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REVIEW ARTICLE

CRISPR-Based Functional Genomics in Antiviral Innate Immune Response

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Abstract

CRISPR (clustered regularly interspaced short palindromic repeats)-based genome-wide functional genomics can be applied to uncover novel genes controlling an antiviral immune response, thereby augmenting antiviral immunity. To date, much progress has been made in understanding the host and viral factors influencing the antiviral immune response since its discovery. However, many of the contributing genetic factors for the antiviral response remain uncharacterized. Using antiviral innate immunity as a model, this study reports the first genome-wide functional genomic approach using an integrated lentivirus CRISPR-based gene knockout (GeCKO) screening library to identify critical genetic factors influencing the antiviral immune response. This functional CRISPR-based genomic regime is intended to enhance the understanding of genes and pathways controlling an antiviral immune response and broaden the use of CRISPR methodologies in antiviral research for therapeutic innovations. The scope of this study is to answer critical questions regarding how innate immunity fights off viral infections. Two hypotheses are investigated experimentally that key genetic factors influencing the immune induction of the interferon response remain to be found and perturbation of the interferon response will reveal a network of previously uncharacterized genes and pathways controlling an antiviral immune response. With systemic and comprehensive efforts to dissect the host-virus molecular arms race, a more nuanced understanding of the host antiviral response will be acquired, including the discovery of novel genes and pathways involved in antiviral immunity.

Keywords: CRISPR; Genomics; Antiviral Innate; Immune Response

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1 Introduction

In recent years, there has been a growing interest in using CRISPR-based functional genomics to study antiviral innate immune response. The last decade has seen rapid advances in genomics technologies including next-generation sequencing and bioinformatics. These technological breakthroughs facilitated the identification of host factors required for the entry and replication of various viruses, leading to an enhanced understanding of underlying mechanisms [1]. There is a burgeoning demand for innovative multifaceted approaches to unveil the complexities of antiviral immune mechanisms, especially in light of the ongoing pandemic, with over 203 million confirmed cases and 4.3 million deaths worldwide as of August, 2021. To this end, the application of CRISPR-based functional genomics is poised to significantly reduce the gap between data generation and actionable biological insights, consequently boosting the discovery of

new antiviral strategies. The exponential growth of next-generation sequencing has revolutionized the field of virology by enabling high-throughput discovery of novel pathogenic viruses. To accelerate the healing process following viral infection, an effective response mechanism has emerged in host cells. The immune response is the first line of defense against invading pathogens, with the innate immune system established at the initial stage of pathogen exposure. Pathogen-associated molecular patterns (PAMPs) on pathogens trigger the host immune response [2]. As the earliest discovered and most direct component of innate immunity, the interferon (IFN) system plays a crucial role in neutralizing intracellular viral infections. As a response to viral infection, host cells recognize viral PAMPs via pattern recognition receptors and further activate several signaling pathways. Eventually, IRF3 and IRF7 are activated and transferred to the nucleus, driving the activation of interferon α and β form genes. Broadly secreted IFN molecules bind to the IFN receptors on the cell surface and activate the JAK-STAT signal transduction pathway to upregulate the expression of hundreds of interferon-stimulated genes (ISGs), ultimately exerting antiviral effects.

1.1 Background and significance

A wide range of mechanisms have evolved in animals to combat viral infections. Innate immune responses can be triggered by viral elements in the absence of prior recognition, playing an important role in restricting viral replication at the initial steps of infection. As viruses co-evolve with their hosts, they have evolved mechanisms to antagonize these responses. Mechanistic understanding of innate immune components might underpin strategies to uncover viral immune-evasive activities. The immune response is complex. The changed geological and ecological environment has been selecting for the immune mechanisms in animals, and the immune system, in turn, has been evolving and might have a wide variety of diverse components and strategies for fighting pathogens. Viruses induce a plethora of mechanisms in mammalian hosts, which, on one hand, denote the activation of the host means of defense, and, on the other hand, underpin active immune-evasive strategies. A fundamental response of the vertebrate host to combat viral infection is the establishment of the antiviral state (IS) mediated by the type I, II, and III interferon (IFN) system. However, animals have a wide set of sensors detecting a plethora of pathogen-associated molecular patterns (PAMPs) derived from pathogens [3].

In light of the recent emergence of antibiotic-resistant bacteria and the re-emergence of viral diseases and appearance of new contagious and severe infections, such as the COVID-19 pandemic, public health might once again be at risk. Emerging/re-emerging viruses threaten public health, and effective therapeutics are needed to combat them [4]. There is a need for novel strategies to understand immune-evasive mechanisms employed by viruses in the animal kingdom. Broad antiviral innate immune responses incorporating a rapid activation of gene expression programs are induced by viral pathogens. Besides their physiological role during an infection, these programs are also modulated as an immune-evading strategy by diverse animal viruses, and combinations of systems and techniques are used [5]. See Figure 1.

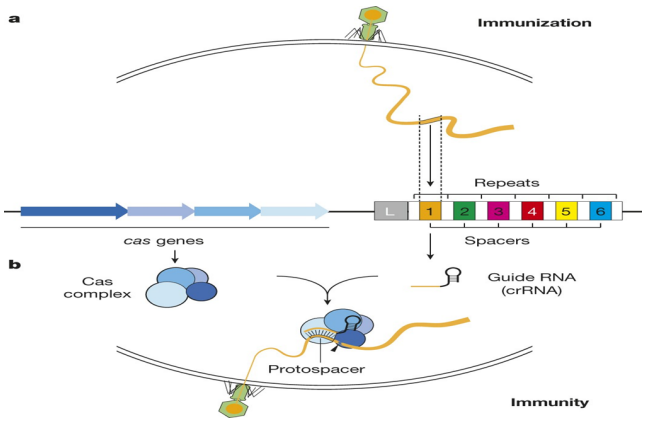

Figure 1: CRISPR [5].

2 The antiviral innate immune response

Since the discovery of the first virus infecting tobacco in 1892, many efforts have been made to understand their classification and structure. Understanding of viral genomes and the biological cycle of viruses required more than half a century. However, the development of antibacterial drugs in the first half of the 20th century provided the opportunity to gain control over serious diseases caused by bacteria [6]. Viruses have much more complicated life cycle compared to bacteria and are incomparably more heterogeneous. Bacteriophages were discovered in early 20th century; however, the originally antibactericidal phage products have not become a competitor to antibiotics. The concept of viruses and their impact on human life was not recognized for a long time, and antibiotics played a dominant role in the treatment of infectious diseases. The foundations for the development of antiviral drugs were laid only at the end of the 20th century due to a better understanding of viral replication [7]. In recent years, development of new antiviral drugs has attracted increased attention, mainly due to a new surge of infectious diseases. Annually, millions of people become infected with viral agents worldwide. Despite

the development of antiviral drugs, vaccination, and various prophylactic measures, viral infections remain a serious danger. The current pandemic caused by SARS-CoV-2 and the complexity of antiviral immunity reveal the urgent need to broaden comprehension of the strategy used by viruses to evade immune defense.

2.1 Overview of antiviral innate immunity

Antiviral innate immunity is the first arm of the immune system that detects and responds to viral infection. Upon viral entry into a host, the own genetic material of viruses is recognized by the host immune system. Such viral components generally consist of double or single-stranded RNA or DNA, which are unusual in uninfected host cells. The host formulates a range of pattern recognition receptors (PRRs) to recognize viral components and activate subsequent immune signaling. The activation of PRR triggered by viral infections results in the release of type I and type III interferons, pro-inflammatory responses, and adaptive immunity [8], as well as directing various immune cells to respond effectively to viral infections. Macrophages and dendritic cells play critical roles in defending the host against various infections, including viruses, and are considered pivotal for the initial detection of invading pathogens. One of the distinguishing features of antiviral innate immune defenses is the dual role of PRRs. Comparative studies on invertebrates and vertebrates have shown that the primary PRRs for antiviral defense are unlike the Toll- and Immune Deficiency (Imd)-mediated pathways. Instead, they appear to rely more on the RNAi pathways or the mimicry and deamination of viral nucleic acids. Despite the differences, invertebrates and vertebrates rely on PRRs, such as RIG-I-like receptors (RIG-Is and MDA5), and TLRs to detect viral infection and initiate appropriate immune responses. These receptors are essential in the detection of false structures present in the viral cell that are lacking when the virus infects the host, triggering one or more signaling pathways and activating NF-κB, IRF3/7, or other transcription factors, subsequently inducing transcription of type I interferon (IFN) and chemokine gene. Activated PRRs initiate the onset of the signaling process to reach the nucleus within several minutes and activate transcription within one or two hours, succinctly clearing the pathogenic stimuli before the virus completes replication and mature infection. In addition to replicating the virus, robust innate immune responses lead to a large number of immune cells, leading to the establishment of a second circle of immune defense. Under certain conditions, this destructive inflammatory feedback possesses the potential to impair host health although most pathogens have been immune decimated. In contrast to the fast and compact nature of innate immunological responses, adaptive immune immunity triggers a defense system in the later phases of infection, the host of itself, and then prepares a large number of specific T/B cells in extremely extraordinary circumstances to actively clone them. Interrelated innate immunity, adaptive immunity, and immunologically deprived mutations all emphasize inflammatory modulatory frequencies to completely understand infection immunology [9]. At present, exist acknowledged studies focused on dissecting the mechanisms of each individual pathway of immune defense, mostly through genetic, cell, and biochemical overexpression and leakage experiments. CRISPR technology has garnered tremendous attention over the last few years and there is a way to integrate CRISPR into existing work to explore megaprime explorations in the antiviral response pathway. To demonstrate the unique usage of utilizing CRISPR technology to probe the cascade and pathway of the immune response to the immune response and to offer future probable study directions, the portion is submitted to explain the fundamental principles of immune responses against antiviral diseases.

2.2 Key players in antiviral immune response

The innate immune response to viral infections is triggered by the detection of PAMPs (Pathogen Associated Molecular Patterns) by host cells. It includes immediate development of an antiviral state, recruitment of immune cells, and activation of adaptive immunity. The innate immune system consists of a variety of cell types that detect and combat pathogens. Viral infection results in the lysis of virus-infected cells and release of viral particles. Free virions activate the humoral immune response. Natural killer (NK) cells are a specialized component of the innate immune system that kills virus-infected cells and cells displaying an abnormal phenotype. Also, the role of dendritic cells, which are antiviral effector cells, has been analyzed. Dendritic cells are professional antigen-presenting cells. They uptake pathogens, process them, and, after transitioning to the lymph nodes, present antigen determinants on major histocompatibility complex molecules to TCD8+ and TCD4+ lymphocytes. Also noteworthy are antiviral effector functions of macrophages [10]. Macrophages are a type of immune cell that engulfs microbiological objects. It is the primary nonspecific cellular immune defense. It combats possible intruders through phagocytosis, in which pathogens are destroyed with the help of lysosomes filled with antimicrobial substances. Effector cells of the antiviral immune response, including macrophages and DC, interact with TCD8+ lympho-

cytes and present them with viral peptides, thereby inducing the lysis of infected cells. The response of effector cells is modulated by cytokines, which mediate cytoplasmatic communication between cells. The main components of the intracellular antiviral immune response are interferons (IFNs). IFN signaling is mediated by a cascade of reactions leading to the nuclear translocation of the complex ISG (interferon stimulated gene) factor driving the expression of IFN stimulated genes (ISG). The latter prevent viral amplification in the infected cells. Not surprisingly, the viral arsenal comprises a number of countermeasures against the IFN response. Some viruses are equipped with proteins that suppress IFN production. Moreover, many viruses encode proteins blocking IFN signaling by specifically targeting transcription factors and pathogenic process mediators. The mechanism underpinning the detection by the innate immune system of thriving intruders is not fully understood, but the likely key modules have been identified. Virus growth results in the accumulation of replicative forms of viral RNA, which are recognized by RIG-1 like RNA Helicases (RLRH) [11]. This findings led to the hypothesis that a long-lasting arms race has developed over the ages between viruses and the host immune system. They have driven the emergence of diversified viral elements ablating detection by counteraction against some types of PRR. On the host side, these elements forced the immune system to evolve into a pleiomorphic assemblage of detectors. This dynamic equilibrium is apparently the optimum solution for the organisms, ensuring the independence of internal homeostatis and exogenous freedom from persistent viral infections. have shown that in conditions of active viral replication PRR rapidly relocated into the viroplasm and associated in large aggregates [12]. At the same time, the subsequent development of the antiviral response was suppressed. Summing up the above, the proposed research is of great interest, since it focuses on the poorly understood process of enhancement of the initial stage of the antiviral immune response. Moreover, there exists a broad exposition of immunobiological materials underlying antiviral response mechanisms, which is a requirement for a CRISPR study, not only in general terms, but also in the context of specific pathogens. At the current level of understanding, CHIME CRISPR-Cas technology might be applied to active studies aimed at identification of the function of new genes in the context of antiviral response. The herpesvirus family, including large DNA viruses, is an especially flexible model for this purpose, as it encodes a broad spectrum of proteins affecting innate and/or adaptive immunity [13]. Additionally, HSV-1, in particular, is an intensively researched pathogen, and its life cycle has been described in exquisite detail. CRISPR-Cas mediated knockout of a variety of cellular and viral factors should be explored to track a decrease in a specific antiviral response, since entry into infected cells is critical and susceptible to regulation by the immune response. Positive results may reveal new approaches to the inhibition of infection by other pathogens [14].

3 CRISPR technology

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and the CRISPR-associated protein (Cas) have been harnessed to create a simple and versatile tool for precision genome editing. The essential components of the type II (CRISPR-Cas9) system are Cas9 protein and a guide RNA that directs Cas9 to a DNA target. The affinity of Cas9 for DNA is driven by bound guide RNA, of which a component known as the spacer region directs sequence-specific recognition of the target gene. The target sequence of the spacer region is used to design a complementary sgRNA termed the guide RNA (gRNA) [15]. The DNA target site must be directly adjacent to a protospacer adjacent motif (PAM) sequence (5'-NGG-3' for the commonly used S. pyogenes Cas9), which is recognized by the Cas9 protein component. The development of the CRISPR technology has been an innovation in the toolbox of functional genomics. Their precise DNA binding enables both gene knockout and endogenous gene activation or repression through the design of mutant cas proteins or the fusion of a cas protein to different effector domains, respectively [16]. With greater understanding of the biochemistry of these systems, numerous adaptations both to the natural systems and the development of artificial systems have now enabled applications to diverse model organisms, although to date most published mammalian applications have utilized the type II CRISPR-Cas9 system [17].

Despite the rapid adoption of CRISPR in many model organisms, well-characterized biological systems often have strong evolved defenses against foreign DNA, including plasmids encoding the guide RNA and Cas9 nuclease. Thus, the toolkit built for these organisms may not directly transfer to other systems. As a result, there remains a need for continued innovation in order to adapt and expand the toolkit of CRISPR-based reagents to enable advanced and high-throughput characterization of more and complex biological systems. Here, recent advances of the adaptation and development of CRISPR-based technologies that have been applied to antiviral innate immune research are discussed. These advances allow for high-throughput RNAi-based genetic screens to be conducted, paving the way for new insights into the study of the biology of the innate immune system [18].

3.1 Principles of CRISPR-Cas system

The DNA targeting class 2 CRISPR-Cas system is a powerful technology represented by the popular type II CRISPR-Cas9 system. At the heart of any CRISPR-Cas system, there is at least one nuclease protein called Cas that is directed to a specific sequence within a target DNA [19].

This is mediated by a second RNA molecule, commonly called the guide RNA (gRNA), which is encoded by the DNA present within the host cell genome. The gRNA consists of a handle sequence required for interaction with structural proteins, and a programable tail sequence called spacer. The spacer is used by the complex, combined with other host RNAs, to direct the various Cas proteins to samples of invader DNA for interrogation [20]. The specific interrogations can be divided in three main events: detection of a foreign DNA, activation of the Cas protein, and cleavage of the foreign DNA.

Detection is made at the transcriptional, or thereabouts, level when the foreign DNA is scanned by the gRNA and the PAM sequence is recognized. The recognition of a suitable PAM is necessary for the system to differentiate between self and invader DNA. The Cas endonuclease is induced, and loaded with energy, and is generally compared with sheer power. The Cas protein somehow jumps across space-time into the invaded cell/replicon, and either generates blunt or staggered double strand breaks. The repair of said breaks causes a mutation in the Invader DNA, and it ceases to be a further threat. The crRNA can act as a pair, or be amplified by another RNA molecule called the tracrRNA, which hosts an additional annealing handle for the Cas9 recruitment [21]. Generally, they both contribute to a more efficient targeting of the desired DNA locus, causing a stronger and more spread-out response. Modern applications of the DNA targeting CRISPR-Cas system have been paramount in shedding light over this intricate mechanism of genomic surveillance. Here-in showcased is the interplay of the fully functional CRISPR-Cas components while engaging in a time sensitive, and destructive, concatenating sequence of events. Overall, novel Cas orthologs, modular Cas architectures, and precise engineering work have been developed to scale down the size of CRISPR class 1 and class 2 systems, and improve understanding [22].

3.2 Applications in functional genomics

CRISPR technology or clustered regularly interspaced short palindromic repeat is a development in the biological field that has enabled easy and precise editing of genomes. It has opened a new door in understanding biology by transforming the way gene functions, interactions, and proteins are investigated within cells and organisms [23]. CRISPR technology has revolutionized genome engineering and expanded the scope of questions biological researchers can address. Its capacity to edit the genome enables manipulation of gene coding regions to alter proteins, characterization of gene regulatory regions, and generation of genetic mutations or insertions at desired locations. Moreover, developed tools and techniques based on CRISPR technology yield a functional analysis of the cellular pathways and genetic networks at scale, ultimately paving the way for the analysis of complex genetic interactions and networks. One of the main methodologies in complex diseases is high-throughput screening. Hence, developing an understanding of how CRISPR technology influences these applications and provides important considerations in developing future high-throughput CRISPR screening strategies [24].

Genetic perturbation screens provide enrichment for unique gene-gene interactions that one-dimensional (1D) screens fail to detect [25]. Case studies are demonstrated in yeast using both 1D and 2D CRISPRi screens to help deconvolute the co-modulators of FLO8 and HMO1, two master regulators of filamentous growth. Together with existing technologies, fine scale multi-gene genetic probing is demonstrated, thereby fostering the development of a powerful and broadly accessible genetic and functional analysis toolkit. By using this technology to identify and validate interaction of genes, several new regulators of filamentous growth are discovered. This 'proof-of-principle' study demonstrates the making of an array of powerful tools and concepts that will facilitate wide adoption of ultra-high-order genetic and functional probing in the broader scientific community, accelerating the discovery of multigene genetic biology and complex pathways. Moreover, its implications on better understanding complex genetics networks, cellular biology, and developing therapeutic strategies are discussed; emphasis is placed on the importance of linking genetic perturbation screens and cellular mechanism in elucidating an intricate genetic networks and pathways landscape, as well as how the study of complex genetic networks is critical for understanding diseases and designing effective therapeutic strategies [26].

4 CRISPR-Based screening approaches

The innate immune system serves as the first line of defense against microbial infection and also plays an essential role in the host antiviral defense. Effector mechanisms of the antiviral innate immune response are mainly driven by a variety of cytokines and involve the concerted activities of numerous antiviral effec-

tor proteins. A comprehensive understanding of these pathways remains an important focal point in antiviral research. Antiviral mechanisms of the type I interferon system have been well documented, many individual effectors have been identified through targeted approaches, and multiple strategies are employed by viruses to counter them. However, the type I interferon system is only one facet of the innate immune response, and recent years have witnessed a surge in the development of powerful approaches for the unbiased study of gene function [27].

The CRISPR/Cas9 platform promises to revolutionize genetic research, accelerating the design and execution of sophisticated high-throughput, functional-genomic library screens that interrogate gene function on a genome-wide scale. This Review covers the rapidly expanding array of CRISPR-based screening approaches and tools. Although still in its infancy for some experimental systems and questions, CRISPR technology already offers a toolbox unparalleled in its ease of use, versatility, and high-throughput scalability. Functional genomics screens have proven particularly transformative and hold much promise for innovative strategies to identify previously unsuspected therapeutic targets [28].

4.1 Genetic screens using CRISPR-Cas9

Genetic perturbation is a powerful tool for understanding a wide range of biological systems and processes. With genetic screens, one can interrogate the functions of thousands of genes in parallel and explore potential mechanisms underlying the biological differences of interest. Major advancements of CRISPR/Cas9 technology have made it possible for the first time to generate most loss-of-function indels at a targeted site in the human genome. As a result, CRISPR-based functional genomics has been developed for a systems-level understanding of mammalian genome regulation. This has enriched antiviral innate immune response studies and further improvements in CRISPR technologies have been recently conducted and applied [29].

Genetic screens using the CRISPR-Cas9 system are unique in consideration of the compatibility between CRISPR and pooled libraries with a broad dynamic range. The initially poorly depleted or enriched sgRNA pools after puromycin selection will maintain remaining target cells, therefore maintaining the level of the initial pool. Meanwhile, the well-depleted sgRNAs that efficiently inhibit the genes are likely to lose target cells in the presence of viral stimulation, resulting in the loss of mature virus progeny. Hence, an antiviral screen has an intrinsically built-in positive selection proxy in contrast to a "typical" genetic screen of cellular behaviors. Interested hits can be shortlisted not only for their FDR values, but also for their characteristics, such as different types of targeting sgRNAs and on-target/in-frame localization [30]. Following the laborious wet-lab validation process using individual gene knockout, it is highly recommended to use orthogonal reagents, such as dCas9-KRAB-mediated silencing, small molecules or multiple sgRNAs for the candidate genes to evaluate the robustness and quality of the screen. Successful CRISPR screens are performed in the context of antiviral response research, revealing the antiviral mechanisms and genes whose functions are more important from the early-to-late stages. Altogether, CRISPR-based functional genomics can identify the regulator of interest with an unbiased approach and explore the potential mechanisms, greatly expanding antiviral innate immune response research [31].

4.2 Advantages and limitations

CRISPR-based approaches have revolutionized the field of functional genomics and open new perspectives both in the fundamental understanding of genotype-phenotype relationships and the development of new strategies and tools to exploit this knowledge for therapeutic purposes. On one hand, the ability of the CRISPR-Cas9 system to target any given loci of the genome allows for the first time to perform high-throughput hypothesis-free genetic screens for various phenotypes. In turn, on the other hand, the same system can deliver a comprehensive functional analysis of high-throughput genomics data sets. CRISPR-based methods are becoming popular tools for functional genomics because they are high-throughput, precise, and cost-effective. They can introduce knockout or knockdown of target genes by blocking mutations or indels in the coding exon [32]. Furthermore, CRISPR can be used as a powerful tool to identify the genetic interaction between targeting gene and coveted pathways by generating high-throughput gene–gene knock-out matrices. CRISPR screens by lentivirus or a pooled library correct wild type Cas9 suffered transcription activation, which provides a high through pathway interactome screening strategy.

Similar to RNAi-mediated genome-wide screening approaches, the discovery allowed easy gene editing by directed DNA cleavage to be carried out in cells. But the precision and efficiency of the CRISPR/Cas9 system greatly surpass those of RNAi-based screening methodologies. A gene knockdown of 80%–90% is usually required for an RNAi experiment to reach phenotype levels considerable enough to be effectively used for arrayed library screening. Instead, the creation of obvious knockout genes caused by knocking out exons stabilized by insertion/indel mutations in genome across various cell lines can be almost 100%

effective with CRISPR/Cas9 [33]. This result has been confirmed with endogenous gene knockout screening of dozens of genes in many different cell lines, which strongly supports the fact that off-target mutagenesis occurred by traditional RNAi library screening that reported hits across many genes are avoided when using CRISPR. However, as with any new technology, it is important to appreciate both the potential and risks of these methods. The widespread adoption of next generation sequencing in genomics has highlighted the surprisingly complex genetic and biological factors influencing the production of high-throughput data [34]. The recent explosion in CRISPR/Cas9 methods has made possible high-throughput gene editing in mammalian systems. However, the production of knockout or knockdown cells or animals by the introduction of exogenous genetic material has highlighted the complexity of biological pathways that were previously assumed to be well understood. And as CRISPR/Cas9 methods increase in complexity, these unexpected results may interfere with the aims and goals of the proposed genomics research. One approach to minimize this risk is to establish validation protocols that can verify the results of high-throughput gene editing-based genomics screens. There are potential factors that could confound a screen's results, and the development of standards that can detect these factors would be beneficial to the field.

5 Key genes identified in antiviral immune response

The discovery of innate immune response to viruses is largely promoted by identification of numerous crucial genes. To deeply uncover the gene interaction network in orchestrating immune protection, a number of genome-scale screenings of antiviral immune response have been performed. There is a collection of key genes, aggregated through these screenings, which plays important roles in antiviral immune responses and cross-species viral resistance. The biological functions and interactions of the identified genes and their cross species viral resistant effect are discussed. The identified genes, especially human and mouse gene orthologues, are significant to generate more targets for screening and development of antiviral drugs and for better understanding of the mechanisms of antiviral immunity. This will be helpful to design more effective antiviral therapeutic strategies [35].

Ever-evolving viruses constantly challenge human health. The discovery of the mechanisms implicated in antiviral immunity is significantly helpful for designing effective antiviral therapeutics. The host's immune system, as the primary defence against various pathogen infections, is able to detect pathogen associated molecular patterns and, in turn, to activate cellular defence mechanisms to restrict virus replication and spreading. The innate immune response to viral infections leads to inducing of hundreds of or even more of genes, participating in biological processes such as blocking the early steps of the viral infectious cycle, increasing the elimination of infected cells, and stimulating the adaptive immune response. It is well known that identification of numerous important crucial genes was conducted greatly facilitated by the discovery of viruses, like in reverse. To deeply and more completely uncover the gene interaction network in orchestrating immune protection against virus, several genome-scale screenings of antiviral immune response have been intensively performed [36].

5.1 CRISPR Screens for antiviral immunity

CRISPR screens supercharge the detective hunt for genes vital for antiviral immunity [37]. Loss-of-function immune-activation screening of eight distinct primary cells, demonstrating they cooperate in a modular way as interferon-inducible immune genes keep various viruses in check 4. Besides finding the components of known ISGs, CRISPR screens revealed orphan genes like FAM111A that potently restricted SARS-CoV-2.

Something was secreted by activated immune genes in the DNA-sensing pathway that broadly suppressed a spectrum of avirulent and virulent viruses, turning genes like CSF1 into overarching virus restrictions. Activated immune signals splicing nontemplated retropseudogenes to generate chimaeric, antiviral long non-coding RNA transcripts, some of which interact with and decimate viral replication centers. Clever technical tricks then identify XRN1 as a key proviral host dependency gene. Translational applications arise: small molecules sculpt the activity of these newly discovered immune genes to enable a broad-spectrum, yet entirely off-target-free, antiviral response; untoward drug toxicities are detected by revealing novel proviral genes like NUP43 that herpesviruses exploit by sequestering otherwise viral-degrading microRNAs. Antiviral drug development might be guided by the path, which unexpectedly pinpoints the lethal stalk region as the prime viral grooming zone when it is associated with dozens of unique herpesvirus genes intertwined with hints of antiviral glycoprotein emergence [38].

5.2 Functional characterization of key genes

Recent advances in molecular tools have made it possible to study the genetic basis of the interac-

tions between pathogens and host cells genome-wide. Genome-wide RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR) screens have been successfully used to identify cellular factors required for viral entry, replication, and egress of both RNA and DNA viruses [34]. Indeed, both Ebola and Zika viruses have undergone RNAi and CRISPR screens to uncover new cellular factors key to their pathogenesis, as well as to the immune pathways responsible for controlling the viral infection. Additionally, genome-wide CRISPR screens have been identified as a major breakthrough for infectious disease research, with the ability to uncover host dependencies or restrictive factors with huge potential for drug development. Understanding population-scale genetic variation in antiviral immune response is key to elucidating dynamic host-pathogen interaction and could offer new insights into therapeutic and vaccine design. However, large-scale functional exploration of antiviral immune traits is challenging due to the complexity of immune system and genetic networks [39].

Advances in molecular tools have transformed the ability to investigate virus–host interactions, leading to the wide application of genome-wide CRISPR screens for the exploitation of genes essential for viral infection 4. However, most phenotypically identified genes remain unexplored with unknown biological functions, especially in complex traits such as the immune response. In order to address this gap and to understand further the genetic basis of antiviral innate immune response, an integrative pipeline between CRISPR screens and follow-up experimental validation is presented. A complex CRISPR screen lineage with the immune challenge of the model virus vesicular stomatitis virus (VSV) is designed, followed by in-depth functional characterization of key genes, contributing and connecting to disparate immunological fields both intracellularly and extracellularly. Case studies taking the findings of immune-associated genes as positive evidence have also highlighted the extensive application of the analytical pipeline in experimental and therapeutic inquiries [40].

6 Implications for therapeutic development

Knowledge from CRISPR screens is a promising treasure trove for the development of antiviral therapies. Specific virus genes were found to impede the antiviral immunity by CRISPR screens targeting the innate immune response in all four sets of data analyzed recently. Moreover, interference with multiple functions of the host cells, which are favorable to viral invasion, was also observed in these studies [41]. The potential therapeutic applications of these findings, which involve combating the viruses via boosting immunity rather than direct interference with the pathogen, are extensively discussed here. The analysis on critical genes assigned by RIG-I and MDA5 KO together with MAVS KO highlighted that boosting intrinsic immunity is a promising therapeutic strategy against SARS-CoV-2. Indeed, recent research reported that administration of coli isolated from the gut of CRISPR KO rats effectively lessened the symptoms of H1N1-infected mice. Since the CRISPR-based screens mostly target ISGs, chemicals that tried to boost the host immune responses, such as interferon stimulators, could be effective therapeutic reagents. Although the CRISPR screen techniques have evolved rapidly since their development, numerous concerns still hamper their application in drug development. Thus, it remains a challenge to fully utilize the CRISPR screening data in drug development. On the other hand, drug companies would substantially speed up the whole process if they collaborate with laboratories proficient in utilizing the CRISPR screens. Recently developed techniques may help tackle these challenges. Since the knockdown efficiency of CRISPRi is relatively low, it may be more challenging to clarify the mechanisms of the highly complex systems with scarce previous knowledge. Further exploration on these directions would serve specify dormant targets for antiviral therapy [42].

6.1 Targeting key genes for antiviral therapies

Concurrent with our transcriptomic studies, we collected a publicly available database of transcriptomic studies of cells/tissues where the DNA harmonized. These studies then were analyzed by a conventional meta-analysis to identify genes significantly regulated upon viral infection. Genes marked in the DNA by promoter marks in cells were then pooled as a null distribution, where genes by chance would be expected to be significantly regulated post-infection. A similar statistical analysis was then performed with the RNA-seq of a cell that was infected with a functioning immune system. The genes that were significantly regulated post-infection in these two settings resulting from the above null distributions were then identified [37].

To be able to draw conclusions from them, the functional term similarity through the enrichment analysis was assessed at various values of the fold-change cutoff to enrich for particularly strong modulators of gene function. This was then tested in a retrospective epigenome editing screen that aimed to enhance antiviral immune response. Through this methodology, it was revealed that targeting genes with promoter marks is an effective prophylactic enhancer

of immune response. The findings suggest that single molecules can be modulated to improve the antiviral immune response and provide a wealth of potential therapeutic avenues. This can include small molecule interventions, CRISPR based editing, epigenome editing or viral inhibition, with many revealed genes having pre-existing drugs that target their product. It is however, challenging to deliver any form of enhancements to already infected cells [43].

Moreover, as exemplified by the ongoing failures to FDA-approve an experimental viral treatment, the ability to specifically deliver molecules to pathogens or infected cells while still preserving patient health is underdeveloped. This CRM-screen presents 85 potential therapeutic avenues, of offering many-of-many potential means of modulating gene function within the antiviral immune response. Nonetheless, due to the diversity of mechanisms in these functionalities, some applications will almost certainly not be feasible in a clinical setting. Some may be possible only in very specific scenarios, but many may find wide applicability in prophylactic settings that could significantly enhance patient profiles. These will most likely require the development of personalized medicine strategies that take into account patients' cell type/factor expression profiles and genetic predispositions, to develop a targeted enhancement strategy against viral infectivity. Overall, the results demonstrate the potential of using a form of CRM screens to identify optimal gene targets in complex responses of tractable biology and provide the initial seeds of new treatment strategies in urgently facing disease [44].

6.2 Challenges and future directions

As the 2010s ushered in an era of genome-wide CRISPR screening, human genetic vulnerabilities to diverse RNA and DNA viruses have emerged through unbiased functional genomic interrogation. For a subset of antiviral gene candidates, end-to-end scrutiny of their potent viral restriction capacity by orthogonal validation remains sparse 6. Antiviral restriction effector mechanism(s) are especially opaque at the stage of hits and leads. A rich tapestry of gene families of mostly unknown mechanism emerged from genome-wide CRISPR-mediated screens for poliovirus, human cytomegalovirus, and influenza A virus among hundreds of antiviral candidates. There is much known to be discovered about essential gene and pathway function throughout viral infections. Some antiviral genes targeting diverse viruses appear to regulate the same process, such as it was for 14-3-3 chaperone proteins. Activation of broad spectrum antiviral mechanism triggered by a specific viral infection can be conferred through genetic perturbation of diverse gene targets, such as mitochondria metabolic stress response triggered by human herpes simplex virus-1 provoked by autoimmune defense nuclease TREX1. Such macroscopic phenotypes are observed when targeting many different genes. Thus, explained suppression of specific virus by a gene can be quantitative in nature, evidenced for MyD88 immune signaling in A549 infection by IAVs of diverse subtypes. There is much to be uncovered concerning the restrictive power of antiviral biological processes, secreted soluble factors, metabolites, and post-translational modifications. The timely translation of these findings into forefront antiviral part treatments is slowed by technical, biological, and regulatory hurdles. The establishment of a viral infection illness might revitalize clinical development of apparently viable genes that have been previously deprioritized. These genes may also let down the hopes for the antidote cure, if CRISPR excision triggers a well-characterized antiviral evasion that annotated genes are unlikely to target. To deliver viral nucleic acids and proteins while keeping viral genome intact implies some stage of viral gene expression. Since this requirement is anathema to some research applications, such a possibility is first ruled out. Needed supporting experimental conditions may exist yet preclude the examination of foreseeable alternative premises. Discovery of PDZD8 as conserved requisite 2-component species for poliovirus infection illustrates such convergence between dissimilar research histories.

7 Conclusion and future perspectives

Understanding genes and their relations to perturbed biological contexts, which is to respond or resist the immune challenge posed by viral infection, allows us a comprehensive view of the organization of antiviral immunity. In Drosophila, a recent study explored combinatorial gene regulatory networks associated with antiviral innate immune response across different cell types, the fat body and hemocytes. Those studies with high-throughput RNAi targeted screens broadened and deepened a view of genes and networks involving antiviral immunity. Broadly antiviral genes were discovered through the disparate basal responses of thousands of genes across experimental treatments and conditions. Further context-specific antiviral genes associated with combinatorial regulation were identified across those two cell types. Even with the dominant-negative and overexpression of a TF, this modulation on antiviral defense genes in the single cell type could give rise to phenotype in the network of combinatorial pan-cell type. Apart from those characterized genes and regulators, approximately 1000 known and novel genes associated with the mitotic cell division processes were discovered as context-specific broad an-

tiviral genes. It is possible a differentiation without mitotic cell division is beneficial during the initial infection of Drosophila cells grown in culture but of little benefit in vivo for either of the two immune relevant cell types. Also with manufacture of these tissues, and cell cultures would be a physically disparate cellular milieu may be the reasoning to net the AR fused peptides did not induce any of the broadly antiviral genes in these screens, directional or not. The large discrepancies in results cast doubt on some or all subsequent results given the lack of independent verification. Using one of the broadly cross-targeted conserved viruses, the two works did not find an activation of antiviral genes in the PNS, AP, and VG, known tissues that support C-like propagation. Nine of 15 genes showed no impact of increasing doses of virus until the 5d post infection timepoint, with ten showing no expression differences during the course of infection. One of the genes upregulated only at 5d was a positive control from the original manuscript, the data from which also showed infection dependent antiviral gene expression changes at non-necrotic doses or timepoints. Considering the results from the work form 2005, viral doses for most viral stock injections ranged from 0 to 6 PFU. The data at these doses provides a minimal number of antiviral and virally induced genes compared to the >300 for these three tissues/cell types here). Given the increasing pandemic threatening and the apocalyptic and avoidless loss of human life worldwide, it becomes significantly urgent to detect and clarify the mechanisms of the global interactions between virus and host. Viral infection activates the host innate immune system through recognizing the viral elements by host sensing proteins. Thereafter, the innate immune system plays essential protective roles to mediate the interferon response and develop the inflammation to resist the spreading of virus within the host and limit the viral proliferation. Conversely, viruses have illustratively evolved various tactics to escape the host immune defense mechanisms. Therefore, understanding the work of virus–host communication is critical not only to explain the antiviral strategies of the host, but also to elucidate the disguises of the pathogens within the host for the treatments of virus infection-related diseases. Recently, it was found that viral infection benefits the aphid by amplifying a glutathione peroxidase-like gene via an RNA intermediary.

Therefore, the coinfection of a benign virus with a virulent virus can have persistent mutualistic effects on host plants by deregulating a gene in a phytohormone-sensitive network. These results demonstrate an unforeseen side effect of polyspecific viral infection of the target host that may have consequences for virus-based biological control as well as plant–virus–insect interactions. Nonetheless, partially because of the lack of suitable models and robust approaches, still less is known about the precise mechanisms of the interactions between virus and host. On the other hand, discovered in recent years, the ingenious mechanisms for the viral immune evasion around some of the identified immune sensors or effectors call for exploring additional antiviral mechanisms of the host. Over the past decade, with a better molecular understanding of the antiviral immune strategies, Drosophila has become a useful model system to elucidate the antiviral mechanisms of innate immunity. To simply and efficiently conduct viral screenings of genes for their impact on viral replication, labelled RFP in the genome combined with a GFP-tagged virus significantly enhanced sensitivity over small-RNA or protein knockdowns used in previous screens. Candidates from genome-wide scrutiny included hits in the TGF-β pathway and innate immunity modulators. Further investigations into genes related to viral entry showed that GFP-tagged virus can shed its envelope and enter the cytoplasm after gaining access to cells. This lead to the finding that fusogenic peptides based on the Dengue virus E glycoprotein relaxed host range against viral infection by disrupting the endosome. Another ground-breaking discovery from in vitro and in vivo experiments was that a Drosophila virus encoding a conserved viperin gene extended survival during lethal challenge with vesicular stomatitis virus. Taken as a whole, these findings provide a systematic foundation for understanding antiviral innate immunity that can develop preventative measures against emergent or persistent viral threats.

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