

A CRISPR toolbox to study virus–host interactions

Andreas S. Puschnik, Karim Majzoub, Yaw Shin Ooi and Jan E. Carette

Abstract | Viruses depend on their hosts to complete their replication cycles; they exploit cellular receptors for entry and hijack cellular functions to replicate their genome, assemble progeny virions and spread. Recently, genome-scale CRISPR–Cas screens have been used to identify host factors that are required for virus replication, including the replication of clinically relevant viruses such as Zika virus, West Nile virus, dengue virus and hepatitis C virus. In this Review, we discuss the technical aspects of genome-scale knockout screens using CRISPR–Cas technology, and we compare these screens with alternative genetic screening technologies. The relative ease of use and reproducibility of CRISPR–Cas make it a powerful tool for probing virus–host interactions and for identifying new antiviral targets.

Pathogen-associated molecular patterns

(PAMPs). Molecules that are expressed by pathogens and recognized by the host innate immune system.

Forward genetic screens

Genetic screens in which mutant genes are identified on the basis of their phenotypes.

Signal-to-noise ratio

The ratio of truly enriched genes (signal) versus random enrichment of genes (noise).

Single-guide RNA

(sgRNA). An artificial fusion of CRISPR RNA (crRNA) and partial transactivating RNA (tracrRNA) through a synthetic stem–loop that mimics the natural crRNA–tracrRNA hybrid and directs Cas9 to its target DNA.

Department of Microbiology and Immunology, Stanford University, Stanford, California 94305, USA.

Correspondence to J.E.C. carette@stanford.edu

doi:10.1038/nrmicro.2017.29
Published online 19 Apr 2017

Viruses are obligate intracellular pathogens that depend on host cellular components for replication. They bind to cell surface receptors to enter cells, and they co-opt cellular functions and organelles to replicate. Host cells can counteract infections by sensing pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids, and by subsequently triggering the expression of antiviral genes. The identification and characterization of host factors that promote and restrict viral replication can provide important insights into basic aspects of cellular biology and virus–host relationships, and can lead to the identification of new targets for antiviral therapeutics.

The use of forward genetic screens has provided an unbiased and comprehensive strategy to uncover host factors that promote or restrict virus replication. Originally, the use of these genetic screens was limited to genetically tractable model organisms, such as yeasts, fruit flies, roundworms and zebrafish, and relied on the use of X-rays or chemical mutagens to introduce mutations. These forward genetic screens have markedly contributed to our understanding of many fundamental biological processes^{1–4}, but their application to cultured mammalian cells was challenging. With technological advances such as RNAi and insertional mutagenesis in human haploid cells, it became possible to disrupt gene expression on a genome scale in mammalian cell culture^{5–7}. Recently, the prokaryotic CRISPR–Cas adaptive immune system has been engineered to efficiently induce knockout mutations in almost any cell type, which has revolutionized biological research^{8–10} (BOX 1). In contrast to gene knockdown approaches, such as RNAi, the knockout of alleles by CRISPR–Cas often results in more marked phenotypes, a greater signal-to-noise

ratio and the identification of fewer false-positives^{11–14}. Knockout alleles are generated by the endonuclease Cas9, which is directed to a specific genomic region by a single-guide RNA (sgRNA) through Watson–Crick base pairing. Cas9 creates a double-strand break (DSB) at the target site, which is then repaired by non-homologous end joining (NHEJ). This often results in a frameshift mutation and the expression of truncated or non-functional proteins. The ease of Cas9 targeting to specific loci, combined with the design of multiplexed pools of sgRNAs that span the entire human genome^{14–17}, has enabled the genome-scale identification of host factors that are crucial for virus replication.

In this Review, we describe how genetic screens have contributed to our understanding of virus–host biology and how CRISPR–Cas screens have been used to expand our toolkit to identify host factors that are important for virus replication. We provide practical advice on how to set up CRISPR–Cas screens and give examples of recent discoveries that have been made using CRISPR–Cas technology for viruses that cause important human diseases, including dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), hepatitis C virus (HCV) and noroviruses. We also discuss the potential for CRISPR–Cas technology beyond genetic screening applications, and how it could advance our understanding of viral pathogenesis and the development of antiviral therapeutics.

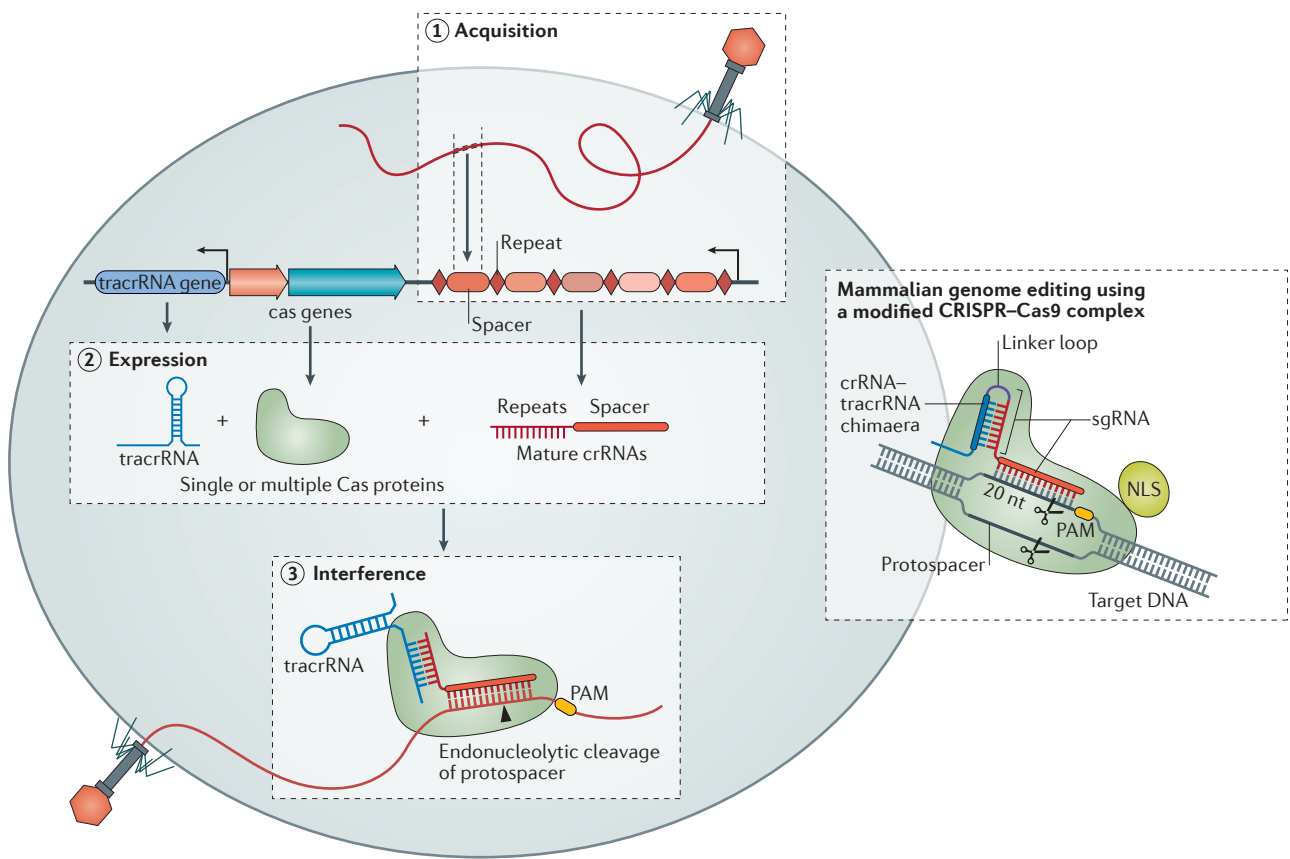
The power of unbiased genetic screens

Historically, loss-of-function screens have lagged behind gain-of-function approaches in mammalian cells owing to the lack of efficient tools that can mutate both alleles in diploid genomes in a high-throughput manner.

Box 1 | CRISPR–Cas-mediated adaptive immunity

The CRISPR–Cas system is an adaptive immune system that protects bacteria and archaea against bacteriophages and plasmids. CRISPR–Cas immunity is mediated by CRISPR RNA (crRNA) and a Cas endonuclease that targets genetic elements¹⁴¹. The mode of action consists of three distinct steps: acquisition, expression and interference (see the figure). In the acquisition step, foreign nucleic acids are directionally integrated, as new CRISPR spacers, into a CRISPR array that is separated by repeat sequences, thus creating a memory of invading genetic elements¹⁴² (see the figure, step 1). In the expression step, the CRISPR locus is transcribed into a pre-CRISPR RNA transcript (pre-crRNA), which is then processed into a mature crRNA that contains partial CRISPR spacer sequences joined to partial CRISPR repeats¹³². The CRISPR locus also encodes a transactivating RNA (tracrRNA) that has complementarity to the repeat regions of crRNA transcripts¹⁴³. In addition to the CRISPR array, a single or multiple Cas nucleases (for example, Cas9) are encoded by the CRISPR locus (see the figure, step 2). In the interference stage,

a crRNA–tracrRNA hybrid is formed through binding of the complementary repeat region sequences, and this RNA hybrid guides the Cas nuclease towards complementary DNA sequences, which leads to the targeting and cleaving of invading genetic elements¹⁴⁴. Most CRISPR effector proteins rely on a protospacer-adjacent motif (PAM; for example, NGG for Cas9) in the targeted nucleic acid. The PAM is essential for recognition, cleavage and the distinction between self and non-self DNA^{145–147} (see the figure, step 3). For Cas9, perfect complementarity will drive a conformational change in the endonuclease that leads to a cleavage-competent structural state^{148–153}. The protein and RNA components of the *Streptococcus pyogenes* class 2 CRISPR system have been adapted to function in eukaryotes, including in human cells. A human-codon-optimized Cas9 is fused to a nuclear localization signal (NLS) to direct Cas9 to the nucleus in mammalian cells^{8–10}. To generate single-guide RNAs (sgRNAs) for genome editing that mimic the natural crRNA–tracrRNA hybrid, crRNA-like sequences are fused to a partial tracrRNA through a synthetic stem-loop.



Non-homologous end joining
A pathway that repairs double-strand breaks (DSBs) in DNA by directly ligating the break ends without the need for a homologous template.

Frameshift mutation
A genetic mutation caused by indels (insertions or deletions) of a number of nucleotides that is not divisible by three, leading to a shift in the ORF of the gene.

Gain-of-function approaches. Gain-of-function approaches rely on the ectopic overexpression of genes and have been successful in identifying cell surface receptors that are required for viral entry and host viral restriction factors. To identify entry receptors, a cell line that is refractory to infection is typically transduced with a complementary DNA library (cDNA library) derived from a cell type that is permissive to infection. For example, claudin 1 (CLDN1)¹⁸ and occludin (OCLN)¹⁹ were identified as entry receptors for HCV by transducing a non-permissive cell line with a cDNA library derived

from hepatocellular carcinoma cells. In addition to discovering receptors, an unbiased expression screen also discovered that SEC14-like protein 2 (SEC14L2), which is a cytosolic lipid-binding protein, enhances the replication of clinical strains of HCV²⁰. Furthermore, a library of ~380 interferon-stimulated genes (ISGs) was used to identify key proteins that are important for innate immune defences against several DNA and RNA viruses^{21,22}. In addition to these screens, in a continuing effort, comprehensive cDNA libraries that contain all annotated ORFs from humans have been cloned

Table 1 | Comparison of mammalian loss-of-function screening methods

	CRISPR–Cas	Haploid	RNAi
Mechanism	Induced DSBs lead to error-prone NHEJ and, frequently, frameshift mutations	Integration of retroviral gene traps that contain a splice acceptor site leads to truncated mRNA transcripts	siRNAs or shRNAs bind to target mRNAs, which leads to their cleavage and degradation
Phenotype	Complete knockout of gene expression leads to strong phenotypes in virus infection assays	Complete knockout of gene expression leads to strong phenotypes in virus infection assays	Partial knockdown of gene expression may not produce a strong phenotype in virus infection assays
Selection of candidate host factors	Strong phenotypes achieved by gene knockouts result in the identification of candidate genes with higher confidence than those identified through RNAi	Strong phenotypes achieved by gene knockouts result in the identification of candidate genes with higher confidence than those identified through RNAi	Incomplete knockdown and the variability of gene expression combined with off-target effects makes the identification of candidate genes more challenging
Coverage	4–12 sgRNAs per gene	~525 independent gene trap insertions per gene (median)	Typically 4–6 shRNAs per gene; up to 30 shRNAs per gene for pooled screening
Off-target effects	Mismatched base pairing may lead to off-target cleavage in the genome (usually in non-coding regions)	No described off-target mechanism	Mismatched base pairing may lead to knockdown of off-target mRNAs
Cell types	Wide range of cell types	Haploid and near-haploid cells (HAP1, KBM7, human and mouse embryonic stem cells)	Wide range of cell types
Analyses	Enrichment of multiple sgRNAs per gene	Enrichment of independent gene trap insertions per gene	Enrichment of multiple siRNAs or shRNAs per gene

DSB, double-strand break; NHEJ, non-homologous end joining; sgRNAs, single-guide RNAs; shRNA, short hairpin RNA; siRNA, small interfering RNA.

into lentiviral expression vectors²³, generating an expression vector library that is likely to improve the utility of gain-of-function screens in the study of host–pathogen interactions.

Loss-of-function genetic screens. Loss-of-function screens are based on the stable knockdown or knockout of genes. Initial approaches that used RNAi have provided valuable insights into virus–host relationships²⁴. In contrast to RNAi, which leads to the partial depletion of expression for a specific gene, recent technological advances have made it possible to completely disrupt gene expression (TABLE 1). One approach, termed haploid genetic screening, relies on insertional mutagenesis of genes in cultured haploid cell lines. For example, retroviral gene traps that contain a splice acceptor site can integrate into the host genome, leading to the expression of truncated mRNA transcripts⁷. The complete ablation of gene expression can have marked effects on virus replication and enables the identification of the most crucial host factors for virus infection. Insertional mutagenesis in haploid cells has been used to discover essential receptors for several viruses, including Ebola virus and Lassa virus^{25,26}. Both viruses use abundant lysosomal proteins as receptors. The interaction between the Ebola virus glycoprotein and its receptor Niemann–Pick C1 protein (NPC1) is triggered by cathepsin cleavage^{27,28}, whereas the Lassa virus glycoprotein interacts with its receptor, lysosome-associated membrane glycoprotein 1 (LAMP1), following acidification of the endosome. Subsequent structural studies defined the binding interface between the viral glycoprotein and NPC1 (REFS 29–31). Interestingly, several mutations arose in the host-binding site of the viral glycoprotein during the 2013–2016 Ebola virus epidemic.

These mutations increased the infectivity of the virus in primate cells but not in rodent cells, which suggests that these mutations contributed to adaptation and spread in humans^{32,33}.

Haploid genetic screens were important for the discovery of a cellular phospholipase that enables viral evasion of an antiviral restriction mechanism that is broadly active against many picornaviruses³⁴. Recently, a haploid screen identified a proteinaceous receptor that enables virus entry for multiple distinct serotypes of adeno-associated virus (AAV)³⁵, potentially affecting the use of AAV as a gene therapy vector. These and other studies have established loss-of-function screens as a reliable strategy to uncover host factors that are crucial for virus replication (TABLE 2).

Practical considerations for screens

Genetic screens enable the identification of virus–host interactions without prior knowledge of the interaction and on a genomic scale. In this section, we describe the different technologies that are currently available to carry out genetic screens, and we highlight important considerations at different stages of the screen, including the generation of the library of mutant cells, the virus infection assay, phenotypic selection, next-generation sequencing and bioinformatic analyses (FIG. 1). We also consider the degree of ‘saturation’ in genetic screening; that is, the fraction of target genes it is possible to identify in a specific screen.

Choice of cell line and screen. Viruses differ in their host range and tissue tropism. Whether a cell is permissive or non-permissive to virus infection is determined by the expression of genes that facilitate virus

Loss-of-function

Stable suppression or disruption of gene expression in a cell or organism.

Gain-of-function

Ectopic overexpression of genes or activation of non-expressed genes in a cell or organism.

Transduced

Cells that are infected with a lentiviral or retroviral vector containing a DNA of interest that is integrated into the genome.

Complementary DNA library

(cDNA library). A library that is prepared from all expressed mRNAs in a cell by reverse transcription into DNA.

Permissive

Pertaining to a host cell: susceptible to infection with a particular virus; permissiveness usually depends on the expression of certain proviral genes and the absence of certain antiviral restriction factors.

Table 2 | Genome-wide knockout screens to identify virus–host interactions

Viruses	Knockout screen	Critical host factors	Refs
Adeno-associated virus	Haploid	AAVR, GARP complex	35
Dengue virus	Haploid and CRISPR–Cas	OST complex (STT3A and STT3B), TRAP complex (SSR1, SSR2, SSR3), EMC, ERAD (SEL1L, AUP1, DERL2)	61
Ebola virus	Haploid	NPC1, HOPS complex	25
Enterovirus 68	Haploid	Sialic acid	163
Hanta virus	Haploid	SREBF2, MBTPS1, MBTPS2, SCAP	164,165
Hepatitis C virus	CRISPR–Cas	CD81, CLDN1, OCLN, miR-122, CYP4, ELAVL1, RFK, FLAD1	61
Human immunodeficiency virus	CRISPR–Cas	CD4, CCR5, ALCAM, SLC35B2, TPST2	88
Lassa virus	Haploid	LAMP1, DAG1	26,166
Murine norovirus	CRISPR–Cas	CD300LF	85,86
Picornaviruses	Haploid	PLA2G16	34
Rift Valley fever virus	Haploid	Heparan sulfate, COG complex	167
West Nile virus	CRISPR–Cas	SPCS1, SPCS3, EMC, OST complex (STT3A), TRAP complex, SEL1L, HRD1	69,70
Zika virus	CRISPR–Cas	EMC, AXL, OST complex (STT3A), TRAP complex	71

AAVR, adeno-associated virus receptor; ALCAM, CD166 antigen; AXL, also known as UFO; CLDN1, claudin 1; COG, conserved oligomeric Golgi; CYP4, cyclophilin A; DAG1, dystroglycan; DERL2, derlin 2; EMC, endoplasmic reticulum membrane protein complex; ERAD, endoplasmic reticulum-associated degradation; FLAD1, FAD synthase; GARP, Golgi-associated retrograde protein; HOPS, homotypic fusion and protein sorting; HRD1, also known as SYVN1; LAMP1, lysosome-associated membrane glycoprotein 1; NPC1, Niemann–Pick C1 protein; OCLN, occludin; OST, oligosaccharyltransferase; RFK, riboflavin kinase; SCAP, SREBP cleavage-activating protein; SLC35B2, adenosine 3′-phospho 5′-phosphosulfate transporter 1; SPCS, signal peptidase complex subunit; SREBF2, sterol-regulatory-element-binding protein 2; TPST2, protein tyrosine sulfotransferase 2; TRAP, translocon-associated protein.

Lentiviral expression vectors

Gene delivery tools that are modified from HIV-1, with most of the viral genes removed and a desired gene inserted, often under the control of a cytomegalovirus (CMV) promoter. The lentiviral vector integrates into the host genomic DNA through long terminal repeats and expresses the inserted gene.

Gene traps

Lentiviral or retroviral constructs that predominantly integrate into the coding regions of genes to disrupt gene expression.

Transformed cell lines

Immortalised cell lines that can proliferate indefinitely owing to one or several mutations. The cells have evaded normal cellular senescence and can be grown for prolonged periods *in vitro*.

Karyotype

The number and appearance of chromosomes in the nucleus of a eukaryotic cell.

Off-target effects

Unwanted knockout or knockdown of a gene, most often as a result of partial complementarity to an unintended target.

replication and genes that restrict virus infection. Genetic screens can uncover genes that promote and restrict virus replication depending on the choice of host cell type (permissive or non-permissive) and the type of screen (loss-of-function or gain-of-function; FIG. 1). CRISPR–Cas genome editing has been reported for a wide range of cell lines that can be infected with many viruses. However, large-scale genetic screens in which the sgRNAs are introduced into the cells in a pooled manner have some limitations. To ensure the appropriate representation of each of the sgRNAs in the pool, numerous cells are transduced and undergo phenotypic selection. In practice, many transformed cell lines will be suitable for generating a mutagenized cell library; however, primary cells have a limited proliferative capacity, and it is therefore more challenging to transduce and expand these cells in large numbers. Pre-arrayed sgRNA formats, in which wells contain individual synthetic sgRNA constructs for reverse transfection³⁶, may therefore be more suitable for primary cells.

Haploid screens are limited to cell types that have a haploid or near-haploid karyotype to achieve insertional mutagenesis of the allele. Commonly used cell lines include the chronic myeloid cell line KBM7 (REF. 7) and its derivative, HAP1 (REFS 25,37), and human³⁸ and mouse embryonic stem cells^{39,40}. Despite this limited choice of cell types, haploid genetic screens have been useful for studying many different virus–host interactions^{41,42} (TABLE 2).

Overall, both CRISPR–Cas and haploid screens are well suited for the identification of host factors if the cell line is permissive to the virus. The two types of screen may even be carried out in parallel for additional validation and comprehensive screening of candidate genes.

CRISPR libraries and mutagenesis. Several CRISPR sgRNA libraries are available as plasmid repositories (see the [Addgene](#) website). The libraries vary in the number of sgRNAs they contain, their target genes (genome wide or a subpool of genes only), the targeted position within the gene (for example, the ORF or the promoter), the targeted species, and their availability as a one-plasmid or two-plasmid system (such that Cas9 is encoded on the same plasmid as the sgRNA or on a second plasmid, respectively). In addition, custom libraries can be constructed for a specific class of gene (for example, kinases) or for validation screens.

The initial genome-scale CRISPR knockout (GeCKO) libraries contained 4–6 sgRNAs per gene and were designed to minimize off-target effects^{14,43}. More recently constructed CRISPR–Cas libraries (for example, the Broad Brunello⁴⁴, Toronto KnockOut¹³ or Sabatini–Lander libraries⁴⁵) contain more sgRNAs per gene (up to 12), which increases the likelihood of statistically significant enrichment of candidate genes. However, larger sgRNA libraries also require larger-scale screening, which could be challenging to achieve, especially in cells that have a limited capacity to divide. Notably, a small sgRNA library that contains a subset of sgRNAs from a larger CRISPR–Cas library was still able to identify the majority of the same hits, albeit with less statistical significance⁴⁴. This suggests that when scaling up is unfeasible owing to cost or cell number, sgRNA libraries that contain fewer sgRNAs per gene can be used in an initial screen, which can then be followed by a secondary screen and/or careful validation.

The more recent CRISPR–Cas sgRNA libraries were constructed to have greater on-target cleavage efficiency than previous sgRNA libraries, in addition

to minimal off-target activity. They have shown consistent on-target cleavage efficiency, therefore reducing the chance of false-negative identifications. For example, in a genome-scale screen selecting for resistance to the toxic effect of thymidine, 11 of the 12 sgRNAs against thymidine kinase 1 (TK1) scored as hits, which indicates that the majority of these sgRNAs were active because TK1 is crucial for mediating thymidine toxicity¹³. In a more systematic study, ~85% of all sgRNA constructs that target essential genes were accurately recalled without false-positive identifications¹¹. Owing to the high efficiency of CRISPR–Cas knockouts, the marked phenotypes that are generated in knockout cells and the reproducibility of CRISPR–Cas screens, these screens have outperformed RNAi library screens for the identification of drug resistance genes¹⁴, modulators of protein stability¹² and essential genes^{11,13}. In addition to creating gene knockouts, CRISPR–Cas technology has been used to modulate the transcription levels of target genes (BOX 2). This approach can be advantageous when studying essential genes because it can decrease gene expression without eliminating it completely. It also enables the role of long non-coding RNAs to be assessed, as small insertions or deletions (indels) do not typically disrupt their biological activity⁴⁶.

To ensure that the sgRNA library is of sufficient quality, it is important to maintain the complexity of the sgRNA pool when expanding the sgRNA plasmid pool in *Escherichia coli*, during transfection or transduction of the target cells and during the extraction of genomic DNA from cells for downstream analyses. For example, we consistently found a good sgRNA representation (>99%) when the number of transduced cells was ~500-fold higher than the total number of sgRNAs in the library. Furthermore, a low multiplicity of infection (MOI; ~0.3) during transduction is advised to ensure that only one integration event takes place per cell⁴⁷.

Phenotypic selection. Many viruses such as poliovirus or DENV are cytotytic, which enables a straightforward selection of virus-resistant cells in cell viability-based screens. This selection recovers mutant cells that do not support viral entry, translation of the viral genome, replication of the viral genome or virus-induced cell death, but typically not mutant cells that do not support virion assembly and egress. In a pooled screen, in which mutant cells are cultured together, the selection can be extremely stringent because of the requirement that resistant cells survive multiple rounds of infection. Therefore, this screening method identifies genes for which disruption causes marked phenotypes. Strong selection conditions in which >99% of cells die from infection are preferred. Although this high stringency increases the confidence in the candidate genes identified, other genes that have subtler effects on virus infection may be missed. Decreasing the stringency could help to identify these genes. Strategies to achieve this include the use of naturally attenuated virus strains or the use of antiviral compounds during selection. However, fine-tuning of the stringency is not always possible in pooled screens, and arrayed screens may be a valuable alternative.

If the virus is not efficient at inducing cell death, then a longer selection period, multiple rounds of virus challenge and larger sgRNA libraries may help to increase the signal-to-noise ratio. As an alternative strategy, fluorescence-activated cell sorting (FACS)-based selection can be used to study persistent or non-cytolytic viruses (for example, hepatitis B virus (HBV), HIV and AAV). This approach relies on genetically engineered viruses that express a fluorescent reporter or on antibody staining³⁵. FACS-based selection enables the isolation of cells that have low or high levels of virus gene expression, making it possible to simultaneously identify factors that enhance virus infection and factors that inhibit virus infection.

It is also possible to identify host factors that are required at specific stages of the viral life cycle; for example, pseudotyped viruses^{48,49}, viral replicons^{50,51} and internal ribosome entry site reporters (IRES reporters)^{52,53} can be used in the virus infection assay to identify host factors that are required for virus entry, genome replication and translation, respectively.

Next-generation sequencing and bioinformatics.

After phenotypic selection, genomic DNA is isolated. Uninfected, mutagenized cells are used as control samples (the starting population either collected at day 0 or grown and harvested in parallel with the virus-selected population). At this step, the total amount of DNA template should be sufficiently high to maintain the complexity of the library. The sgRNA integrations are PCR-amplified and sequenced by next-generation sequencing to quantify their relative abundances. The level of sgRNA enrichment in phenotypically selected cells compared with that in unselected cells is determined by comparing the number of reads that map to specific sgRNAs in the different cell populations. To normalize for differences in sequencing depth between populations, the number of reads that map to each specific sgRNA is divided by the total number of reads. Bioinformatic tools can help to determine whether a gene is significantly enriched over background by assessing the level of enrichment of multiple sgRNAs against the same gene. Analysis tools that were developed for RNAi screens, such as RNAi gene enrichment ranking⁵⁴ (RIGER) and redundant siRNA activity⁵⁵ (RSA), can be repurposed for this task. More recently, scoring algorithms, such as model-based analysis of genome-wide CRISPR–Cas9 knockout (MAGeCK)⁵⁶ and STARS⁴⁴, have been developed to improve the bioinformatic analyses of CRISPR–Cas screen data sets, taking into account the increasing number of sgRNAs that are used per gene.

Validation of candidate genes and off-target effects.

An important step after any genetic screen is the validation of the candidate genes and the consideration of off-target effects. Gene editing at off-target loci has been reported^{57–59}, and if these off-target sites are within exons, they have the potential to cause false-positive results. The use of multiple sgRNAs per gene combined with the implementation of sgRNA sequence design

Pseudotyped viruses

Viruses or viral vectors that are packaged with envelope proteins from another virus.

Viral replicons

Self-replicating subgenomic viral RNAs that originate from viral genomes. These replicons contain viral genes that encode non-structural proteins that are critical for viral genome replication, but the genes that encode structural proteins are either deleted or replaced by foreign genes.

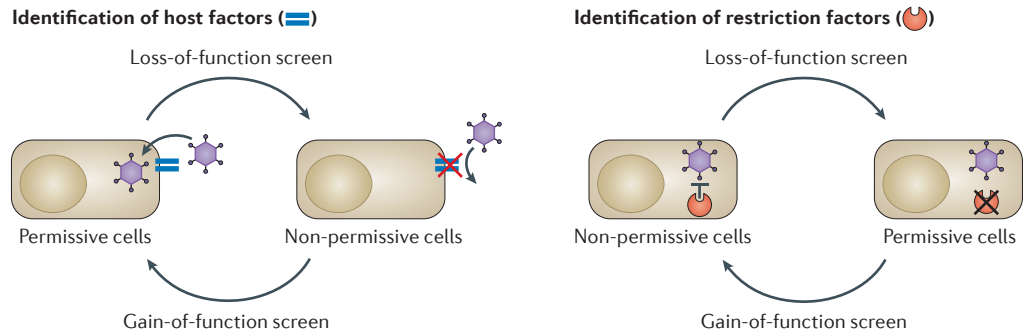
Internal ribosome entry site reporters

(IRES reporters). Reporter constructs that consist of a viral IRES (an RNA element that allows for translation initiation in a cap-independent manner) fused to a reporter gene, such as a gene that encodes luciferase or a fluorescent protein.

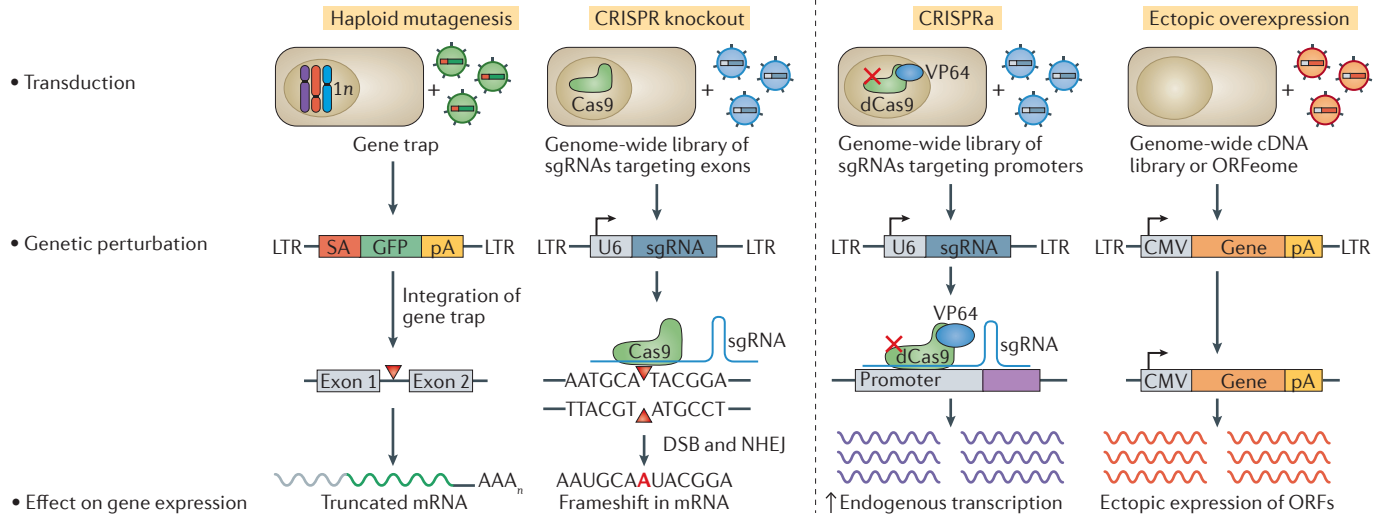
Background

During a CRISPR–Cas screen: random, low-level detection of single-guide RNAs that are not causal to a knockout phenotype.

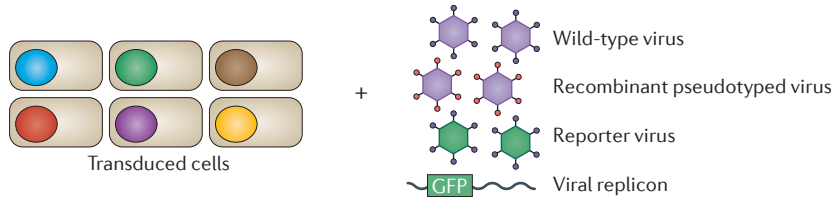
Screen for proviral host factors or restriction factors



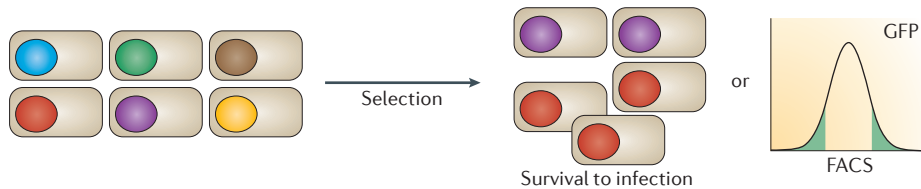
Mutagenesis



Virus infection assay



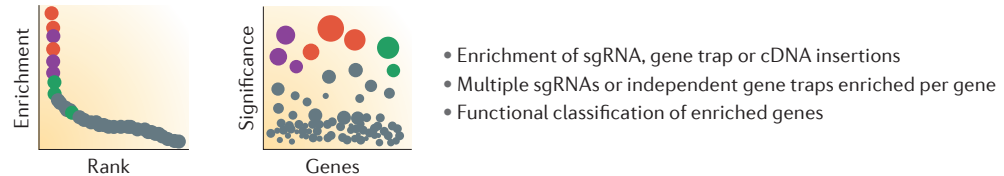
Phenotypic selection



Next-generation sequencing



Bioinformatics



◀ **Figure 1 | Genome-wide screening strategies to investigate host factors that are involved in virus infection.** Genetic screens can identify host factors that promote virus replication, as well as antiviral restriction factors. For example, in a loss-of-function screen, knockout of a viral receptor in a permissive cell line will make the cell resistant to the virus infection. By contrast, in a gain-of-function screen, overexpression of the viral receptor in a non-permissive cell line will enable virus infection. Various technologies are available for genome-wide screening. Loss-of-function screens can be carried out using a haploid mutagenesis (in a 1n cell type) or CRISPR–Cas knockout approaches, whereas gain-of-function screens use CRISPR activation (CRISPRa) or ectopic overexpression. Target cells are mutated by delivering retroviral gene trap or lentiviral expression constructs, which can either disrupt or lead to gene expression. The pooled mutagenized cell population is then subjected to a virus infection assay, in which either the cells are infected by the virus of interest, or a subgenomic viral reporter is introduced by transduction or transfection. Virus-resistant cells are selected either by surviving virus-induced cell death or by fluorescence-activated cell sorting (FACS). Next-generation sequencing and bioinformatic analyses enable the enrichment of CRISPR single-guide RNAs (sgRNAs), gene traps or complementary DNA (cDNA) insertions to be determined. CMV, cytomegalovirus; dCas9, catalytically inactive Cas9; DSB, double-strand break; LTR, long terminal repeat; NHEJ, non-homologous end joining; pA, poly(A) tail; SA, splice acceptor; VP64, herpes simplex virus VP16 activation domain.

rules can help to reduce this risk. In side-by-side comparisons with RNAi-based approaches, CRISPR–Cas screens typically have fewer false-positive identifications^{11–13,44}. Nevertheless, a thorough validation of candidate genes is still essential. Individual knockout cell lines should be generated using CRISPR–Cas methods and start from a single cell clone. After confirming that the gene has been knocked out by genotyping and immunostaining, the effect of the knockout on virus replication can be measured, and genetic complementation experiments can confirm that the effect was due to the knockout.

Essential genes and genome coverage. It is challenging to identify all of the genes that affect virus replication because a proportion of them are essential for cell growth and viability and will therefore be excluded from downstream analyses. CRISPR–Cas screens and haploid screens have enabled the systematic and comprehensive identification of a core set of ~2,000 human genes that are essential for optimal cellular growth and viability^{13,45,60}, corresponding to ~10% of human genes.

Owing to the essential roles that these genes have in cell physiology, it is challenging to determine whether they directly influence virus replication. The remaining ~90% of genes can be tested in genetic screens because they do not affect cell growth or viability; however, this figure is likely to be an underestimate because the list of ‘essential genes’ includes genes that only moderately affect cell growth and could therefore be included in the screens. For example, in haploid and CRISPR–Cas screens for host factors that are crucial for DENV replication, multiple subunits of the oligosaccharyltransferase complex (OST complex) were identified⁶¹ despite the genes that encode these subunits being classified as essential genes⁶⁰.

Another important consideration is the coverage of the genome. Notably, sgRNA libraries are designed according to the presence of annotated genes in reference genomes (~20,000 genes in humans). The increasing number of independent sgRNAs (now at 4–12 per gene) and improved sequence rules for cleavage efficiency make current sgRNA libraries more reliable than early libraries for probing the entire human genome, thus minimizing false-negative results. By contrast, haploid genetic screens rely on retroviral insertional mutagenesis and do not require genome annotation. Retroviral integration is not random and occurs more frequently in actively transcribed chromatin⁶², which biases the insertions towards genes. Indeed, mapping of insertion sites in gene trap screens revealed insertions in ~70% of all annotated genes and ~98% of expressed genes⁶³. Recently, more extensive mapping efforts in HAP1 cells showed that >90% of all annotated genes contained insertions, with a median of 525 independent gene trap insertion events per annotated gene⁶⁰. This high number of knockout events increases the power of identifying signal over noise.

Despite the fundamental differences between CRISPR–Cas and haploid genetic screens, both approaches have been equally powerful in identifying core essential human genes^{45,60}, endoplasmic reticulum-associated protein degradation (ERAD) components⁶⁴ and host factors that are required for DENV replication⁶¹.

Oligosaccharyltransferase complex

(OST complex). A protein complex in the endoplasmic reticulum (ER) membrane. This complex transfers a lipid-linked oligosaccharide precursor to asparagine residues on nascent proteins in the lumen of the ER.

Endoplasmic reticulum-associated degradation

(ERAD). A process by which the endoplasmic reticulum (ER) recognizes misfolded proteins and directs their degradation.

Synergistic activation mediator

A CRISPR–Cas-based engineered protein complex that activates transcription from endogenous genes.

Box 2 | CRISPR–Cas approaches to regulate transcription

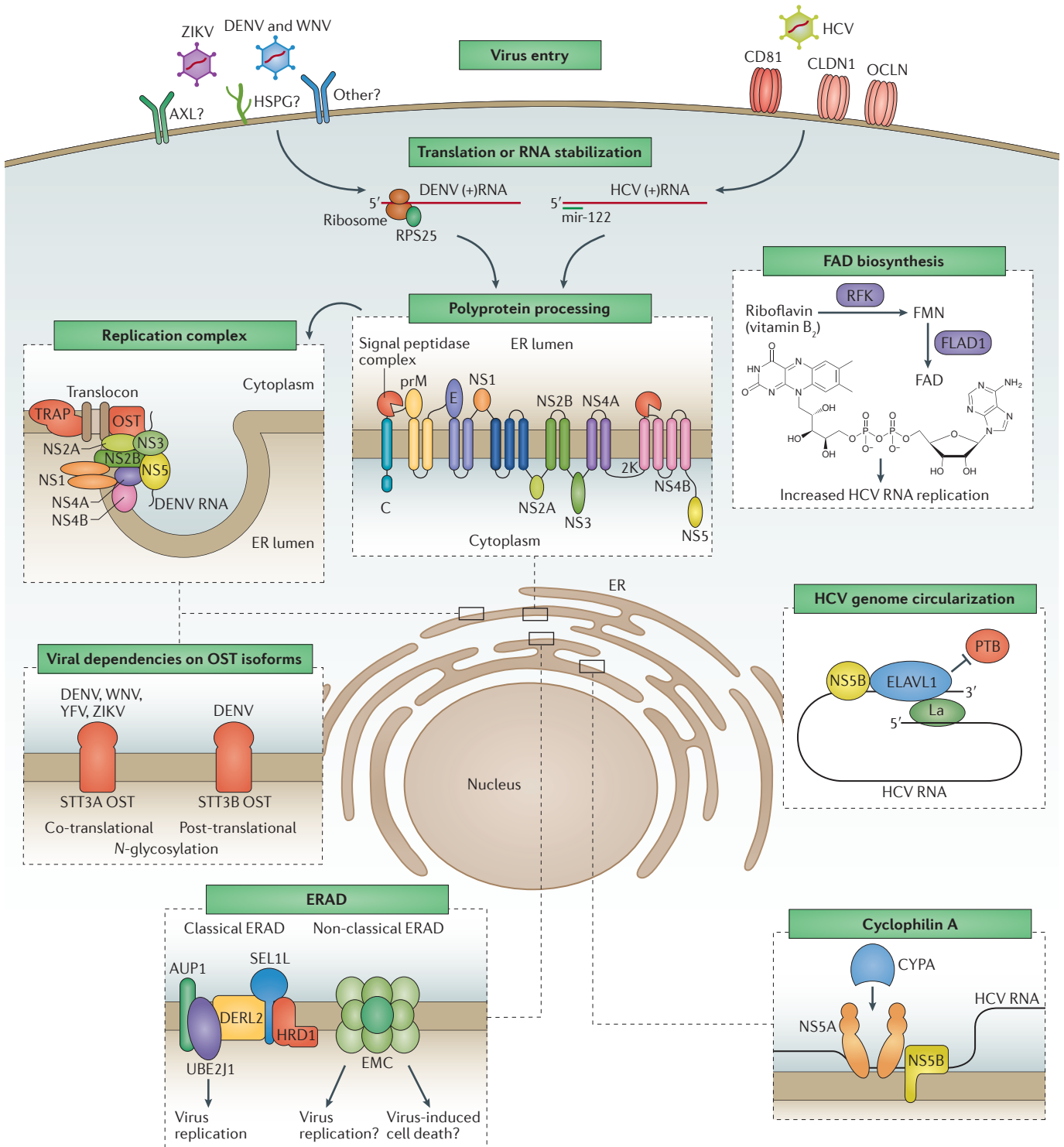
Catalytically inactive Cas9 (dCas9) can be fused to transcriptional activators or repressors to modulate gene expression without introducing irreversible mutations into the genome^{154,155}. Approaches that use dCas9 for this purpose are commonly referred to as CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi)¹⁵⁶. To achieve transcriptional repression (that is, CRISPRi), chromatin-modifying repressor domains, such as the Krüppel-associated box (KRAB) domain, are fused to dCas9 and are recruited to transcription start sites. CRISPRi-mediated and RNAi-mediated knockdown both lead to the downregulation of gene expression, albeit through different molecular mechanisms. CRISPRi occurs by inhibiting transcription, whereas RNAi degrades mRNAs in the cytoplasm. CRISPRa relies on the fusion of dCas9 to multiple repeats of the herpes simplex virus VP16 activation domain (VP64 or VP160) to enhance transcription at target sites. Alternatively, in the synergistic activation mediator (SAM) library, MS2 RNA aptamers added to the tetraloop and second stem–loop of the sgRNA additionally recruit a fusion construct of the bacteriophage MS2 coat protein (MCP), the nuclear factor-κB subunit p65 and heat shock factor protein 1 to enhance Cas9-mediated gene expression¹⁵⁷. CRISPRa and SAM libraries are an alternative approach for gain-of-function screens (FIG. 1), which traditionally rely on complementary DNA (cDNA) overexpression libraries²³. The activation of endogenous gene expression has the advantage of not being limited by conventional molecular cloning techniques to generate cDNA constructs and can be used to increase the expression of different isoforms from the same gene. Although Cas9-mediated gene activation has not been used in the context of virus–host studies, it may be used to identify antiviral restriction factors or host factors that are expressed at very low levels and are required for virus replication.

The high concordance in identified genes between the two different technologies underscores the power and reliability of knockout screens.

Insights from CRISPR–Cas screens

The potential for CRISPR–Cas screens to discover host factors that are crucial for viral pathogenesis is great and may lead to the development of new antivirals⁶⁵. Several viruses have been studied using CRISPR–Cas screens.

Mosquito-borne flaviviruses. The mosquito-borne flaviviruses include important pathogens such as DENV⁶⁶. More recently, ZIKV has emerged in Brazil and is spreading at a rapid pace throughout South America⁶⁷, causing severe congenital abnormalities in the unborn children of pregnant mothers who are infected⁶⁸. The biogenesis and membrane topology of mature flavivirus proteins is complex and involves the translation of a polyprotein at the ER membrane,



Signal peptide

A short peptide that is present at the amino terminus of the majority of newly synthesized proteins that are targeted towards the secretory pathway.

Translocon

A protein complex in the endoplasmic reticulum membrane that directs the translocation of nascent polypeptides from the cytosol into the endoplasmic reticulum lumen.

Retrotranslocation complex

A membrane protein complex in the endoplasmic reticulum that mediates transport of misfolded proteins from the endoplasmic reticulum lumen into the cytosol.

the co-translational and post-translational insertion of several membrane-spanning hydrophobic helices, and polyprotein cleavage by a viral protease and several host proteases into the mature viral proteins. Despite this knowledge of these processes, a detailed understanding of the host proteins that are involved is lacking.

CRISPR–Cas screens that were carried out independently using DENV⁶¹, WNV^{69,70} and ZIKV⁷¹ have each identified a number of ER proteins that are required for virus replication (FIG. 2). Many of these proteins are involved in the biosynthesis of membrane and secretory proteins, a core function of the ER. In particular, the proteins that were identified have described roles in *N*-linked glycosylation, ERAD, and signal peptide insertion and processing. Notably, the identification of these proteins was reproduced in replica screens in the same laboratory and in independent screens in different laboratories using different cell lines and different virus strains. There was also a substantial overlap with results from haploid genetic screens. This reproducibility is remarkable and a major advantage of this technology.

CRISPR–Cas technology also provides a reliable way to validate candidate genes and measure the effects of knockouts on virus replication. In contrast to knockdown approaches, such as RNAi, gene knockouts are absolute and do not result in the variable levels of depletion seen with RNAi. This enables a faithful comparison between genes when quantitative assays for virus replication are used, such as quantitative PCR, immunostaining or plaque assays. Remarkably, flavivirus replication was decreased 100–10,000-fold when the most significantly enriched host factors from the screens were knocked out⁶¹. This demonstrates that pooled sgRNA screens have the potential to identify host factors that are essential for virus replication.

Moreover, CRISPR–Cas knockout cells can be used to understand the molecular basis of knockout phenotypes and to help identify the stage of the virus life cycle in which the host factor is involved. For example, the OST complex was found to be required for viral RNA synthesis, but not for viral entry and translation⁶¹. The OST complex catalyses the *N*-linked glycosylation of newly synthesized proteins. In mammalian cells, two distinct OST multiprotein complexes are formed, each composed of a catalytic subunit (one of two paralogues, STT3A or STT3B) and accessory subunits⁷². Both isoforms are individually required for the replication of DENV, as knockout of either STT3A or STT3B resulted in complete abrogation of DENV replication. Other mosquito-borne flaviviruses, including ZIKV, are exclusively dependent on the STT3A isoform for viral RNA replication, which indicates a specific but divergent virus–host interaction (FIG. 2). Surprisingly, the catalytic activity of STT3A and STT3B was dispensable for virus replication, because catalytically inactive mutant proteins were able to restore DENV replication in the knockout cells, which indicates that the OST complex has an unconventional role in DENV replication. The OST complex was found to bind to multiple non-structural viral proteins that form the RNA synthesis complex at the ER⁶¹, which suggests that the OST complex acts as a scaffold to coordinate the assembly of a functional DENV RNA replication complex.

Other host factors that were found to be required for flavivirus replication include SEC61A1 and SEC63, which form the translocon channel in the ER membrane; the translocon-associated protein (TRAP) complex, which stimulates co-translational translocation of polypeptides into the ER⁷³; and the signal peptidase complex that cleaves signal peptides in the ER lumen. Knockout of a subset of signal peptidase complex subunits (SPCSs) revealed severe defects in the polyprotein cleavage of multiple flaviviruses. In particular, cleavage of the structural proteins prM and E from the polyprotein was affected, leading to marked defects in the release of virus particles⁷⁰.

Components of the ERAD pathway were also found to be important for flavivirus replication. This protein quality control mechanism targets incorrectly folded proteins in the ER lumen for retrotranslocation through the ER membrane to the cytosol, in which proteasomal degradation occurs⁷⁴. Two categories of ERAD components were found in the CRISPR–Cas screens: first, components of the classical ERAD machinery, including SEL1L, derlin 2 (DERL2) and ubiquitin-conjugating enzyme E2 J1 (UBE2J1), which are part of the retrotranslocation complex⁷⁵; and second, components of the ER membrane complex (EMC), an evolutionarily conserved complex that has less-well-understood roles in ERAD⁷⁶. Knockout of ERAD components led to substantial decreases in viral-RNA accumulation, particle formation and virus-induced cell death for DENV, ZIKV, Japanese encephalitis virus and WNV^{61,69,70}. However, how ERAD functions promote flavivirus replication remains to be fully understood.

◀ **Figure 2 | Host factors that have been identified by CRISPR–Cas screens as important for infection and replication of viruses in the family *Flaviviridae*.** The flaviviruses Zika virus (ZIKV), dengue virus (DENV) and West Nile virus (WNV) enter the cell by attachment to cell surface molecules, including heparan sulfate proteoglycans (HSPG) and potentially other protein receptors^{61,71,77}. After uncoating, viral (+)RNA is translated by host ribosomes. The ribosomal subunit 40S ribosomal protein S25 (RPS25) is important for DENV infection and for translation of hepatitis C virus (HCV) RNA, but is dispensable for host mRNA translation^{61,158}. The flavivirus polyprotein is inserted into the endoplasmic reticulum (ER) membrane and cleaved by viral and host proteases, including the host signal peptidase complex⁷⁰. The viral proteins assemble a replication complex in close association with several ER-resident host protein complexes: the oligosaccharyl-transferase (OST) complex, the translocon-associated protein (TRAP) complex and components of the ER-associated protein degradation (ERAD) pathway^{61,69–71}. Notably, different flaviviruses have different dependencies on the two distinct OST multiprotein complexes, which contain either an STT3A or an STT3B catalytic subunit. The ERAD-related host factors belong to the classical ERAD complex and the ER membrane protein complex (EMC). HCV enters hepatocytes through the receptors CD81, occludin (OCLN) and claudin 1 (CLDN1)^{61,159}, and the host microRNA miR-122 binds to and stabilizes the 5' UTR of the HCV RNA^{61,160}. FAD biosynthesis, catalysed by riboflavin kinase (RFK) and FAD synthase (FLAD1), is important for HCV RNA synthesis⁶¹. ELAVL1 binds to the 3' UTR of HCV to circularize the viral genome by interacting with La protein (also known as SSB) and displacing polypyrimidine-tract-binding protein 1 (PTB) to stimulate virus replication^{61,161}. Cyclophilin A (CYPA) is required for HCV replication through its interaction with NSSA^{61,83}. UBE2J1, ubiquitin-conjugating enzyme E2 J1; YFV, yellow fever virus.

It is important to note that in contrast to genetic screens with several other viruses (for example, Ebola virus), screens with WNV and DENV have not been able to identify a specific receptor that is required for viral entry into host cells. This is most probably due to redundancy in entry routes, such that knockout of one virus receptor still leaves cells susceptible through a different route. Indeed, several receptors have been reported for DENV⁷⁷. Nevertheless, CRISPR–Cas screens have contributed to our understanding of flavivirus biology, revealing a central role for several ER complexes in promoting flavivirus infection.

HCV. Another important pathogen that has been investigated using CRISPR–Cas screens is HCV, which causes chronic liver disease in ~160 million infected individuals worldwide⁷⁸. Whereas mosquito-borne flaviviruses have a dependence on ER proteins, screening with HCV, which is a more distantly related member of the family *Flaviviridae*, revealed non-overlapping hits, including entry receptors CD81, OCLN and CLDN1, the liver-specific microRNA miR-122 and several RNA-binding proteins and metabolic enzymes⁶¹ (FIG. 2). One of the most significant hits was ELAVL1, an RNA-binding protein that is involved in mRNA stabilization⁷⁹. HCV RNA replication was markedly reduced in ELAVL1-knockout cells, whereas RNA replication for other RNA viruses (for example, DENV and poliovirus) was unaffected. The HCV screens also uncovered an unexpected link between intracellular FAD levels and HCV RNA replication. The enzymes riboflavin kinase (RFK) and FAD synthase (FLAD1), which are involved in the conversion of riboflavin (vitamin B₂) to FAD, were found to be crucial for the replication of HCV. Lumiflavin, an inhibitor of cellular uptake of riboflavin, potently inhibited viral RNA replication, which indicates that the modulation of intracellular FAD levels could be explored as an antiviral treatment. Host-targeted antiviral therapeutics may become an effective strategy to control virus replication because they may present a higher genetic barrier for resistant mutants to evolve than virus-targeting antivirals, and they have the potential to inhibit a broader range of viruses⁶⁵. For example, cyclophilin A (CYPA) is a host factor that is required for HCV replication and also promotes HIV infection^{80–82}. CYPA inhibitors have advanced to phase II/III clinical trials for the treatment of HCV infection, and their use is also being explored to treat other viral infections⁸³.

Noroviruses. Human noroviruses are a leading cause of gastroenteritis globally. Although their mechanism of entry and cellular receptor remain unknown, carbohydrates — in particular, the histo-blood group antigens (HBGAs) — have been shown to have a role in human norovirus entry⁸⁴. Unbiased genetic CRISPR–Cas screens led to the discovery of CD300LF (also known as CLM1) as a proteinaceous receptor for murine norovirus^{85,86}. CD300LF knockout abolished murine norovirus infection in mouse cell lines and in a mouse model of murine norovirus infection. Moreover,

the expression of mouse CD300LF in human cells made them susceptible to murine norovirus infection. The discovery of a key host receptor that is necessary and sufficient for the binding of murine norovirus raises the possibility that human noroviruses also require a specific proteinaceous receptor or receptors. The recent development of a more reliable *in vitro* infection model for human noroviruses⁸⁷ combined with CRISPR–Cas technology could lead to a better understanding of the entry pathway that is used by human noroviruses and to the development of entry inhibitors.

HIV. To identify host factors that are required for HIV replication, a CRISPR–Cas screen was carried out in a physiologically relevant CD4⁺ T cell line⁸⁸. In addition to the T cell surface glycoprotein CD4 and the co-receptor CC-chemokine receptor type 5 (CCR5) that are required for entry of CCR5-tropic viruses, a cell adhesion molecule named CD166 antigen (ALCAM) and two proteins, protein tyrosine sulfotransferase 2 (TPST2) and adenosine 3'-phospho 5'-phosphosulfate transporter 1 (SLC35B2), that are involved in tyrosine sulfation were found to be important for HIV infection. To validate these findings, electroporation was used to introduce Cas9–sgRNA ribonucleoprotein complexes into CD4⁺ T cells that were isolated from the blood of healthy human donors, and these cells were then challenged with CCR5-tropic HIV. This demonstrates that CRISPR–Cas technology can be used to study host factors in primary cells.

Bacteria, parasites and immune signalling. Genome-scale knockout screens have also been used to uncover immune-regulatory networks⁸⁹, the pyroptosis pathway⁹⁰ and host requirements for bacterial pathogenesis^{91–93}. CRISPR–Cas screens in *Toxoplasma gondii* have also identified genes that are essential for the fitness of apicomplexan parasites⁹⁴. In bacteria, partial knockdowns using CRISPR interference enabled the systematic phenotypic identification of essential bacterial genes in *Bacillus subtilis*⁹⁵.

Emerging CRISPR–Cas tools

CRISPR–Cas technology has broad applications in the study of viruses, extending beyond host factor screens. CRISPR–Cas methods are being used to generate both *in vitro* and *in vivo* models to study viral pathogenesis, to edit and image viral genomes, in the development of gene drive systems that have the potential to eradicate viral disease vectors, and to advance the development of antiviral therapeutics (FIG. 3).

Generation of *in vitro* and *in vivo* models to study viral disease. Traditionally, *in vitro* systems using cell lines have been invaluable tools to study virus infections. However, these systems have limitations in providing comprehensive insights into host physiology, immunity, pathology and transmission during infection. CRISPR–Cas technology has been used to generate advanced *in vitro* and *in vivo* knockout models to study viral pathogenesis, such as primary cells,

miR-122

A liver-specific microRNA that is required for hepatitis C virus replication by interacting with its 5' UTR.

FAD

A redox cofactor that is involved in several important reactions in metabolism.

Pyroptosis

A caspase 1-dependent form of programmed cell death that is inflammatory and crucial for controlling microbial infections.

Gene drive

A technique that promotes the inheritance of a particular gene to increase its prevalence in a population.

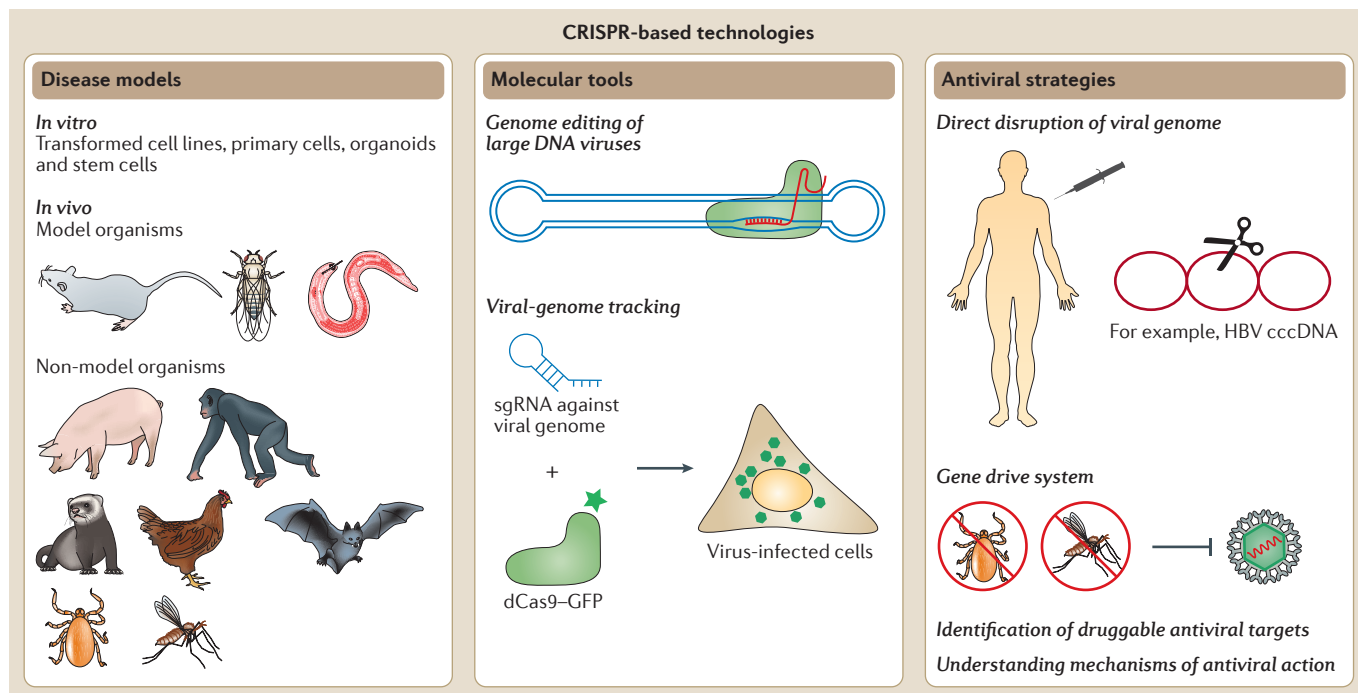


Figure 3 | CRISPR–Cas applications beyond genetic screening. CRISPR–Cas genome editing enables the generation of *in vitro* and *in vivo* models to study viral pathogenesis. The technology is not limited to engineering model organisms, such as mice, fruit flies and roundworms, but can also be applied to non-model organisms, such as pigs, macaques, ferrets, chickens, ticks, bats and mosquitoes. CRISPR–Cas technology is also useful for engineering the genomes of large DNA viruses, such as poxviruses. Catalytically inactive Cas9 (dCas9) proteins that are fused to fluorophores may be useful to track viral nucleic acids in cells¹⁶². CRISPR–Cas technology could lead to the development of new approaches to treat virus infections and prevent transmission, including the development of gene drive systems to eradicate viral disease vectors, the direct targeting to inactivate viral gene expression, the identification of druggable host proteins that are required for virus replication, and elucidating the mechanisms of action of antivirals. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; sgRNA, single-guide RNA.

organoids, induced-pluripotent stem cells (iPSCs)^{96–98} and animal models⁹⁹. CRISPR–Cas methods have expedited the process of generating knockout animal models. In addition to genome engineering of laboratory animals, such as roundworms^{100,101}, fruit flies^{102,103} and mice^{104,105}, CRISPR–Cas approaches can be applied to non-model organisms, such as mosquitoes¹⁰⁶, ticks, bats, pigs^{107,108}, macaques¹⁰⁹, ferrets¹¹⁰ and chickens¹¹¹, which are important vectors or reservoirs of viruses. For example, bats are reservoirs for rabies virus, Nipah virus, Ebola virus and severe acute respiratory syndrome-related coronavirus, whereas mosquitoes transmit DENV, ZIKV, WNV and chikungunya virus¹⁰⁴. Ferrets are a suitable animal model to study influenza viruses¹¹². Previously, it was challenging to genetically engineer ferrets. Ferrets that have been genetically engineered using CRISPR–Cas technology have recently been reported and may substantially broaden the application of the ferret model in the study of influenza virus pathogenesis and transmission¹¹⁰.

CRISPR–Cas tools for studying large DNA viruses. Efficient genetic modification of large viral genomes has been limited by conventional molecular cloning techniques, especially for DNA viruses that belong to

the proposed order *Megavirales*. For example, it has been challenging to edit the genomes of human poxviruses, which range from 130 kb to 375 kb in size. However, CRISPR–Cas technology has been used to efficiently edit the genomes of large DNA viruses, such as vaccinia virus, Epstein–Barr virus and adenoviral vectors^{113–115}.

CRISPR–Cas antiviral strategies. There is also potential for the application of CRISPR–Cas technology in the prevention and treatment of diseases by targeting viruses and their vectors. Vector control has been used as a strategy to limit the transmission of vector-borne viruses, including ZIKV, DENV and yellow fever virus. For example, several attempts have been made to introduce genetically modified, sterile mosquitoes into the environment in an attempt to eradicate wild-type mosquito populations that transmit viral diseases^{116–119}. CRISPR–Cas tools have been used to generate gene drives that have the potential to diminish mosquito populations^{120,121}. Furthermore, CRISPR–Cas technology could be used to treat persistent virus infections, such as infections with HIV, HBV, HCV and herpes simplex virus^{122–125}. Recently, HBV covalently closed circular DNA (cccDNA), the hallmark of persistent HBV infection, has been successfully targeted in cell culture

Organoids

3D, miniaturized and simplified versions of organs, produced *in vitro*.

Covalently closed circular DNA

(cccDNA). The replicative form of the hepatitis B virus DNA, which persists within the nuclei of infected liver cells and produces viral RNA transcripts.

C2c2

A ribonuclease that is guided by a single CRISPR RNA and can be programmed to cleave single-stranded RNA targets.

Epistatic relationships

The relationships between genes in which one gene influences the phenotypic expression of another gene.

and in animal models^{126–129}. In addition, CRISPR–Cas screens can be used to understand the mode of action of antivirals. For example, CRISPR–Cas and short hairpin RNA (shRNA) screens carried out in parallel uncovered the mechanism of action of GSK983, an antiviral drug that may prove effective in the treatment of a wide range of RNA and DNA viruses^{130,131}. GSK983 was found to block virus replication by inhibiting the cellular pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase, thus reducing intracellular levels of nucleotides, which are needed for viral nucleic acid synthesis.

Conclusions and perspectives

The repurposing of the CRISPR–Cas system as a genome-engineering tool is starting to transform biomedical research in several areas, including infectious diseases, cancer and gene therapy. This new approach is also being used to gain a better understanding of how viruses exploit their host and to develop new antiviral therapeutics. Since its discovery, CRISPR–Cas technology has already advanced our understanding of the life cycles of noroviruses and flaviviruses. Future screens will undoubtedly shed light on commonalities and differences in how viruses have evolved to exploit and subvert host functions, and may provide potential targets for antiviral therapy. Together with advances in the genetic engineering of animal models using

CRISPR–Cas and improvements in field applications such as gene drive systems, these new CRISPR–Cas technologies will help us to tackle current and future viral epidemics.

Continued efforts to develop and enhance CRISPR–Cas systems will expand the toolbox that enables us to gain a greater understanding of complex biological and disease processes. Engineering of Cas nucleases will make DNA and RNA targeting more versatile. For example, *Staphylococcus aureus* Cas9 is smaller than most Cas9 nucleases that have been used to date, making *in vivo* delivery of Cas9–sgRNA complexes more feasible^{132,133}; and the CRISPR-associated endoribonuclease C2c2 could lead to the development of new RNA-targeting tools^{134,135}. Moreover, CRISPR–Cas systems can be combined with other technologies to develop more sophisticated screening approaches. Combining CRISPR–Cas technology with advances in single-cell profiling could lead to better measurements of virus replication dynamics^{136–140}. Furthermore, a screening strategy that investigates epistatic relationships (for example by combining haploid and CRISPR–Cas mutagenesis⁶⁰), will enable the systematic analysis of functional interdependencies between the host genes that are most crucial for virus infection. We expect that refining and expanding the genetic toolbox for manipulating host cells will lead to novel insights into the ‘arms race’ between viruses and their hosts.

- Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94 (1974).
- Hartwell, L. H., Culotti, J. & Reid, B. Genetic control of the cell-division cycle in yeast. I. Detection of mutants. *Proc. Natl Acad. Sci. USA* **66**, 352–359 (1970).
- Rutschmann, S. *et al.* Role of *Drosophila* IKK γ in a toll-independent antibacterial immune response. *Nat. Immunol.* **1**, 342–347 (2000).
- Nusslein-Volhard, C. & Wieschaus, E. Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795–801 (1980).
- Berns, K. *et al.* A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* **428**, 431–437 (2004).
- Paddison, P. J. *et al.* A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428**, 427–431 (2004).
- Carette, J. E. *et al.* Haploid genetic screens in human cells identify host factors used by pathogens. *Science* **326**, 1231–1235 (2009). **This publication demonstrates the first comprehensive, genome-wide knockout screen in human cell culture by using insertional mutagenesis in a haploid cell line.**
- Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
- Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).
- Jinek, M. *et al.* RNA-programmed genome editing in human cells. *eLife* **2**, e00471 (2013).
- Evers, B. *et al.* CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nat. Biotechnol.* **34**, 631–633 (2016).
- DeJesus, R. *et al.* Functional CRISPR screening identifies the ufmylation pathway as a regulator of SQSTM1/p62. *eLife* **5**, e17290 (2016).
- Hart, T. *et al.* High-resolution CRISPR screens reveal fitness genes and genotype-specific cancer liabilities. *Cell* **163**, 1515–1526 (2015). **This study and reference 11 provide side-by-side comparisons of CRISPR knockout, CRISPR interference and RNAi-based genetic screens to identify essential genes.**
- Shalem, O. *et al.* Genome-scale CRISPR–Cas9 knockout screening in human cells. *Science* **343**, 84–87 (2014).
- Wang, T., Wei, J. J., Sabatini, D. M. & Lander, E. S. Genetic screens in human cells using the CRISPR–Cas9 system. *Science* **343**, 80–84 (2014). **This study and reference 14 are the first studies to demonstrate the power of genome-scale CRISPR–Cas knockout screens.**
- Shalem, O., Sanjana, N. E. & Zhang, F. High-throughput functional genomics using CRISPR–Cas9. *Nat. Rev. Genet.* **16**, 299–311 (2015).
- Zhou, Y. *et al.* High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature* **509**, 487–491 (2014).
- Evans, M. J. *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805 (2007). **This investigation is a good example for a gain-of-function cDNA expression screen to identify a receptor for viral entry.**
- Ploss, A. *et al.* Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* **457**, 882–886 (2009).
- Saeed, M. *et al.* SEC14L2 enables pan-genotype HCV replication in cell culture. *Nature* **524**, 471–475 (2015).
- Schoggins, J. W. *et al.* Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* **505**, 691–695 (2014).
- Schoggins, J. W. *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* **472**, 481–485 (2011).
- ORFeome Collaboration. The ORFeome Collaboration: a genome-scale human ORF-clone resource. *Nat. Methods* **13**, 191–192 (2016).
- Ramage, H. & Cherry, S. Virus–host interactions: from unbiased genetic screens to function. *Annu. Rev. Virol.* **2**, 497–524 (2015).
- Carette, J. E. *et al.* Ebola virus entry requires the cholesterol transporter Niemann–Pick C1. *Nature* **477**, 340–343 (2011).
- Jae, L. T. *et al.* Lassa virus entry requires a trigger-induced receptor switch. *Science* **344**, 1506–1510 (2014).
- Miller, E. H. *et al.* Ebola virus entry requires the host-programmed recognition of an intracellular receptor. *EMBO J.* **31**, 1947–1960 (2012).
- Cote, M. *et al.* Small molecule inhibitors reveal Niemann–Pick C1 is essential for Ebola virus infection. *Nature* **477**, 344–348 (2011).
- Bornholdt, Z. A. *et al.* Host-primed Ebola virus GP exposes a hydrophobic NPC1 receptor-binding pocket, revealing a target for broadly neutralizing antibodies. *mBio* **7**, e02154-15 (2016).
- Gong, X. *et al.* Structural insights into the Niemann–Pick C1 (NPC1)-mediated cholesterol transfer and Ebola infection. *Cell* **165**, 1467–1478 (2016).
- Wang, H. *et al.* Ebola viral glycoprotein bound to its endosomal receptor Niemann–Pick C1. *Cell* **164**, 258–268 (2016).
- Diehl, W. E. *et al.* Ebola virus glycoprotein with increased infectivity dominated the 2013–2016 epidemic. *Cell* **167**, 1088–1098.e6 (2016).
- Urbanowicz, R. A. *et al.* Human adaptation of Ebola virus during the West African outbreak. *Cell* **167**, 1079–1087.e5 (2016).
- Staring, J. *et al.* PLA2G16 represents a switch between entry and clearance of Picornaviridae. *Nature* **541**, 412–416 (2017).
- Pillay, S. *et al.* An essential receptor for adeno-associated virus infection. *Nature* **530**, 108–112 (2016).
- Schmidt, T., Schmid-Burgk, J. L. & Hornung, V. Synthesis of an arrayed sgRNA library targeting the human genome. *Sci. Rep.* **5**, 14987 (2015).
- Essletzbichler, P. *et al.* Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res.* **24**, 2059–2065 (2014).
- Sagi, I. *et al.* Derivation and differentiation of haploid human embryonic stem cells. *Nature* **532**, 107–111 (2016).
- Elling, U. *et al.* Forward and reverse genetics through derivation of haploid mouse embryonic stem cells. *Cell Stem Cell* **9**, 563–574 (2011).
- Leeb, M. & Wutz, A. Derivation of haploid embryonic stem cells from mouse embryos. *Nature* **479**, 131–134 (2011).
- Wutz, A. Haploid mouse embryonic stem cells: rapid genetic screening and germline transmission. *Annu. Rev. Cell Dev. Biol.* **30**, 705–722 (2014).
- Pillay, S. & Carette, J. E. Hunting viral receptors using haploid cells. *Annu. Rev. Virol.* **2**, 219–239 (2015).
- Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* **11**, 783–784 (2014).

44. Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* **34**, 184–191 (2016). **This report shows how to apply sgRNA sequence rules to improve on-target cleavage efficiency and reduce off-target effects in order to design more powerful CRISPR-Cas libraries.**
45. Wang, T. *et al.* Identification and characterization of essential genes in the human genome. *Science* **350**, 1096–1101 (2015).
46. Liu, S. J. *et al.* CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science* **355**, aah7111 (2016).
47. Joung, J. *et al.* Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. *Nat. Protoc.* **12**, 828–863 (2017).
48. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. & Yee, J. K. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl Acad. Sci. USA* **90**, 8033–8037 (1993).
49. Wool-Lewis, R. J. & Bates, P. Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. *J. Virol.* **72**, 3155–3160 (1998).
50. Xiong, C. *et al.* Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* **243**, 1188–1191 (1989).
51. Lohmann, V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113 (1999).
52. Trono, D., Pelletier, J., Sonenberg, N. & Baltimore, D. Translation in mammalian cells of a gene linked to the poliovirus 5' noncoding region. *Science* **241**, 445–448 (1988).
53. Simoes, E. A. & Sarnow, P. An RNA hairpin at the extreme 5' end of the poliovirus RNA genome modulates viral translation in human cells. *J. Virol.* **65**, 913–921 (1991).
54. Luo, B. *et al.* Highly parallel identification of essential genes in cancer cells. *Proc. Natl Acad. Sci. USA* **105**, 20380–20385 (2008).
55. Konig, R. *et al.* A probability-based approach for the analysis of large-scale RNAi screens. *Nat. Methods* **4**, 847–849 (2007).
56. Li, W. *et al.* MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biol.* **15**, 554 (2014).
57. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 (2013).
58. Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013).
59. Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843 (2013).
60. Blomen, V. A. *et al.* Gene essentiality and synthetic lethality in haploid human cells. *Science* **350**, 1092–1096 (2015). **Together with reference 45, this paper defines a core set of essential human genes and identifies synthetic-lethal interactions using genetic knockout approaches.**
61. Marceau, C. D. *et al.* Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* **535**, 159–163 (2016). **This paper describes a comprehensive analysis of host factors that are crucial for DENV and HCV infections using haploid and CRISPR-Cas genetic knockout screens.**
62. Wu, X., Li, Y., Crise, B. & Burgess, S. M. Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**, 1749–1751 (2003).
63. Carette, J. E. *et al.* Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat. Biotechnol.* **29**, 542–546 (2011).
64. Timms, R. T. *et al.* Genetic dissection of mammalian ERAD through comparative haploid and CRISPR forward genetic screens. *Nat. Commun.* **7**, 11786 (2016).
65. Bekerman, E. & Einav, S. Combating emerging viral threats. *Science* **348**, 282–283 (2015).
66. Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496**, 504–507 (2013).
67. Lessler, J. *et al.* Assessing the global threat from Zika virus. *Science* **353**, aaf8160 (2016).
68. Cao-Lormeau, V. M. *et al.* Guillain-Barre syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *Lancet* **387**, 1531–1539 (2016).
69. Ma, H. *et al.* A CRISPR-based screen identifies genes essential for West-Nile-virus-induced cell death. *Cell Rep.* **12**, 673–683 (2015). **This study is the first to use a CRISPR-Cas screen to study virus-host interactions.**
70. Zhang, R. *et al.* A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature* **535**, 164–168 (2016). **This work uses a CRISPR-Cas genetic screen to reveal host factors that are important for flavivirus polyprotein processing.**
71. Savidis, G. *et al.* Identification of Zika virus and Dengue virus dependency factors using functional genomics. *Cell Rep.* **16**, 232–246 (2016).
72. Cherepanova, N. A. & Gilmore, R. Mammalian cells lacking either the cotranslational or posttranslational oligosaccharyltransferase complex display substrate-dependent defects in asparagine linked glycosylation. *Sci. Rep.* **6**, 20946 (2016).
73. Fons, R. D., Bogert, B. A. & Hegde, R. S. Substrate-specific function of the translocon-associated protein complex during translocation across the ER membrane. *J. Cell Biol.* **160**, 529–539 (2003).
74. Olzmann, J. A., Kopito, R. R. & Christianson, J. C. The mammalian endoplasmic reticulum-associated degradation system. *Cold Spring Harb. Perspect. Biol.* **5**, a013185 (2013).
75. Christianson, J. C. *et al.* Defining human ERAD networks through an integrative mapping strategy. *Nat. Cell Biol.* **14**, 93–105 (2011).
76. Wideman, J. G. The ubiquitous and ancient ER membrane protein complex (EMC): tether or not? *F1000Res.* **4**, 624 (2015).
77. Cruz-Oliveira, C. *et al.* Receptors and routes of dengue virus entry into the host cells. *FEMS Microbiol. Rev.* **39**, 155–170 (2015).
78. Lavanchy, D. Evolving epidemiology of hepatitis C virus. *Clin. Microbiol. Infect.* **17**, 107–115 (2011).
79. Brennan, C. M. & Steitz, J. A. HuR and mRNA stability. *Cell. Mol. Life Sci.* **58**, 266–277 (2001).
80. Madan, V., Paul, D., Lohmann, V. & Bartenschlager, R. Inhibition of HCV replication by cyclophilin antagonists is linked to replication fitness and occurs by inhibition of membranous web formation. *Gastroenterology* **146**, 1361–1372.e9 (2014).
81. Nag, A., Robotham, J. M. & Tang, H. Suppression of viral RNA binding and the assembly of infectious hepatitis C virus particles *in vitro* by cyclophilin inhibitors. *J. Virol.* **86**, 12616–12624 (2012).
82. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V. & Goff, S. P. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**, 1067–1078 (1993).
83. Lin, K. & Gallay, P. Curing a viral infection by targeting the host: the example of cyclophilin inhibitors. *Antiviral Res.* **99**, 68–77 (2013).
84. Tan, M. & Jiang, X. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. *Trends Microbiol.* **13**, 285–293 (2005).
85. Orchard, R. C. *et al.* Discovery of a proteinaceous cellular receptor for a norovirus. *Science* **353**, 933–936 (2016).
86. Haga, K. *et al.* Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells. *Proc. Natl Acad. Sci. USA* **113**, E6248–E6255 (2016).
87. Jones, M. K. *et al.* Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* **346**, 755–759 (2014).
88. Park, R. J. *et al.* A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. *Nat. Genet.* **49**, 193–203 (2017).
89. Parnas, O. *et al.* A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks. *Cell* **162**, 675–686 (2015). **This is the first investigation to carry out a genome-wide CRISPR-Cas screen in primary cells and provides a comprehensive view on components of Toll-like receptor 4 signalling.**
90. Shi, J. *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665 (2015).
91. Popov, L. M. *et al.* The adherens junctions control susceptibility to *Staphylococcus aureus* α -toxin. *Proc. Natl Acad. Sci. USA* **112**, 14337–14342 (2015).
92. Virreira Winter, S., Zychlinsky, A. & Bardoe, B. W. Genome-wide CRISPR screen reveals novel host factors required for *Staphylococcus aureus* α -hemolysin-mediated toxicity. *Sci. Rep.* **6**, 24242 (2016).
93. Blondel, C. J. *et al.* CRISPR/Cas9 screens reveal requirements for host cell sulfation and fucosylation in bacterial type III secretion system-mediated cytotoxicity. *Cell Host Microbe* **20**, 226–237 (2016).
94. Sidik, S. M. *et al.* A genome-wide CRISPR screen in toxoplasma identifies essential apicomplexan genes. *Cell* **166**, 1423–1435.e12 (2016). **This study applies genome-scale CRISPR-Cas screening in the eukaryotic parasite *T. gondii* to investigate its fitness genes.**
95. Peters, J. M. *et al.* A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* **165**, 1493–1506 (2016). **This paper describes the use of genome-scale CRISPR-Cas knockdown screens to uncover bacterial genes that are important for growth and cell morphology.**
96. Hou, P. *et al.* Genome editing of *CXCR4* by CRISPR/Cas9 confers cells resistant to HIV-1 infection. *Sci. Rep.* **5**, 15577 (2015).
97. Li, C. *et al.* Inhibition of HIV-1 infection of primary CD4⁺ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. *J. Gen. Virol.* **96**, 2381–2393 (2015).
98. Wells, M. F. *et al.* Genetic ablation of AXL does not protect human neural progenitor cells and cerebral organoids from Zika virus infection. *Cell Stem Cell* **19**, 703–708 (2016).
99. Tschaharganeh, D. F., Lowe, S. W., Garippa, R. J. & Livshits, G. Using CRISPR/Cas to study gene function and model disease *in vivo*. *FEBS J.* **283**, 3194–3203 (2016).
100. Friedland, A. E. *et al.* Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nat. Methods* **10**, 741–743 (2013).
101. Dickinson, D. J., Ward, J. D., Reiner, D. J. & Goldstein, B. Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat. Methods* **10**, 1028–1034 (2013).
102. Bassett, A. R., Tibbit, C., Ponting, C. P. & Liu, J. L. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep.* **4**, 220–228 (2013).
103. Gratz, S. J. *et al.* Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* **194**, 1029–1035 (2013).
104. Bean, A. G. *et al.* Studying immunity to zoonotic diseases in the natural host — keeping it real. *Nat. Rev. Immunol.* **13**, 851–861 (2013).
105. Li, D. *et al.* Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 681–683 (2013).
106. Kistler, K. E., Vosshall, L. B. & Matthews, B. J. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Rep.* **11**, 51–60 (2015).
107. Lei, S. *et al.* Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. *Sci. Rep.* **6**, 25222 (2016).
108. Hai, T., Teng, F., Guo, R., Li, W. & Zhou, Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res.* **24**, 372–375 (2014).
109. Niu, Y. *et al.* Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843 (2014).
110. Kou, Z. *et al.* CRISPR/Cas9-mediated genome engineering of the ferret. *Cell Res.* **25**, 1372–1375 (2015).
111. Oishi, I., Yoshii, K., Miyahara, D., Kagami, H. & Tagami, T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Sci. Rep.* **6**, 23980 (2016).
112. Belsler, J. A., Eckert, A. M., Tumpey, T. M. & Maines, T. R. Complexities in ferret influenza virus pathogenesis and transmission models. *Microbiol. Mol. Biol. Rev.* **80**, 733–744 (2016).
113. Yuan, M. *et al.* Efficiently editing the vaccinia virus genome by using the CRISPR-Cas9 system. *J. Virol.* **89**, 5176–5179 (2015).
114. Bi, Y. *et al.* High-efficiency targeted editing of large viral genomes by RNA-guided nucleases. *PLoS Pathog.* **10**, e1004090 (2014).
115. Yuen, K. S. *et al.* CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells. *J. Gen. Virol.* **96**, 626–636 (2015).
116. Harris, A. F. *et al.* Field performance of engineered male mosquitoes. *Nat. Biotechnol.* **29**, 1034–1037 (2011).
117. Burt, A. Heritable strategies for controlling insect vectors of disease. *Phil. Trans. R. Soc. B* **369**, 20130432 (2014).

118. Carvalho, D. O. *et al.* Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Negl Trop. Dis.* **9**, e0003864 (2015).
119. Adelman, Z. N. & Tu, Z. Control of mosquito-borne infectious diseases: sex and gene drive. *Trends Parasitol.* **32**, 219–229 (2016).
120. Hammond, A. *et al.* A CRISPR–Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat. Biotechnol.* **34**, 78–83 (2016).
121. Hall, A. B. *et al.* A male-determining factor in the mosquito *Aedes aegypti*. *Science* **348**, 1268–1270 (2015).
122. Price, A. A., Sampson, T. R., Ratner, H. K., Grakoui, A. & Weiss, D. S. Cas9-mediated targeting of viral RNA in eukaryotic cells. *Proc. Natl Acad. Sci. USA* **112**, 6164–6169 (2015).
123. van Diemen, F. R. *et al.* CRISPR/Cas9-mediated genome editing of herpesviruses limits productive and latent infections. *PLoS Pathog.* **12**, e1005701 (2016).
124. Dong, C. *et al.* Targeting hepatitis B virus cccDNA by CRISPR/Cas9 nuclease efficiently inhibits viral replication. *Antiviral Res.* **118**, 110–117 (2015).
125. Kaminski, R. *et al.* Excision of HIV-1 DNA by gene editing: a proof-of-concept *in vivo* study. *Gene Ther.* **23**, 690–695 (2016).
126. Zhen, S. *et al.* Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. *Gene Ther.* **22**, 404–412 (2015).
127. Liu, X., Hao, R., Chen, S., Guo, D. & Chen, Y. Inhibition of hepatitis B virus by the CRISPR/Cas9 system via targeting the conserved regions of the viral genome. *J. Gen. Virol.* **96**, 2252–2261 (2015).
128. Kennedy, E. M. *et al.* Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease. *Virology* **476**, 196–205 (2015).
129. Ramanan, V. *et al.* CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci. Rep.* **5**, 10833 (2015).
130. Deans, R. M. *et al.* Parallel shRNA and CRISPR–Cas9 screens enable antiviral drug target identification. *Nat. Chem. Biol.* **12**, 361–366 (2016).
131. Harvey, R. *et al.* GSK983: a novel compound with broad-spectrum antiviral activity. *Antiviral Res.* **82**, 1–11 (2009).
132. Barrangou, R. Diversity of CRISPR–Cas immune systems and molecular machines. *Genome Biol.* **16**, 247 (2015).
133. Ran, F. A. *et al.* *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nature* **520**, 186–191 (2015).
134. Abudayyeh, O. O. *et al.* C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* **353**, aaf5573 (2016).
135. East-Seletsky, A. *et al.* Two distinct RNase activities of CRISPR–C2c2 enable guide-RNA processing and RNA detection. *Nature* **538**, 270–273 (2016).
136. Klein, A. M. *et al.* Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. *Cell* **161**, 1187–1201 (2015).
137. Macosko, E. Z. *et al.* Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* **161**, 1202–1214 (2015).
138. Adamson, B. *et al.* A multiplexed single-cell CRISPR screening platform enables systematic dissection of the unfolded protein response. *Cell* **167**, 1867–1882.e21 (2016).
139. Dixit, A. *et al.* Perturb-Seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell* **167**, 1853–1866.e17 (2016).
140. Jaitin, D. A. *et al.* Dissecting immune circuits by linking CRISPR-pooled screens with single-cell RNA-Seq. *Cell* **167**, 1883–1896.e15 (2016).
141. Marraffini, L. A. CRISPR–Cas immunity in prokaryotes. *Nature* **526**, 55–61 (2015).
142. Pourcel, C., Salvignol, G. & Vergnaud, G. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* **151**, 653–663 (2005).
143. Deltcheva, E. *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602–607 (2011).
144. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71 (2010).
145. Marraffini, L. A. & Sontheimer, E. J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **463**, 568–571 (2010).
146. Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733–740 (2009).
147. Horvath, P. *et al.* Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. *J. Bacteriol.* **190**, 1401–1412 (2008).
148. Jiang, F., Zhou, K., Ma, L., Gressel, S. & Doudna, J. A. A Cas9-guide RNA complex preorganized for target DNA recognition. *Science* **348**, 1477–1481 (2015).
149. O’Connell, M. R. *et al.* Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* **516**, 263–266 (2014).
150. Jinek, M. *et al.* Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* **343**, 1247997 (2014).
151. Wiedenheft, B. *et al.* RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. *Proc. Natl Acad. Sci. USA* **108**, 10092–10097 (2011).
152. Haurwitz, R. E., Jinek, M., Wiedenheft, B., Zhou, K. & Doudna, J. A. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science* **329**, 1355–1358 (2010).
153. Hale, C. R. *et al.* RNA-guided RNA cleavage by a CRISPR RNA–Cas protein complex. *Cell* **139**, 945–956 (2009).
154. Gilbert, L. A. *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451 (2013).
155. Gilbert, L. A. *et al.* Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* **159**, 647–661 (2014).

This publication demonstrates the development of genome-wide CRISPR interference and activation screens.

Acknowledgements

The authors thank M. Diamond and C. Dovey for critically reading the manuscript and for helpful comments.

Competing interests statement

The authors declare competing interests: see [Web version](#) for details.

FURTHER INFORMATION

Addgene: <http://www.addgene.org/crispr/libraries>

ALL LINKS ARE ACTIVE IN THE ONLINE PDF