.5.1	Arrayed	Dharmacon siGENOME pooled siRNA library	72 h	48 h	% Infectivity (HCV anti-core antibody)	Yes	$\geq 3\times$ median absolute deviation	Yes	$\geq 3\times$ median absolute deviation	12 interferon effector genes	Various	Western blot; qRT-PCR; shRNA KDs; overexpression; microarray analysis

Panda et al. (2013)	Sindbis virus SINV (HRsp)	DL1	Arrayed	Ambion *Drosophila* genome wide	72 h	36 h	% Infectivity (GFP)	Yes 57 genes validated	Robust *Z* score < −2; <40% viability decrease	Yes 37 genes validated	Robust *Z* score > 2; <40% viability decrease	SEC61A VCP	Entry/early stage	Gene ontology (GO) enrichment analysis; dsTE12H strain; independent dsRNAs; small-molecule Eeyarestatin 1, NH_4Cl; Western blot analysis; *in vivo* assay; localization microscopy
Lavanya, Cuevas, Thomas, Cherry, and Ross (2013)	Junin virus GP pseudotyped Moloney Leukemia virus MLV-Lac-Z	U2OS	Arrayed	Ambion druggable genome RNAi library	72 h	48 h	% Infectivity (anti-Lac-Z)	Yes 89 genes	Robust *Z* score ≤ −1.5; viability *Z* score decrease < 2	Yes 13 genes	Robust Z score ≥ 1.5; viability Z score decrease < 2	CACNA2D2	Entry	Independent siRNAs; luciferase assay; RT-qPCR; small molecules—U73122, U73343, BCECF-AM, BAPTAAM, gabapentin, nifedipine, verapamil, bafilomycin A; binding assay; *in vivo* assay C57BL/6 mice; molecular function (GO) analysis for enrichment; KD-related proteins
Hopkins et al. (2013)	Rift Valletty Fever virus RVFV (MP12)	DL1	Arrayed	Ambion genome-wide dsRNA library (13,073 genes)	72 h	30 h	% Infectivity (anti-RVFV N)	Yes 7 validated genes	Robust *Z* score ≤ −1.3; viability *Z* score > −2	Yes 124 validated genes	Robust *Z* score ≥ 1.3; viability *Z* score > −2	Dcp2	Decapping	Other RNA viruses DCV, SINV, LACV, VSV; colocalization; *in vivo* infectivity; Northern blot; RT-PCR; Aag-2 cells; Western blot

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Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Zhu et al. (2014)	HIV-1-IIIB	P4-P5 MAGI cells	Arrayed	Ambion Silencer Select (21,584 siRNA pools)	72 h	48 h	% Infection (anti-p24 capsid antibody)	Yes	Infectivity $\leq 50\%$; viability $\geq 50\%$	Yes	Infectivity $\geq 200\%$; viability $\geq 50\%$	UMPS; ATIC; RRM	Pyrimidine and purine metabolism	MORR analysis; RIGER analysis; gene expression filtering; literature comparison; reagent redundancy; enrichment analysis ConsensusPath DB-human; microarray analysis; genome-wide enrichment of seed sequence matches (GESS); network analysis; lifecycle assays
				Sigma esiRNA (15,300 siRNA pools)								THOC2	Replication	
												COG complex	Glycosylation	
				Dharmacon SMARTpool RefSeq27, Revision Human 5 (4506 siRNA pools)								GOLGI49	Entry	
												SEC13	Nuclear	
Yasunaga et al. (2014)	West Nile virus WNV	DL1	Arrayed	Ambion *Drosophila* library (13,071 genes)	72 h	48 h	% Infection (anti-WSN-NS1)	Yes	Robust Z score < -2; Z score < -2	Yes	Robust Z score > 2; Z score < -2	dRUVBL1	Antiviral	Repeat for validation with dsRNA against different region of gene; other viruses: WNV-KUN, DENV, SINV, VSV, RVFV MP12; functional annotation and clustering using DAVID bioinformatics resource; *in vivo* assay; Northern blot; RT-qPCR; small molecule: Leptomycin B, dichloroacetic acid, hexokinase II; other cell lines U2OS, Aag-2
								376 genes		161 genes		dXPO1	Innate immune response	

Balistreri et al. (2014)	Semliki Forest virus SFV-ZsG	HeLa	Arrayed	Dharmacon human ON-TARGET plus (4 pooled siRNAs/gene)	72 h	6 h	% Infection (*Zoanthus* species G, ZSG) viability (Hoechst)	No	N/A	Yes	Top hit	UPF1	Early cytosolic	Specific validated shRNA; Western blot analysis; rescue with shRNA-resistant UPF1; immunofluorescence microscopy of viral components
Wen, Ding, Hunter, and Spearman (2014)	HIV-1 NL4-3-EGFP	HeLa	Arrayed	Dharmacon-Thermo Fisher cellular membrane trafficking genes (140 genes)	24 h	48 h	Particle production in supernatants	Yes	Particle output $<50\%$; viability $>60\%$ control	No	N/A	24 genes overlap	Particle production	STRING—Search tool for retrieval of interacting genes; shRNA validation; Western blot analysis
	Mason-Pfizer monkey virus pSARMX-EGFP + pTMO-Env	Cos-1						24 overlap hits; HIV-1 NL4-3 41 candidates (8 known); pSARMX 52 candidates						
Kwon et al. (2014)	Dengue virus DENV2 (BR DEN2 01-01)	Huh7	Arrayed	Dharmacon siGENOME kinase library (G-003500-05) (779 genes) (4 siRNA/gene) (2 siRNAs/well)	48 h	48 h	% Infection (4G2 antibody)	Yes	−2 standard deviations of mean	Yes	+2 SDs from mean	SHPK	Macrophage polarization	8 candidates—6 cherry picks; individual siRNAs; U937 DC-SIGN cell line; flow cytometry; gene expression analysis; qRT-PCR
												ETNK2	Entry/cellular trafficking	
								22 candidates—6 cherry picks		8 candidates—6 cherry picks		EIF2AK	Unfolded protein response	22 candidates—16 cherry picks—6 validated; individual siRNAs; Western blot; flow cytometry; U937 DC-SIGN cell line; gene expression analysis; qRT-PCR
												SMAD7	Prolong cell survival	

Continued

Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
Pohl, Edinger, and Stertz (2014)	Influenza A virus IAV VLP	A549	Arrayed	Custom library (169 siRNAs)	48 h	30 h	Renilla luciferase	Yes 43 candidates—22 related to entry	2 siRNA 50% reduction in infection, cell viability 70%	No	N/A	PEPD	Early endosomal block	Control VLPs (LASV and MLV); compare to previous screens; Western blotting; WT virus (A/WSN/33); strains: FPV/Dobson (H7N7), A/Hong Kong/68 (H3N2), A/Netherlands/602/2009 (H1N1), A/Panama/2007/99 (H3N2); WI38 primary cells; cell cycle assay; fusion assay; colocalization
Beard et al. (2014)	Vaccinia virus VACV-A5eGFP	HeLa	Arrayed	Dharmacon druggable genome siRNA SMARTpool library (6719 genes) (4 siRNAs/gene)	48 h	48 h	Infection (GFP fluorescence)	Yes 153 candidates—35 cherry picks—24 validated	eGFP ≤ -2 *Z* score; cell number > -2 SDs from plate mean	Yes 149 candidates—24 cherry picks—7 validated	eGFP ≥ 2 *Z* score; cell number > -2 SDs from plate mean	AMPK Septins; MAZ; DNA replication/repair pathway	Regulation actin cytoskeleton Unknown	RT-PCR; individual siRNAs; comparison to known data; transcriptional profiling comparison; pathway analysis
Lee, Burdeinick-Kerr, and Whelan (2014)	Vesicular Stomatitis virus rVSV-EGFP	HeLa	Arrayed	Dharmacon SMARTpools (21,121 pools)	48 h	7 h	% Infectivity (EGFP+); EGFP intensity	Yes 405 candidates—305 confirmed—29 further evaluated	>3.0 SDs from mean for % infected or intensity; <3.0 SDs alteration for viability	No	N/A	GPR149 PSCA	Entry Entry	Individual siRNAs; Western blot; RNP cores

Aydin et al. (2014)	Human Papillomavirus HPV16-GFP	HeLa MZ	Arrayed	Qiagen druggable genome version 2 +siRNA#3 from Qiagen druggable genome version 3 (6979 genes)	60 h	36 h	% Infectivity (GFP)	Yes	Reduction in Z score >3	Yes	Increase in Z score >3	AURKB; ANAPC; INCENP	Mitosis regulators	Reagent redundancy; literature review; enrichment analysis; network analysis; lifecycle assay; other cell lines primary human keratinocytes; small molecules: aphidicolin, CPG74514A, NH$_4$Cl; localization assays; immunofluorescence analysis
Schreiber et al. (2015)	Adeno-associated virus AAV9 CMV-Luc	HeLa	Arrayed	SMARTpool siRNA library: Human siGENOME ubiquitin conjugation subsets #1 (89 genes), #2 (115 genes), and #3 (396 genes)	Unknown	48 h	Luciferase expression	No	N/A	Yes	10-fold increase	PHF5A; RAB40B; PRICKLE4	Transduction efficiency	12 candidate genes—3 confirmed hits: Verification with distinct siRNAs and lenti-shRNAs; rescue with PHF5A-HA-escape vector; small-molecule meayamycin B; immunoprecipitation
Sivan, Ormanoglu, Buehler, Martin, and Moss (2015)	Vaccinia virus VACV C7L−K1L−/+GFP	HeLa; BS-C-1	Arrayed	Ambion Silencer Select genome siRNA library version 4 (~21,500 genes) (3 siRNA/gene)	Unknown	18 h	% Infection (GFP)	No	N/A	Yes	4 siRNAs >3% GFP$^+$ cells	SAMD9; WDR6; FTSJ1	Unknown	Immunoprecipitation; CRISPR/Cas9; rescue of CRISPR; Western blotting
			Arrayed	Dharmacon On-Target Plus SMARTpool siRNA (17,320 genes) (4 siRNAs pooled/gene)										

Continued

Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
de Wilde et al. (2015)	SARS-Coronavirus SARS-CoA-GFP	293/ACE2	Arrayed	Dharmacon ON-TARGET plus SMARTpool protein kinases siRNA library (779 genes) (4 siRNAs pooled/gene)	48 h	24 h	GFP expression	Yes	Proviral hits $<50\%$ control; normalized viability >0.85	Yes	Antiviral hit $>150\%$ control; normalized viability >0.85	PKR	Translation initiation	Individual siRNAs; Western blot; 90 candidates—mapped to cellular pathways
								90 candidates		40 candidates		COPB2	COPI-coatomer	Specific shRNAs; viral protein expression; KD of related/complex proteins; 40 candidates—mapped to cellular pathways
												PRKCι	Unknown	Small-molecule sodium aurothiomalate; 40 candidates—mapped to cellular pathways
Williams, Abbink, Jeang, and Lever (2015)	HIV-1 VSV-G pseudotyped	HeLa	Arrayed	Library against 59 RNA helicases (3 siRNAs/gene)	Unknown	96 h	Intracellular p24 capsid levels; infectious virion production; luciferase expression	Yes	Decrease all 3 parameters $>20\%$	No	N/A	DDX5; DDX10; DDX17; DDX28; DDX52	Viral replication	Cherry picks screened with WT-HIV-1 (pLAI) virus; Western blot; cell viability
								48 candidates—42 repeat—8 cherry picks—5 confirm WT-HIV-1						
Poenisch et al. (2015)	Hepatitis C virus JcR2a	Huh7.5 Firefly luciferase	Arrayed	Ambion Silencer Select extended druggable genome library V3 (9102 genes) (3 siRNAs/gene)	48 h	72 h	Luciferase expression; production	Yes	<-2 Z score for 2/3 siRNAs	Yes	>2 Z score for 2/3 siRNAs	HNRNPK	Entry/early replication	Meta-analysis with other studies; Dharmacon validation screen; pathway enrichment analysis; known to interact with virus core and related proteins; RT-qPCR; IF/subcellular localization
								78 candidates—40 validate		29 candidates—16 validated			Production	
								263 siRNA pools		130 siRNA pools				

	Perreira et al., (2015)	Human Rhinovirus HRV14	HeLa-H1	Arrayed	SMARTpool Dharmacon (21,121 pools, 3 oligos/pool)	72 h	14 h	% Infectivity (antibody to HRV14 V1 CA protein)	Yes	Infectivity < 50%; viability > 40%	Yes	Infectivity > 150%; viability > 40%	RNASEK	Entry	MORR analysis; RIGER analysis; gene expression filtering; pathway/complex enrichment analysis; other viral analysis IAV (X31H3N2) (WSN/33), DENV (2, 3, 4), YF17D, MLV-VSV, HIV-1-IIIB, MLV-CMV; lifecycle assay; mass spec; immunoprecipitation; acidification studies; immunofluorescence assay; cellular localization assay
				Arrayed	Ambion Silencer Select (21,584 pools, 3 oligos/pool)										
				Arrayed	Sigma esiRNA (15,300 siRNA pools, complex pools)										
				Arrayed	Dharmacon RefSeq27 Revision Pools (4506 siRNA pools/4 oligos/pool)										
shRNA	Yeung, Houzet, Yedavalli, and Jeang (2009)	HIV-1 NL4-3	Jurkat	Pooled	SBI Feline immuno-deficiency virus vector-based shRNA library (54,509 transcripts)	1 week	4 week	Survival	Yes	Survival	No	N/A	NRF1	Entry—Affects co-receptor CXCR4	Reagent redundancy; individual shRNAs; pathway analysis; qPCR; flow cytometry; lifecycle assay
													STXBP2	Viral reverse transcription	
													PRDM2; NCOA2	Transcription	
													EXOSC5	Gag-trafficking	
	Su et al. (2013)	Influenza A virus IAV A/WSN/33	A549	Pooled	TRC RNAi Consortium (81,925 shRNAs) (16,368 genes)	5 days	2 weeks	Survival	Yes 110 genes—38 selected	Survival with 2 unique shRNAs per gene	No	N/A	Itch	Exit endosomes	Western blot; immunofluorescence; RT-qPCR; cellular localization; ubiquitin assay; EST analysis; microarry analysis

Continued

Table 1 Functional Genomic Screens for Elucidating Host–Viral Interactions—cont'd

	Citation	Virus	Cell Line	Pooled/ Arrayed	Library	Knockdown/ Out Time	Challenge Time	Readout	Viral Dependency Factors	Viral Dependency Factor Selection Criteria	Viral Competitive or Restriction Factors	Viral Competitive or Restriction factors Selection Criteria	Main Candidates	Stage of Viral Lifecycle Impacted	Candidate Validation and Follow up Assays
	Tran et al. (2013)	Influenza A virus IAV A/NY/ 55/2004	A549	Pooled	7 decode RNA GIPZ lentiviral positive screening library pools (Thermo)	48 h	72 h	Survival	Yes	Survival	No	N/A	TNFSF12-13; TNFSF13	Late viral replication	Reagent redundancy; RT-qPCR; viability; lifecycle assay; immunofluorescence; flow cytometry; Western blot; other viruses: PR8 (H3N2), pandemic California (H1N1); GO analysis
									1256 candidates—127 selected		20 confirmed		USP47	Entry	
CRISPR/ Cas9	Ma et al. (2015)	West Nile virus WNV	293FT	Pooled	Custom array library oligo pool—PCR amplified-cloned into plasmids—lentiviral vectors—transduced—transfected with Cas9	Expansion time	12 days	Survival	Yes	Multiple independent sgRNAs	No	No	EMC2	WNV-induced death	sgRNA sequences amplified w/nested PCR + sequenced; Western blot; flow cytometry; other viruses WNV-NY99, SLEV
									28,429 sgRNAs with reads more than 10 identified				EMC3		
													SEL1L		

We searched the literature for large-scale genetic screens using human viruses (or components of human viruses) and any of the three functional genomic screening strategies covered in this review. We then provided some of the major characteristics of each individual screen, including the virus, cell line, format, library, screen timelines, selection criteria, any main candidate focused upon, and the assays used for follow up and mechanistic validation if applicable. Not applicable (N/A).

3.1 RNAi Pooled Screening

Retroviral expression of complex cDNA libraries in tissue culture cells predated the arrival of RNAi and was readily adapted to stably express short hairpin RNAs (shRNAs) that were subsequently processed into dsRNAs suitable for directing the destruction of target mRNAs by RISC. Three major pooled retroviral shRNA libraries were initially constructed, the Hannon–Elledge Open Biosystems shRNA library (Paddison et al., 2004; Silva et al., 2005), the RNAi Consortium (TRC) library (Root et al., 2006), both of which are lentiviral and have whole-genome coverage, and a smaller subgenomic gamma-retroviral library, the Bernards shRNA library (Berns et al., 2004), with additional libraries following (Boettcher & Hoheisel, 2010). While differing in their design (Hannon–Elledge-OB being comprised of microRNA-context shRNAs vs. TRC and Bernards being made up of simple shRNAs) these reagents all produce siRNAs resulting in alterations in target gene mRNA expression. Each gene is typically targeted by three or more distinct shRNAs resulting in library complexities of 100K+ unique shRNAs. These pooled shRNA retroviral vectors are then packaged into complex populations of retroviruses (Fig. 1). A population of cells is transduced with the retroviral pools and then the cells are placed under selection to identify any modulations in viral replication conferred by the integrated provirus shRNA. For all pooled library screens, a key point is that each distinct shRNA vector should be overrepresented by ≥ 1000-fold in the selected cell population to minimize bottle neck effects during the screening process; this tenet is also important for the pooled CRISRP/Cas9 screens to be discussed below.

Pooled shRNA screens for host–virus interactions include an early effort to identify HIV-1 host factors required for replication in a T cell line, as well as two screens for IAV host factors (Su et al., 2013; Tran et al., 2013; Yeung et al., 2009). Advantages of pooled screening are its relative low cost and the higher knockdown efficiencies realized using retroviral transduction of cell types that are not readily transfected with siRNAs, e.g., primary cells or suspension cells. In addition longer term screening assays that may require weeks to run are best performed with stably expressed shRNA libraries since transient transfection of siRNAs in dividing cells peaks and falls quickly >7 days posttransfection. The lack of published pooled shRNA screens for virus–host interactions is noticeable and likely stems from the limitations in readout when using a pooled strategy, as well as the issue of phenotypic penetration in the setting of partially decreased gene expression or

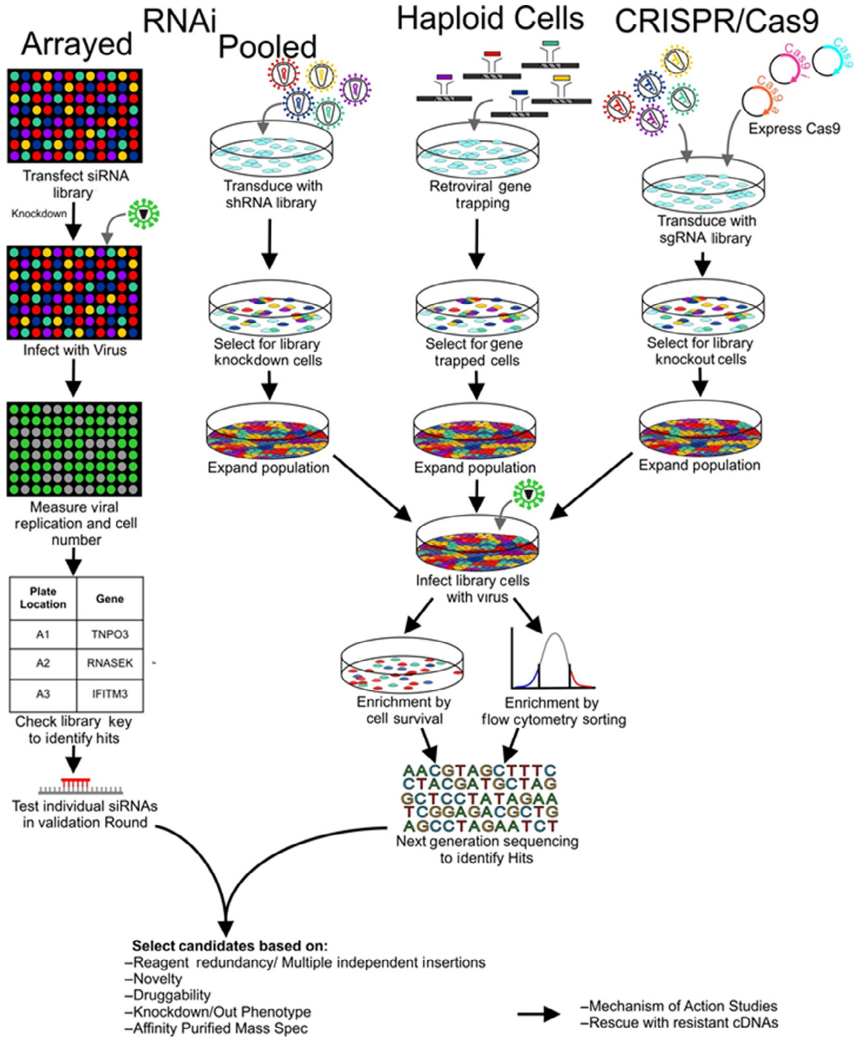


Figure 1 Functional genomic strategies for elucidating host–virus interactions. Schematic of the workflow for each of the three functional genomic screening strategies discussed in this review, RNAi (left) using either arrayed (siRNA) or pooled (shRNA) approaches, haploid cells with retroviral gene trapping (haploid cells, middle), and CRISPR/Cas9, using conventional catalytic (Cas9), CRISPR activators (CRISPRa, Cas9a), or CRISPR repressors (CRISPRi, Cas9i, right). Typical validation and mechanistic studies are outlined at bottom.

hypomorphism. Two prevailing readouts have been used for pooled shRNA screening, flow cytometry-based sorting of cell populations, e.g., high and low expression of viral proteins or a fluorescent marker protein, as a surrogate for infection, as well as survival screens where a cytopathic virus destroys all of the cells that it can infect and spares any cells which are missing a critical host factor, with the survivors undergoing expansion and gene enrichment. The complete loss of gene expression (null phenotype) is unlikely to be achieved using RNAi, and in particular in a population of cells stably transduced with complex shRNA library. This stems from each cell in the screened population expressing only a single shRNA-expressing provirus. Even if a cell is transduced by more than one shRNA-expressing virus, it is highly improbable that both shRNAs will have the same target. It is difficult for a single proviral shRNA to have enough expression to efficiently deplete the mRNA for its intended target. Accordingly, a pooled shRNA screen using a cytopathic virus and cell survival as a means of gene enrichment might not find the host receptor for the virus because there will be some low level of receptor expression remaining (hypomorphism) that could render the cell susceptible to infection and death.

Detecting the shRNAs enriched for at the end of a pooled screen is done using next-gen sequencing technologies which specialize in short reads, combined with informatics programs such a bowtie to assign and quantitate the number of sequencing reads per shRNA in comparison to the starting population. Candidates are selected for follow up based on novelty and on the reagent redundancy principle which states that the likelihood of a gene being a true positive increases as the number of enriched orthologous shRNAs targeting that gene increases (Echeverri et al., 2006). For example, a gene targeted by three independent shRNAs that are enriched in the next-gen sequencing readout is more likely to be a true positive than a gene targeted by only one enriched shRNA. As we will see, the reagent redundancy principle is also important for selection of candidates using all of these functional genomic screening strategies, including the haploid cell screens (number of independent retroviral insertions) (Carette et al., 2009).

3.2 Arrayed RNAi Screening

The high-throughput transfection of arrayed cDNA libraries into mammalian cells for screening predates RNAi and this approach was readily emulated once large-scale arrayed RNAi reagents and appropriate transfection lipids were developed. Pioneering work defining human pathogen interactions was done first using insect cell lines and arrayed siRNA libraries targeting the *Drosophila* mRNA transcriptome (Cherry, 2011; Hao et al., 2008; Sessions et al., 2009). Advantages in using the *Drosophila* system are that the insect cells take up the siRNAs without the need for transfection reagents and that their simpler genetic repertoire may lack functional redundancies which could resist resolution in the more complex human system. Obvious shortcomings are that the findings in the fly cell screens require confirmation in human cells by targeting homologs and that there are human pathogenic viruses that cannot infect fly cells. Thus, a need arose for arrayed RNAi reagents for investigating human pathogenic cells using a human cell-based *in vitro* system. This need was addressed by four life sciences companies; Dharmacon, Ambion, Sigma, and Qiagen, which each introduced their own independently designed whole-genome siRNA libraries.

Methods for performing an arrayed siRNA library screen have been reviewed by us and others in detail elsewhere (Barrows et al., 2014; Chin & Brass, 2013; Panda & Cherry, 2015). Briefly, the project begins with optimizations of both siRNA transfection and infection conditions in the plate format chosen for the screen, with 384-well plates being strongly preferred due to lower amounts of siRNA library needed and the decreased costs and work load using this smaller scale. Once optimized the screen begins with the transfection of the arrayed library in either duplicate or triplicate (Fig. 1); this is usually done in a reverse transfection format with the siRNAs and lipid mixture added to the well first, followed by the cells added in suspension. Target mRNA depletion and decreased protein expression occurs over 1–4 days depending on assay conditions. The longer knockdown periods prior to viral challenge likely improve the observed phenotypes because of increased levels of target protein decay and the dilution effect of added cell divisions. The siRNA-transfected cells are then infected with virus for typically one or two viral lifecycles followed by an assessment of viral replication using either a microscope or plate reader. After the primary arrayed whole-genome screen, the individual siRNAs in the pools of select candidate genes are then rescreened individually in the validation round and the reagent redundancy principle used to select higher confidence genes for follow up.

Arrayed siRNA screening has several advantages over a pooled shRNA approach. For instance, employing an arrayed siRNA library permits shorter term transient transfection-based screens (Fig. 1; Table 2). Additionally the introduction of large effective concentrations of siRNAs into the cells using high efficiency lipid-mediated transfection improves target mRNA depletion producing enhanced phenotypic penetrance. Moreover, by depleting just one-gene-per-well an arrayed screen permits the selection of candidate genes based on more subtle gradations in phenotypes than when using pooled screening readouts. For instance using this format, readouts of viral

Table 2 Strengths and Weaknesses of Functional Genomic Screening Strategies for Human–Virus Interactions

	RNAi Arrayed (siRNA)	RNAi Pooled (shRNA)	Haploid Cells Pooled	CRISPR/Cas9 Pooled
Strengths	• Can use diverse cell lines • High transfection efficiency of adherent cells • Increased sensitivity: arrayed format permits selection of a gradation of phenotypes • Library key permits rapid gene identification • Arrayed format permits screening for viral budding/production • Can perform image-based screens and investigate cell biology phenotypes • Creates hypomorphs permitting many essential genes to be screened • Readily validated using reagent redundancy • Short-term screens <10 days	• Can use diverse cell lines • Viral transduction works better for suspension cells • Good format for suspension cells • Long-term screens (>10 days) • Lower cost than siRNA once the shRNA library is purchased	• Finds receptors, entry factors, and associated genes • High specificity: less false positives • Generates null phenotype • Long-term screens (>10 days) • Low cost to perform survival screens	• Can use diverse cell lines • High specificity: less off-target effects • Generates null phenotype • Viral transduction works better for suspension cells than transfection • Good format for suspension cells • Finds receptors, entry factors, and associated genes • High specificity • Long-term screens (>10 days) • Can inhibit or activate gene expression (CRISPRa and CRISPRi) • Active in the nucleus • Can remove large sections of a targeted locus (e.g., inactivate lncRNA genes) • First-generation reagents graciously shared at low cost on Addgene • Low cost to perform survival screens

Continued

Table 2 Strengths and Weaknesses of Functional Genomic Screening Strategies for Human–Virus Interactions—cont'd

	RNAi Arrayed (siRNA)	RNAi Pooled (shRNA)	Haploid Cells Pooled	CRISPR/Cas9 Pooled
Weaknesses	• Off-target effects • False negatives • Hypomorphs can produce false negatives • Loss-of-function only • RISC has questionable or limited activity in the nucleus • Difficult to transfect primary cells or suspension cells • Difficult to use suspension cells in an arrayed format • Expensive to purchase, use, and maintain libraries • Requires expensive high-throughput microscope or plate reader for analysis	• Off-target effects • False negatives • PCR/next-gen sequencing needed to identify hits • Loss-of-function only • RISC has questionable or limited activity in the nucleus • Cannot do cell biology or imaging screens • Target knockdown more difficulty due to only one shRNA-producing provirus per cell	• Random insertion mutagenesis cannot specifically target a gene • Only two available haploid cell lines • PCR/next-gen sequencing needed to identify hits • Loss-of-function only • Retroviral insertion bias may not permit saturation • Cannot do cell biology or imaging screens • Arrayed format is subgenomic and requires long-term culturing and storage of many thousands of cell lines with likely high cost	• PCR/next-gen sequencing needed to identify hits • Relatively slower validation • Cannot do cell biology or imaging screens • Arrayed lentiviral format will be cumbersome • Arrayed transfectable CRISPR components (sgRNAs, Thermo, and IDT) are subgenomic at present with whole-genome reagents likely obtained at high cost

protein expression, or the expression of a luciferase reporter gene, can be assessed with great sensitivity using high-throughput microscopes or plate readers. Having each gene targeted in its own designated well also creates a homogenously genetically altered population of cells that can be assessed using high content imaging, thus allowing cell biology phenotypes involved in host virus interactions (i.e., RNA virus replication complex morphology) to be screened for in great detail, something which is not possible using a pooled screening strategy. Last, using arrayed annotated libraries allows the immediately identification of which gene may underlie the observed phenotype. Disadvantages of using such an approach include the increased expense of having to purchase, array and maintain these large-scale resources, the analytical machinery needed to carry out and analyze the great number of plates produced by the screen, and the added costs for transfection and screening reagents. Finally, both the siRNA and shRNA screens have major limitations due to their high rates of false positives and false negatives; this last concern regarding the significant caveats of siRNA screening, as well as some corrective measures, are more fully discussed below.

The original Dharmacon arrayed human siRNA library, siGENOME, consists of pools of four 19-mer siRNAs (SMARTpools) designed against each of the 21,141 annotated human genes in RefSeq5–8, one gene per well. A later version, On-target-plus (OTP), was similarly constructed but with selective modification of some of the siRNA's base pairs with the intent of minimizing OTEs created by the first eight base pairs of the antisense, the seed sequence, or the sense-strand pairing with microRNA elements thereby unintentionally altering gene expression. Although useful, the antisense OTP reagents likely have a lower affinity for their intended targets which may explain their loss of efficacy compared to matched siGENOME reagents tested side-by-side for depletion of known positive controls (our unpublished data). An updated SMARTpool siGENOME library based on Refseq27 (Dharmacon 6–16) was constructed in a similar manner and has recently replaced the earlier library. An advantage of the SMARTpool library is that four siRNAs are available for validation round screening. A shortcoming is that the available siRNAs for reorder postscreening are continually changing over making it costly to order the exact siRNAs that scored in the original screen.

The Ambion Silencer Select library targets 21,584 genes using three siRNAs in an arrayed format, one siRNA per well with three total wells for each gene. The arrayed library can be readily converted to pools based on the way it is plated, with the same well on three matching plates (A, B, C) containing a different siRNA targeting the same gene. An advantage of individual siRNA arrayed screening is that candidate selection for follow up can be done immediately after the primary screen based on reagent redundancy, the disadvantage is that three times more reagents are needed to screen the individual siRNA arrayed Silencer Select library. Importantly, Silencer Select siRNAs mark a major advancement in siRNA design as they incorporate locked nucleic acids (LNAs) which increase antisense strand binding affinity to designed targets and inhibit sense-strand binding thereby decreasing OTEs (Puri et al., 2008). As with the SMARTpool library the three individual siRNAs available for the validation round are useful and Ambion maintains a consistent supply of the library oligos that can be reordered, with new potentially improved siRNAs being added without replacing the original library set.

Endonuclease processed siRNA (esiRNA) pools against most human genes are available individually as well as in genome-wide libraries from Sigma. esiRNA pools were originally developed by the Buckholz lab and consist of complex heterogeneous mixtures of overlapping siRNAs (18–25 base pairs in length) targeting the same mRNA sequence (Kittler et al., 2007). esiRNA pools are created using endoribonuclease to digestion of RNA transcribed *in vitro* from 200–400 base pair cDNA templates. Using this strategy concentration-dependent OTEs are anticipated to be less than using conventional siRNA pools or individual oligos. Since the pools cannot be deconvoluted into a few known components, validation is carried out using a distinct esiRNA pool against the same gene. While useful this approach is limited in terms of its level of reagent redundancy. Furthermore, although the relative concentrations of the individual esiRNA pools in the library are closely matched, the final sizes of the digested product vary leading to an induction of dsRNA-mediated antiviral response that precludes their use with some viruses which are vulnerable to such a defense, e.g., dengue virus.

3.3 RNAi Screening Problems and Some Solutions

RNAi screens are powerful and readily implemented discovery tools but suffer from shortcomings arising from their high levels of false negatives and false positives (OTEs) as can be seen when comparing the low concordance among the candidate genes detected in different screens using the same species of virus, e.g., HIV-1, HRV, or IAV (Booker et al., 2011; Bushman et al., 2009; Hao et al., 2013; Perreira et al., 2015; Zhu et al., 2014).

To address these concerns, improvements in the design and synthesis of next-gen RNAi library reagents have been implemented including the elimination of siRNAs with seed sequences that are complementary to microRNA binding sites (Knott et al., 2014; Mohr & Perrimon, 2012; Petri & Meister, 2013). As noted, the seed sequences of the nontargeting siRNA sense strands have had their binding affinity decreased by selectively incorporating methylated or LNA nucleotides. Significant efforts have also been put into validating the siRNAs to find and remove ones that are ineffective and contribute to false negatives.

OTEs in particular must be rigorously controlled for by using reagent redundancy combined with complementation or rescue experiments and an assessment that target depletion and phenotype are proportional (Echeverri & Perrimon, 2006; Echeverri et al., 2006; Mohr & Perrimon, 2012). While a consistently low number of exact genes overlap across related siRNA screens, it is nonetheless clear that similar screens find bioinformatically related genes, e.g., genes that cluster in common pathways and complexes like the nuclear pore complex (NPC) with HIV-1 and the vacuolar ATPase (V-ATPase) for IAV or HRV (Bushman et al., 2009; Hao et al., 2013; Perreira et al., 2015; Stertz & Shaw, 2011; Zhu et al., 2014). With closer study it became readily apparent that this low level of saturation within the dataset of each primary screen was due to a high level of false negatives (Hao et al., 2013; Meier et al., 2014; Zhu et al., 2014). False negatives with RNAi may come about for several reasons including difficulty in targeting a protein (prolonged protein half-life or sufficient remaining catalytic activity), nonspecific toxicity of siRNAs, and plate edge effects. These interscreen comparisons also highlight the importance of a post hoc bioinformatic analysis across multiple related screens (meta-analysis) to provide a systems level understanding of viral dependencies. Additionally, candidate genes that score poorly in reagent redundancy validation assays, e.g., only confirming the phenotype with one of four possible siRNAs, are more likely to represent true positives if they physically or functionally interact with candidate genes that are members of enriched clusters. Consequently, bioinformatics can find useful associations that may save a potentially informative candidate gene from down selection.

RNAi screens have revealed the host cell requirements of many human viruses (Table 1), however, they are beset by false positives and false negatives. We reasoned that by using multiple orthologous RNAi reagents (MORR) in parallel we could take advantage of each large-scale reagent's best characteristics while minimizing their worst. With this in mind, we used

MORR screens (Silencer Select, SMARTpool, and esiRNA libraries) to identify high-confidence HIV-1 dependency factors (HDFs) or HRV host factors (HRV-HFs) (Perreira et al., 2015; Zhu et al., 2014); these three libraries are >90% orthologous based on a comparison of siRNA sequences. We then traditionally validated the candidates from each of the primary screens. In addition, we integrated the primary MORR datasets, and those of earlier studies in the case of HIV-1, by adapting an established analysis method, RNAi gene enrichment ranking (RIGER) (Luo et al., 2008). RIGER uses a weighted likelihood ratio to calculate a gene-specific enrichment score based on the rank distribution of each individual RNAi reagent across all of those screened. The RIGER enrichment score is expressed as a *p* value assigned to each gene which represents the likelihood that the gene plays a role in viral replication. By integrating the entire primary screen datasets RIGER also decreases false negatives created by the combination of hypomorphism and the use of absolute cutoffs for candidate selection. Both these projects represented two of the most comprehensive siRNA screening efforts to date and produced quantitatively integrated datasets for each virus which highly ranked both known viral dependency factors and previously unappreciated ones. To assess if MORR/RIGER improves the yield from the screen as compared to a more traditional screening approach, we assessed each respective dataset (RIGER (all screens integrated) and each of the individual MORR screens) for their enrichment of a set of annotated gene complexes or pathways. The annotated gene sets were selected because there was significant enrichment of their components across the individual screens (e.g., the NPC for HIV-1 or the 80S ribosome for HRV (Perreira et al., 2015). These comparative enrichment analyses quantitatively demonstrated that the MORR/RIGER approach produces a data set which is statistically better in its enrichment for expected host factors than any of the individual screens on their own. Since this approach is more sensitive and specific in finding known host factors, we conclude that it would also be the best method for detecting previously unappreciated host–virus interactions.

To further improve siRNA screening, we and others have decreased OTEs by using the method of gene expression filtering to remove candidates that are not found to be expressed in the cell line used for the screen based on either microarray assays or next-gen sequencing (Perreira et al., 2015; Zhu et al., 2014). OTEs in siRNA screens are also detected and removed using OTE identification programs, for instance, the genome-wide enrichment of seed sequence matches (GESS) method (Sigoillot et al., 2012). GESS is premised on the knowledge that OTEs are the result of siRNA seed sequences binding to mRNAs other than the intended target or by siRNAs inadvertently binding to microRNA sites. GESS detects prominent OTEs by searching for matches between the RefSeq mRNAs and the seed sequences of the siRNAs that confirm in the validation round. The negative control consists of a scrambled set of the validation round seed sequences. mRNAs that are more often complementary to the validation round siRNA seed sequences than the scrambled sequences are flagged as suspicious for being an OTE and removed from further evaluation. Collectively, MORR/RIGER screening combined with gene expression filtering, and OTE identification minimizes the caveats of RNAi screening thus improving its efficiency and yield.

4. HAPLOID CELL GENETIC SCREENING TECHNOLOGY AND APPROACH

The creation of haplo-insufficiencies using retroviral gene trapping has been and continues to be useful for mammalian genetic screening (Dziuba et al., 2012; Evans, Carlton, & Russ, 1997; Organ, Sheng, Ruley, & Rubin, 2004; von Melchner & Ruley, 1989); however, this approach is limited due to its inability to produce homozygous null mutations. This shortcoming was overcome through the introduction of a near-haploid cell line, KBM-7, for use in genetic screens where the remaining allele is inactivated using random retroviral insertion mutagenesis (Carette et al., 2009). KBM-7 cells originated from a 39-year-old gentleman with chronic myelogenous leukemia (CML) and were first reported by the McCredie lab (Andersson et al., 1987), with later isolation of a clonal population of near-haploid cells (2 copies of chromosome 8 and partial disomy of chromosome 15) by Kotecki, Reddy, and Cochran (1999). Haploid cell screens concerned with human–virus interactions have primarily been used in pooled screening approaches involving strong selective pressure by cytopathic viruses, either wild type or recombinant (Table 1). After transduction and selection for a retrovirally expressed selection marker, the cells are cultured to permit phenotypic penetrance via protein turnover and divisional dilution then infected with a cytopathic virus with the rolling infection leading to the destruction of any permissive cells (Fig. 1). The surviving cells are then expanded and the respective integration site of the proviruses are determined using PCR and next-gen sequencing. Genes which are found to have multiple independent insertions are selected as high-confidence candidates using a rationale similar to the reagent redundancy principle employed for selecting candidates in RNAi screens. While powerful, an acknowledged shortcoming of this approach is that it can only be done using a haploid cell line, which may not be readily infected by a human pathogen of interest, e.g., HBV. In an effort to overcome this limitation the KBM-7 cells were genetically reprogrammed, and while the result was not the desired induced pluripotent stem cell line, this work nevertheless gave rise to a more fibroblast like cell line, HAP1 (Carette et al., 2010), that demonstrates adherent growth as compared to the KBM-7 cells, which grow in suspension. The class of host factors predominantly found by the haploid cell screens to date is discussed below.

5. CRISPR/Cas9 GENETIC SCREENING TECHNOLOGIES AND APPROACHES

To defend themselves, bacteria and archaea employ an adaptive immune response using short guide RNAs (sgRNAs) to target and destroy the DNA of invading pathogens (Doudna & Charpentier, 2014). This protective response, known as the CRISPR/Cas9 system, has been adapted for genome editing and the regulation of gene expression in multiple model systems including genome-wide mammalian *in vitro* genetic screening (Cong et al., 2013; Doudna & Charpentier, 2014; Shalem et al., 2014; Wang, Wei, Sabatini, & Lander, 2014). Because Cas9 acts on genomic DNA and not mRNA like RISC, this permits the generation of a permanent homozygous null phenotype. The CRISPR/Cas9 system works in all mammalian cells exogenously expressing Cas9, this combined with its gene targeting specificity make this approach more generalizable than haploid cell screens (Ran et al., 2013). Importantly, because Cas9 locates and binds to a determined DNA target via the complementary base pairing of a short guide RNA (sgRNA), a catalytically inactive Cas9 fused to an activation or repressor domain can bind a desired locus and modulate its gene expression, this capability is extremely powerful and has not been possible using RNAi or haploid cell-screening approaches (Gilbert et al., 2014; Qi et al., 2013) (Table 2). What's more, because a single integrated provirus expressing a sgRNA can, together with Cas9, permanently extinguish a gene's expression, it avoids the same mass action handicap that confronts a single shRNA-expressing provirus whose task is never completed as it must continually silence the products of ongoing transcription. It follows then that under pooled genetic screening conditions, where only one provirus is present per cell, CRISPR/Cas9 will produce greater phenotypic penetrance (Table 2). Several studies have found that while OTEs do occur using CRISPR/Cas9 they appear to be less prevalent than the levels of OTEs encountered with RNAi (Cho et al., 2014; Wang et al., 2015; Wu et al., 2014). Engineered Cas9 proteins with improved specificity also promise to make false positives even rarer (Slaymaker et al., 2016). In order to control for OTEs produced by inadvertent gene editing events the standard for validation of CRISPR/Cas9 results has become similar to RNAi's reagent redundancy principle with the results from two or more orthologous sgRNA against the same gene or two or more clones required. As with RNAi the most convincing confirmation is phenotypic restoration via the expression of a resistant cDNA.

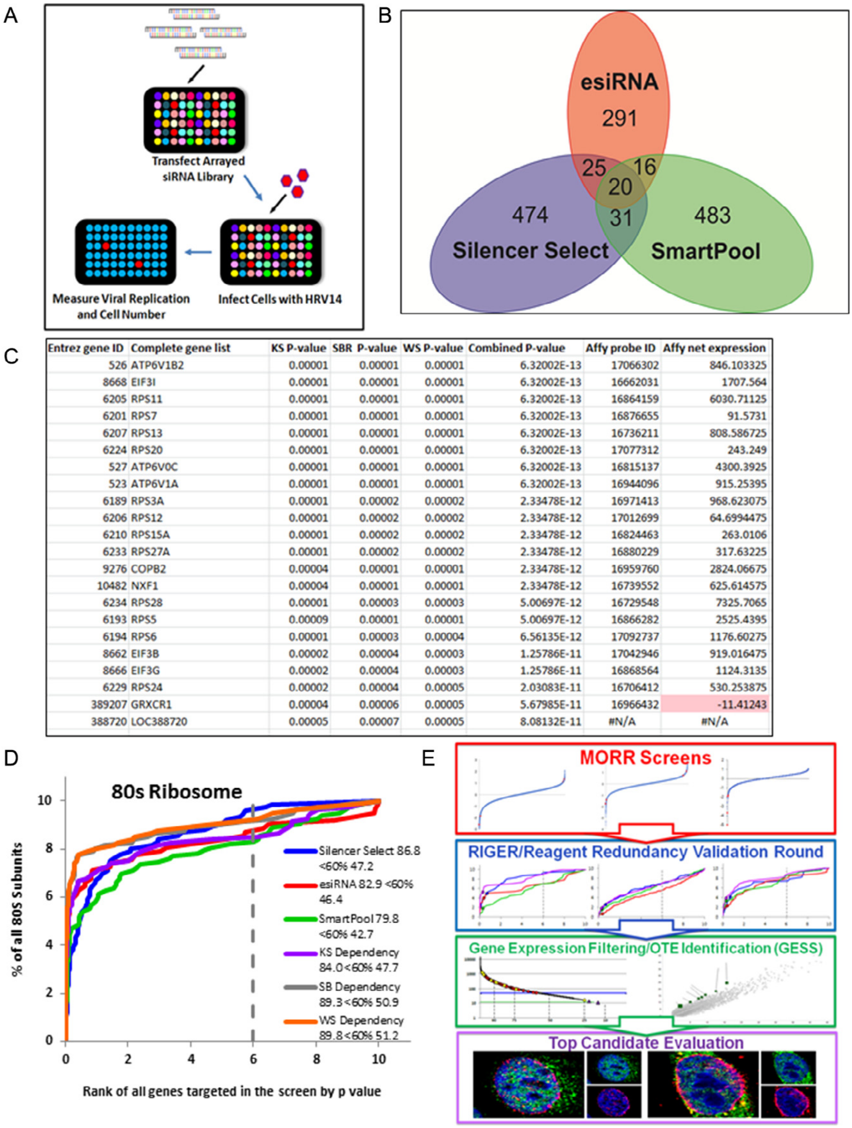
CRISPR/Cas9 screens require the expression of Cas9 in the target cells (Fig. 1). Cas9 expression can be transient, inducible, or stable. If transient expression is chosen then the cells must already express the sgRNA library (Shalem et al., 2015; Wang et al., 2014). The exogenously expressed Cas9 can be either catalytically active and create null alleles, or a catalytically inactive protein fused to one of several transcription factor domains for activation or repression of the sgRNA-targeted locus (Gilbert et al., 2014; Qi et al., 2013). Pooled sgRNA retroviral vectors designed to target every human gene are then packaged into retroviruses and used to stably transduce the Cas9-expressing target cells at a high representation (goal of 1000-fold, Fig. 1). The transduced cells are placed under selection for two weeks to permit the phenotypic maturation. The gene-edited cells are then challenged with the virus of interest, with either cell survival or protein expression based selection or readout. The selected cells are expanded and the identities of enriched sgRNAs are obtained using next-gen sequencing of PCR products amplified from genomic DNA.

CRISPR/Cas9 promises to revolutionize genetic screening, however, due to its recent arrival published screens for host–virus interactions have been limited, but will likely expand greatly in short time. An early effort used CRISPR/Cas9 strategy to identify host factors that govern West Nile virus' (WNV's) cytopathic effect (Ma et al., 2015). An earlier WNV host factor arrayed siRNA screen had discovered a few hundred high-confidence candidates using viral protein expression (GFP transgene) as a readout (Krishnan et al., 2008). This much earlier siRNA screen was also stopped well before any cytopathic effect was appreciated. Not surprisingly the candidate gene overlap between the two efforts was small in part arising from the different endpoints, cell survival versus viral protein expression. Interestingly, the

CRISPR/Cas9 screen found that the EMC complex, a conserved set of ER-associated proteins implicated in transmembrane protein expression and lipid trafficking was required for WNV's cytopathic effect but not its replication (Wideman, 2015).

6. COMPARISON OF HRV-HF SCREENS: ARRAYED MORR RNAi VERSUS POOLED CRISPR/Cas9

To date, RNAi screens have been the primary method used for human–virus loss-of-function genetic screens (Table 1). CRISPR/Cas9 is a newly arrived powerful functional genomic technology which can create homozygous null alleles for each human gene. We wished to compare these two approaches, arrayed MORR RNAi versus pooled CRISPR/Cas9, using the same screening platform involving a fully infectious cytopathic HRV strain, HRV14, and H1-HeLa cells that endogenously express the HRV host receptor, ICAM1. We first performed an image-based MORR/RIGER screen to find HRV14-HFs that modulate replication using viral V1 capsid (CA) expression as determined by an immunofluorescence readout (Fig. 2A). For the screens, we transfected a final concentration of each siRNA pool at 50 nM final concentration for 72 h then challenged the cells with HRV14 at an multiplicity of infection (moi) of 0.3 for 12 h at 33 °C. The replication cycle of HRV14 is approximately 8 h. To evaluate cell numbers the HeLa cell nuclear DNA was stained with Hoechst 33342. Magnified images of each well were captured in two wavelengths (FITC and DAPI) using a high-throughput microscope (ImageXpress Micro-XL, Molecular Devices) and the percent infected H1-HeLa cells calculated using image analysis software. These parallel efforts identified >160 high-confidence candidates across the MORR screens using the Silencer Select, SMARTpool, and esiRNA libraries (Perreira et al., 2015). As seen with ours and others previous siRNA functional genomic screens, the number of exact genes identified across more than one primary screen dataset was low (Fig. 2B). Of interest is that in this instance the only factor that was different between the compared screens was the different siRNA libraries we used, demonstrating the marked influence of the targeting reagents in the observed lack of interscreen concordance. The primary screen candidates were traditionally validated using their respective deconvoluted individual siRNAs (Silencer Select pools with three siRNAs and SMARTpools with four siRNAs), or by retesting the esiRNA pools, in a manner identical to the primary screen (viral capsid expression). As is outlined above, we addressed



Entrez gene ID	Complete gene list	KS P-value	SBR P-value	WS P-value	Combined P-value	Affy probe ID	Affy net expression
526	ATP6V1B2	0.00001	0.00001	0.00001	6.32002E-13	17066302	846.103325
8668	EIF3I	0.00001	0.00001	0.00001	6.32002E-13	16662031	1707.564
6205	RPS11	0.00001	0.00001	0.00001	6.32002E-13	16864159	6030.71125
6201	RPS7	0.00001	0.00001	0.00001	6.32002E-13	16876655	91.5731
6207	RPS13	0.00001	0.00001	0.00001	6.32002E-13	16736211	808.586725
6224	RPS20	0.00001	0.00001	0.00001	6.32002E-13	17077312	243.249
527	ATP6V0C	0.00001	0.00001	0.00001	6.32002E-13	16815137	4300.3925
523	ATP6V1A	0.00001	0.00001	0.00001	6.32002E-13	16944096	915.25395
6189	RPS3A	0.00001	0.00002	0.00002	2.33478E-12	16971413	968.623075
6206	RPS12	0.00001	0.00002	0.00002	2.33478E-12	17012699	64.6994475
6210	RPS15A	0.00001	0.00002	0.00002	2.33478E-12	16824463	263.0106
6233	RPS27A	0.00001	0.00002	0.00002	2.33478E-12	16880229	317.63225
9276	COPB2	0.00004	0.00001	0.00001	2.33478E-12	16959760	2824.06675
10482	NXF1	0.00004	0.00001	0.00001	2.33478E-12	16739552	625.614575
6234	RPS28	0.00001	0.00003	0.00003	5.00697E-12	16729548	7325.7065
6193	RPS5	0.00009	0.00001	0.00001	5.00697E-12	16866282	2525.4395
6194	RPS6	0.00001	0.00003	0.00004	6.56135E-12	17092737	1176.60275
8662	EIF3B	0.00002	0.00004	0.00003	1.25786E-11	17042946	919.016475
8666	EIF3G	0.00002	0.00004	0.00003	1.25786E-11	16868564	1124.3135
6229	RPS24	0.00002	0.00004	0.00005	2.03083E-11	16706412	530.253875
389207	GRXCR1	0.00004	0.00006	0.00005	5.67985E-11	16966432	-11.41243
388720	LOC388720	0.00005	0.00007	0.00005	8.08132E-11	#N/A	#N/A

Figure 2 MORR/RIGER screen for HRV host factors. (A) The HRV-HF siRNA screen workflow showing the transfection of the arrayed MORR libraries, the challenge with HRV14 and the assessment of viral capsid expression and cell number using high-throughput imaging (Perreira et al., 2015). (B) The total number of primary screen candidates found in each of the MORR screens along with the number of exact genes that overlap across two or three of the screens is provided. (C) The ranked RIGER weighted sum (WS), second best (SB), and Kolmogorov–Smirnov (KS) analyses of the MORR HRV screen datasets with their respective individual and combined *p* values. The gene

(Continued)

the problems with siRNA screening by using these three libraries together with the RIGER analysis method to integrate all of the HRV-HF primary screen data sets; this permitted us to assign a numeric value for the likelihood that each gene was important for HRV replication (*p* value, Fig. 2C). KS, SBR, and WS represent three different RIGER methods; we found that the SBR and WS methods performed the best across multiple gene test sets (Fig. 2D). Our MORR screening approach was validated by the significant enrichment of multiple pathways and protein complexes in the respective screens (e.g., the 80S ribosome), as well as an improvement in these benchmarks when the datasets were integrated using RIGER (Fig. 2D) (Perreira et al., 2015). We also used gene expression filtering to remove candidates that were not expressed in the cells used for the screens, e.g., GRXCR1, whose net expression value is highlighted in red (Fig. 2C). The complete MORR/RIGER work flow extending from the primary screens through to top candidate evaluation is shown (Fig. 2G).

To compare screening strategies, as well as perform an orthologous investigation of HRV14's human cell requirements, we next carried out a CRISPR/Cas9 screen using the exact same cell line and virus. We report this CRISPR/Cas9 HRV14 screen here for the first time. We stably expressed a human codon-optimized cDNA of *S. pyogenes* Cas9 in a population of HeLa-H1 cells (Fig. 3A) (Shalem et al., 2014). After selection with hygromycin, the cell population was tested for Cas9 expression by immunoblotting as well as the ability to satisfactorily extinguish the expression

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Figure 2—Cont'd expression data (Affy net expression) is also given based on a microarray analysis of mRNA from the H1-HeLa cells used in the screen. The filled box indicates a gene, GRXCR1, whose expression was found to be below the lower cutoff for candidate selection and thus represents an OTE. (D) The RIGER analyses (WS, SB, and KS) and the individual MORR screen datasets were assessed by determining their respective levels of enrichment for an annotated list of 80S ribosome protein components. A numeric enrichment score was calculated by determining the area under the curve (AUC) produced by plotting the percent fraction of 80S component proteins (% of all 80S subunits) encountered moving from the lowest to highest *p* value on the ranked gene lists (rank of all genes targeted in the screen by *p* value). Numbers represent the percent enrichment of the total gene set at <60% of the ranked gene list (Perreira et al., 2015). (E) A schematic of the workflow for the MORR/RIGER screening approach with the primary MORR screens, integrative RIGER analysis, and traditional reagent redundancy validation round shown. False positives are decreased using gene expression filtering and OTE identification using GESS (Sigoillot et al., 2012). This combined strategy minimizes both false positive and false negatives and is useful for identifying high-confidence HRV-HFs.

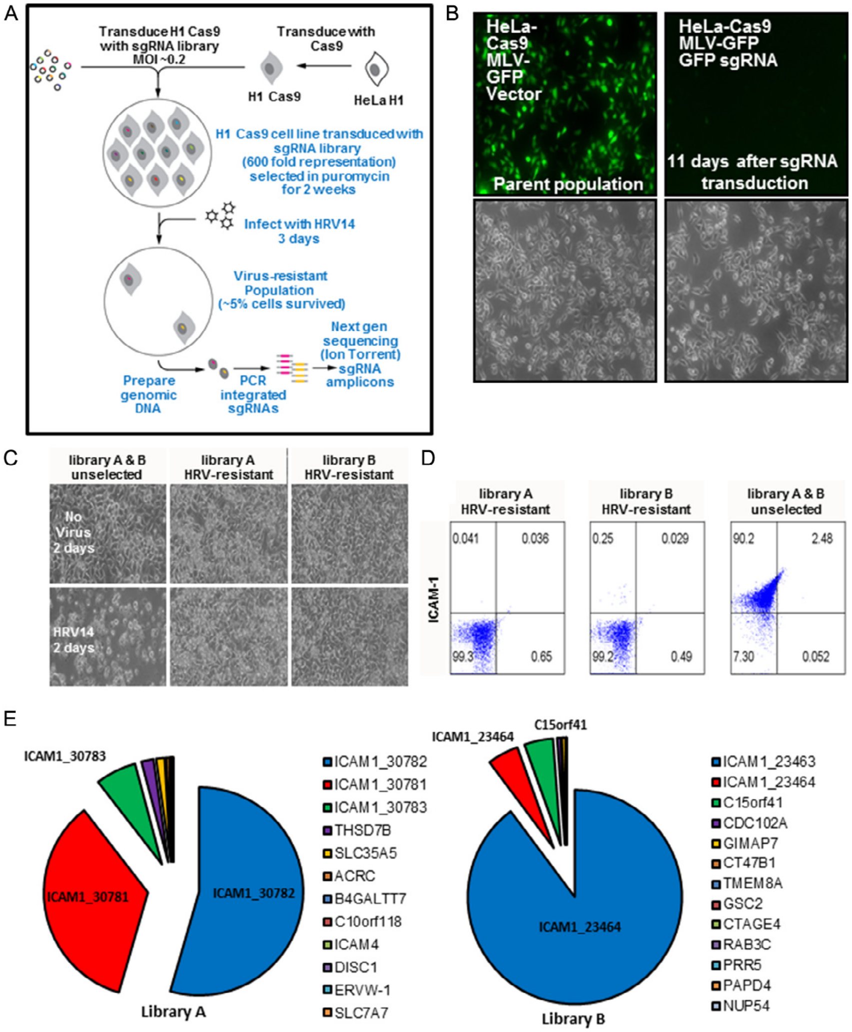

Figure 3 CRISPR/Cas9 screen for HRV host factors. (A) The HRV-HF CRISPR/Cas9 screen workflow showing the generation of the Cas9 expressing H1-HeLa cells containing the sgRNA libraries followed by their subsequent challenge with HRV14 and the assessment of the enriched sgRNAs using next-gen sequencing. (B) HeLa-H1-Cas9 cells were transduced with Moloney Leukemia virus (MLV)-GFP, then supra-transduced with either an empty vector control (parent population) or one expressing a sgRNA against GFP. The cells were selected for puromycin resistance and cultured for 11 days then fixed and imaged for GFP expression. Differential interference contrast (DIC) images are provided below. 4 × magnification. (C) DIC images of cells transduced with either library A or B that survived the HRV14 challenge were expanded and tested for their susceptibility to HRV14's cytopathic effect over 2 days (bottom row) compared to the unselected parent cell population and the respective uninfected cell populations (top row). (D) Cells

(Continued)

of the endogenous HRV14 receptor, ICAM1, and a provirus expressing green fluorescent protein (GFP) using a sgRNA against each respective target (Fig. 3B, data not shown). Next, we stably transduced the H1-HeLa-Cas9 cells at a moi of 0.2 with a complex lentiviral pool expressing the human GeCKO v.2 sgRNA library (Addgene #1000000049), which targets 19,052 genes in the human genome with six sgRNAs per gene across two half-libraries (library A and B) (Shalem et al., 2014). Libraries A and B each possess three unique sgRNA per gene and we used the two half-libraries to screen for HRV14-HFs independently. For each library, we plated 4×10^7 cells onto two 15-cm dishes to achieve a 600-fold representation of each sgRNA in the final cell population. We empirically determined this level of representation using a series of titration plates that were infected and processed side-by-side with the sgRNA library-expressing cells. We then selected the cells in puromycin for 11 days, a period of time which we had empirically determined to result in >80% of cells losing expression of a sgRNA-targeted marker protein (GFP, Fig. 2B) The selected cells were then infected with HRV14 and cultured at 33 °C for ~7 days. To follow the progress of the infection, cytopathic effect (CPE) was monitored by eye using light microscopy. Control plates were run in parallel using the H1-HeLa-Cas9 cell parent population which does not contain the GeCKO library. About 7 days after infection the majority of cells, >95%, had died. The remaining surviving cells were washed extensively and transferred to 37 °C with fresh medium.

The surviving cells were expanded and genomic DNA prepared. No surviving cells were recovered from the control parental cell plates. Proviruses containing the sgRNA stably integrated into each of the surviving cells were amplified and identified from genomic DNA using PCR and next-gen sequencing using an Ion Torrent sequencer. Sequencing reads (reads) were trimmed at their sgRNA boundaries and mapped back to the complete sgRNA entries for both library A and B using Cutadapt, Bowtie2, and Samtools. This process allowed us to map and rank the frequency of 1153 unique reads from a total of 3,961,083 total reads. We also tested the

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Figure 3—Cont'd from (C) were fixed and immunostained for ICAM1 surface expression by flow cytometry. (E) A chart showing the relative proportion of total sequencing reads for the recovered sgRNAs from the HRV14 CRISPR/Cas9 pooled screen based upon the analysis of genomic DNA from the surviving cells from library A or B. Gene names are provided for each sgRNA with the associated numbers designating their unique identifying library number.

expanded surviving cells for their susceptibility to HRV14 infection and found that the postscreen population of cells was highly resistant to viral CPE (Fig. 3C). Analysis of the resistant cell populations by flow cytometry showed the near complete absence of the HRV14 receptor, ICAM1, on the cell surface, which is in stark contrast to the pre-screen parent cell population (Fig. 3D). Similar to RNAi screens, we next used the reagent redundancy principle to select for candidate genes which had >6 sequencing reads for two or more independent sgRNAs. Among the unique sgRNAs detected by next-gen sequencing only two genes presented with more than two independent sgRNAs, ICAM1 (five of six total sgRNAs recovered) and EXOC4 (two of six total sgRNAs, Fig. 3E). Of the 3.9 million total reads >95% mapped to one of the five sgRNAs targeting ICAM1. Of these two candidates only ICAM1 overlapped with the MORR/RIGER screen HRV-HF candidate list (Fig. 4).

The comparison of these two screening approaches side-by-side, using the same cells and virus, raises an interesting point. The number of host factors found for HRV14 was far greater using the MORR/RIGER approach and is approaching a systems level understanding based on bioinformatic analyses and the near saturation of, or enrichment for, multiple complexes and pathways (Fig. 4) (Perreira et al., 2015). By comparison our matched pooled CRISPR/Cas9 screen for HRV-HFs yielded two high-confidence candidates based on reagent redundancy, ICAM1, the known receptor for HRV14, and EXOC4, a gene involved in exocyst targeting and vesicular transport (He & Guo, 2009). Given the known role of ICAM1 as the host receptor for most HRVs, these results point to entry as the major viral lifecycle stage interrogated by a pooled functional genomic screening approach using a population of randomly biallelic null cells infected by a cytopathic virus.

Our CRISPR/Cas9 screen results are not surprising given the predilection of earlier pooled haploid cell survival screens for finding viral entry-associated factors, including host receptors, genes required for receptor modification or endosomal trafficking (for example, the HOPS tethering complex, Table 1) (Carette et al., 2011). Therefore, while conventional catalytic CRISPR/Cas9 and haploid cell-screening technologies use different strategies for creating loss-of-function alleles, their shared method of screening complex pools of cells for survival likely leads to similar results. For an illustration, we note the IAV haploid cell screen and two additional haploid cell survival screens which identified the host receptors for Lassa virus and Ebola virus using similar pooled strategies to those being employed with

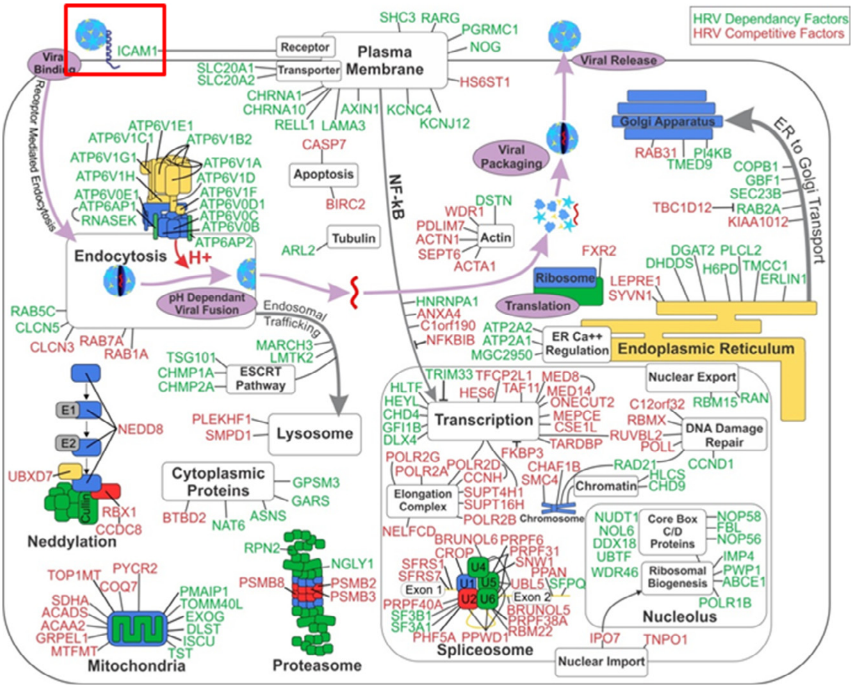

Figure 4 MORR and CRISPR/Cas9 HRV-HF screen candidate overlap. We used the RIGER analysis of the HRV-HF MORR screens to produce a speculative model cell showing the HRV lifecycle overlayed with where the top 164 high-confidence candidate HRV-HFs are likely to act based on available published data (Perreira et al., 2015). A single HRV-HF candidate, ICAM1, shared between the arrayed MORR/RIGER siRNA screen and the matched pooled CRISPR/Cas9 screen, is highlighted with a box. *The authors own all the figures included from published work (Perreira et al., 2015), under a creative commons license agreement with* Cell Reports.

CRISPR/Cas9 screens (Carette et al., 2009, 2011; Jae et al., 2013). Interestingly, the latter two haploid cell screens used identical recombinant vesicular stomatitis viruses (rVSVs) with the exception of their respective envelope proteins, Lassa virus or Ebola virus. Notably there was not a single candidate gene that was found in common between these two pooled screens, arguing that under such conditions only a total block to VSV entry can confer cell survival. A factor which may cause pooled screens to strongly enrich for entry-associated host factors is the intense selective pressure that the cells are subjected to as the levels of virus surge during the course of the screen. It is possible that even with the loss of a reasonably important postentry viral dependency factor that at such a high moi the overwhelming entry of so many viruses alone, even with some diminishment of their replication, would be sufficient to elicit apoptosis or exit from the cell cycle. This last notion is supported by two independently performed arrayed siRNA screens which respectively reported 301 and 72 high-confidence candidates necessary for VSV replication, many of which were involved in postentry phases of the viral lifecycle; none of these candidates were found in the rVSV-based haploid cell screens. Interestingly one of the screens found that coatomer (COP1) and the V-ATPase were required for VSV replication. COP1 and the V-ATPase are essential complexes which would be not be recovered in a haploid cell or CRISPR/Cas9 screen using cells with null phenotypes.

In the exemplary study by Petersen et al. for Arena virus (ANDV) host factors, the authors performed matching haploid cell and arrayed RNAi screens (Petersen et al., 2014). As with the Ebola and Lassa haploid cell screens above, the researchers engineered an rVSV which expressed the ANDV glycoprotein receptor (rVSV-ANDV) on its surface. One billion HAP1 cells were retrovirally mutagenized and screened for survival after infection with either rVSV-ANDV or a matched control virus, rVSV-G, which expressed the VSV-G receptor. After selection, the group expanded the surviving cells and used their pooled genomic DNA to identify 676 independent integrations sites. Of these sites, 37% occurred within four genes: regulatory element binding protein 2 (SREBF2), sterol regulatory element-binding protein cleavage-activating protein (SCAP), site 1 protease (S1P), and site 2 protease (S2P), all of which belong to the sterol regulatory element-binding protein pathway. A nearly identical haploid cell pooled screen was also completed by another group with similar results (Kleinfelter et al., 2015).

Petersen et al. also carried out a matched RNAi screen using an rVSV pseudoparticle (pp) which contains a luciferase transgene and expresses the ANDV glycoprotein on its surface. The VSV-ANDV pp was used to infect an arrayed panel of cells that had been previously transfected in a well by well manner with a first-generation subgenomic Ambion siRNA library targeting 9102 human genes. After VSV-ANDV pp challenge a plate reader was used to quantify pp replication based on relative light units (RLUs). Genes were selected as candidates if they met criteria for significantly decreasing RLUs as compared to the control with two or more unique siRNAs. Follow up involved an identical screen using additional orthologous siRNAs. Thirty three genes were ultimately selected as high-confidence candidates with only one, SREBF2, being shared in common with the companion haploid cell screen. Further mechanistic studies demonstrated that loss of the sterol regulatory element-binding protein pathway prevented ANDV glycoprotein-mediated entry. Given the greater number of high-confidence candidates found in the RNAi screen, it would be interesting to determine if they were also all acting at entry or were instead required for the early postentry replication and expression of the luciferase transgene within the rVSV genome. Therefore, as with the other haploid cell screen noted above, this approach excels at finding entry factors. In this instance the paired RNAi arm of the study showed itself to be more sensitive because it found more high-confidence host factors using viral replication (RLUs) and not survival as a readout.

While the haploid cell screens have been useful in defining host–virus interactions they predominantly select for host genes that play critical early roles in viral replication, e.g., the host receptor(s), proteins that modify receptors, or endosomal trafficking factors (Tables 1 and 2). Based on our experience using pooled CRISPR/Cas9 to screen for host factors required by cytopathic viruses (HRV and IAV, Fig. 3 and our unpublished data) it appears that this approach will produce similar results to those seen with the pooled haploid cell survival screens, with only very early factors associated with viral entry, or genes need for the expression or activity of such genes, being enriched for in the surviving cell populations. One approach for recovering a deeper set of viral host factors may lie in halting the cytopathic virus pooled screen at intermediate stages of CPE, however, in our experience screening with HRV using shifts to nonpermissive temperatures and incubation with neutralizing antibodies, the practical execution of this idea is difficult. An arrayed haploid cell or CRISPR/Cas9 approach would permit more subtle selection criteria to be used such as those employed with arrayed siRNA screens. With this in mind, recent efforts have resulted in 3396 clonal HAP1 cell populations being characterized and arrayed with each one lacking the expression of a single gene due to retroviral insertion (Petersen et al., 2014). Unfortunately, because retroviral insertion is a random process it is not possible to selectively inactivate one class of gene or pathway, making the assembly of specialty libraries a matter of hunt and peck. This expanding arrayed HAP1 null allele cell resource would allow detailed investigation of single clones or focused subsets of clones, although the long-term culturing of such large numbers of distinct cell lines simultaneously will present significant challenges. Similar concerns for whole-genome arrayed CRISPR/Cas9 cell lines or lentiviruses would also present similar hurdles. Price permitting, this limitation might be avoided using large-scale arrayed sgRNA oligos or gene blocks that can be introduced into cells in a one-gene-per-well manner via lipid-mediated transfection along with Cas9 mRNA; these are arrayed sgRNA libraries are presently on hand in smaller gene sets but will undoubtedly become available in druggable or whole-genome versions in the near future. Care will need to be taken to allow sufficient time to elapse posttransfection for the generation of biallelic null mutations and phenotypic maturation prior to screening.

How else might the sensitivity and yield of pooled screens using CRISPR/Cas9 or haploid cells be improved upon? One possibility is the use of less stringent selection criteria such as selecting cells from a pool based on their relative expression of a marker protein. An elegant example of such a strategy for gene enrichment using pooled screening was recently done using flow cytometry to sort cells based on their expression of tumor necrosis factor (Tnf), which is elaborated in primary dendritic cells (DCs) after exposure to the bacterial product, lipopolysaccharide (LPS) (Parnas et al., 2015). The DCs were transduced so as to express Cas9 together with a complex sgRNA library of 125,793 sgRNAs directed against 21,786 mouse genes (Sanjana, Shalem, & Zhang, 2014). The pooled screen was performed three times using >60 million DCs stimulated with LPS. After LPS stimulation, the DCs were fixed, permeabilized, and immunostained for Tnf. Based on anti-Tnf antibody-associated immunofluorescence both high and low expressing Tnf populations were sorted using flow cytometry. The identities of the enriched sgRNAs were determined using PCR amplification of genomic DNA followed by next-gen sequencing. The authors arrived at >100 high-confidence candidates, several of which were previously known to be involved in DC responses to LPS, thus validating their approach and demonstrating its sensitivity.

While most current CRISPR/Cas9 pooled screens lack sensitivity, they nonetheless appear to have fewer false positives than RNAi screens, lowering the work load and increasing the efficiency of validation (Table 2). In our HRV-HF CRISPR/Cas9 screen, we detected a number of single sgRNAs for multiple genes with the majority having <6 reads. This may represent background PCR contamination or the facilitated carryover of phenotypically inconsequential sgRNAs by cells with intrinsic genetic resistance, e.g., cells that inherently lack ICAM1 expression. Therefore, all three genetic screening strategies benefit from the use of reagent redundancy, in the form of orthologous siRNAs and sgRNAs or multiple independent retroviral insertions, as a guiding principle for finding true positives.

To summarize, siRNA screens using arrayed one-gene-per-well format with moderate selection criteria, e.g., percent infected cells, permit the detection of a larger number of viral dependency factors, with the significant tradeoff being a greater number of false positives or OTEs. In contrast, pooled screens using cell survival as a readout as seen with the majority of haploid cell, and likely with additional CRISPR/Cas9 pooled screens to come, display limited sensitivity but excellent specificity in finding host genes that act very early in viral replication, for instance host factors needed for viral entry (ICAM1) (Tables 1 and 2). As can be seen in many of the arrayed siRNA screens, including our screens for HIV-1, HCV, and HRV14, host receptors and viral entry factors are also found with this approach, however, since these screens yield much greater lists of candidates, which include OTEs, any novel host receptors may not immediately jump to the fore. Therefore, given the currently available functional genomic strategies if the goal is to find viral entry factors (e.g., host receptors) with high specificity its best to use a pooled survival screen, but alternatively if the aim is to obtain with relative ease a more comprehensive set of host factors, albeit with more prevalent false positives, than an arrayed siRNA screen would be the preferred method.

7. FUTURE DIRECTIONS

While much has been learned about host–virus interactions there is still a great deal more to be achieved using functional genomic screens. Based on the greater adaptability of CRISPR/Cas9 for gene activation or inactivation/repression, all using a single sgRNA-expressing provirus, it seems likely that pooled shRNA screening will wane, given its comparatively poor phenotypic penetrance and greater burden of OTEs. Pooled haploid cell screens also appear vulnerable to displacement by CRISPR/Cas9 pooled approaches because of their dependence on only two transformed haploid cell lines, in conjunction with their more laborious identification of candidate genes. What's more, based on the established preference of retroviral insertion it is improbable that haploid cell screens will approach the saturation or representation produced with CRISPR/Cas9 methods.

The unique versatility of CRISPR/Cas9 technology to modulate gene expression using activation domain (CRISPRa) or repressor domain (CRISPRi) chimeras will assuredly give rise to many more notable discoveries. However, candidates found in screens using such synthetic transcription factors will need to be confirmed with rescue experiments given the questionable value of reagent redundancy approaches. This concern arises because of the potential for shared long distance OTEs being produced by orthologous sgRNAs designed against the same gene which will be binding relatively close to one another. Arrayed CRISRP/Cas9 screens using oligonucleotides (sgRNA and Cas9 mRNA) introduced into cells via lipid-mediated transfection may also rival or surpass established siRNA arrayed approaches, and while the current offerings of these reagents consist of smaller subgenomic gene sets it is anticipated that whole-genome versions will be commercially available shortly. That said, until the widespread implementation of arrayed CRISPR/Cas9 whole-genome screening, it seems likely that RNAi will continue to be the workhorse of functional genomic screening given its (i) first to market status, (ii) ease of use for arrayed screening, and (iii) high sensitivity and strong yields. However its prominent caveats increase the workload for validation substantially and may help to usher in an arrayed CRISPR/Cas9 screening era. We anticipate that approaches to minimize RNAi's problems, in combination with the expansion and adoption of CRISPR/Cas9 strategies, will continue to accelerate our understanding of human–virus interactions.

ACKNOWLEDGMENTS

We thank S. Whelan, M.C. Smith, and J. Smith for helpful discussions, L. Brass and W. McDougall for their critical reading of the chapter, and University of Massachusetts Medical School colleagues (B. Hobbs, C. Barry, L. Benson, T. Brailey, and R. Fish). A.L.B. is grateful to Bill and Melinda Gates Foundation, the Burroughs Wellcome Fund, and the NIH (1R01AI091786) for their support.

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