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Genetic Screens Using CRISPR-Cas9

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김 헌 석

Abstract

Genetic Screens Using CRISPR-Cas9

Heon Seok Kim

Department of Chemistry

The Graduate School

Seoul National University

Human Genome Project (HGP) which launched in 1990 and completed in 2003, revealed the whole sequence of human DNA. After this triumphs, functional genomics, which is a field that attempts to identify which genes contribute to each biological phenotypes including diseases, has attracted much attention based on the rich data given by the HGP and others.

For functional genomics, tools for genetic perturbations are required to connect the phenotypes to genotypes. Especially for forward genetic approaches, which modify expressions of many genes in a time and selecting and characterizing the cells and genes with desired phenotypes, genetic perturbation systems that enable many modifications easily are essential. As a result, programmable nucleases, CRISPR (clustered regularly interspaced short palindromic repeat) – Cas9 system has been spotlighted as a tool for functional genomics because of its simplicity and

robustness. Although RNA interference (RNAi) has been major tool for functional genomics, it has been criticized for its drawbacks including high off-target effects and incomplete knock-down. On the other hands, RNA guided Cas9 nuclease has much higher specificity than RNAi system and induce complete knock-out of genes. In this respect, CRISPR-Cas9 system could be an excellent substitute for previous RNAi system in functional genomics.

In this thesis, I will describe two genetic screen approaches that I developed using CRISPR-Cas9 and the result of them. In Chapter 1, I will present new pooled CRISPR screen method using 30,840 single-guide RNAs (sgRNAs) combined with whole genome sequencing to directly identify causal genotype of viral resistance. Compared to screens based on lentivirus integration system, this method is quick, virus-free, and confirms authentic mutations.

Second, in Chapter II, I will describe arrayed CRISPR screen which incorporates a single genetic perturbation in each well, using individually cloned 4,542 sgRNA plasmids targeting 1,514 genes encoding potential host factors for viral infection. Combined with image-based assay detecting viral infection, this arrayed screen could reveal new host factors for coxsackievirus-B3 infection to human cells that are missed in pooled CRISPR screens, demonstrating higher sensitivity of arrayed CRISPR screen.

Keywords: Functional genomics, Genetic screen, Pooled screen, Arrayed screen, CRISPR-Cas9, picornavirus

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Chapter 1. Pooled CRISPR Screens and Target Identification via Whole Genome Sequencing

Introduction

Genome-scale libraries of transcriptional activator-like effector nucleases (TALENs) (Kim et al., 2013a; Kim et al., 2013b) and RNA-guided endonucleases (RGENs) (Chen et al., 2015; Koike-Yusa et al., 2013; Ma et al., 2015; Shalem et al., 2014; Wang et al., 2014; Zhou et al., 2014), which consist of the Cas9 protein and guide RNAs (sgRNAs) originated from the type II clustered regularly interspaced repeat (CRISPR)-CRISPR-associated (Cas) prokaryotic adaptive immune system, are now available for forward genetic screens in human and other mammalian cells.

Several groups have constructed genome-wide libraries of lentiviruses encoding sgRNAs by cloning oligonucleotides synthesized in situ on a microarray and used them to search for genes whose disruption in human and murine cells gave rise to oncogenesis (Chen et al., 2015), drug resistance (Koike-Yusa et al., 2013; Shalem et al., 2014; Wang et al., 2014; Zhou et al., 2014), viral replication (Ma et al., 2015), etc. In these systems, target genes are identified indirectly by comparing the number of each sgRNA sequence before and after selection via high-throughput sequencing.

In this study, I sought to develop a new method for genome-wide knockout screens using sgRNA-encoding plasmids rather than lentiviruses and target gene identification via WGS rather than targeted amplicon sequencing.

Here, I chose two human pathogenic viruses, poliovirus (PV) and enterovirus-D68 (EV-D68), which are members of the *Picornaviridae* family that causes a wide range of illnesses such as poliomyelitis and childhood respiratory

diseases (Fields et al., 2007; Liu et al., 2015). In 2014, outbreaks in USA and severe respiratory illness caused by EV-D68 raised a concern about this emerging pathogen (Principi and Esposito, 2015). These viruses are non-enveloped and single-stranded positive-sense RNA viruses. Currently, there are no approved therapies.

Infection by these viruses is a complex process that includes many steps such as receptor binding, viral entry, translation, replication and viral release. Each step requires host factors and interactions between host and viral factors are essential to fulfill successful infection. Identification of these host factors could help to elucidate novel molecular mechanism of viral infection and to facilitate the development of novel target for antiviral drugs.

These viruses are highly cytopathic so infected host cells are eventually killed. Only cells lack key host factors essential for viral infection can survive. CRISPR system induced permanent knockout on target gene. By combining these two features, it is possible to screen key host factors for virus infection by selection of survived cells after virus challenge and target identification.

Materials and Methods

1. sgRNA oligonucleotide preparation.

Oligonucleotides were purchased from Bioneer. In each well in a 96-well plate, two complementary 24-nt oligonucleotides were mixed at 100 μM in a total volume of 100 μl. Each oligonucleotide pair was diluted to 50 μM in TES buffer (1 mM EDTA, 10 mM Tris-HCl (pH 7.5), and 100 mM NaCl) and annealed to form a duplex by heating to 80°C and cooling to room temperature in a water bath.

2. sgRNA library construction.

An empty sgRNA expression vector cleaved using BsaI (New England Biolabs) was ligated with annealed oligonucleotide mixtures. In this vector, sgRNA is transcribed under the control of the U6 promoter; the sequence is 5'-NNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT-3'. Ligation products were electroporated into DH5α-E competent cells (Invitrogen). Transformed E. coli cells were plated on a 24-cm LB plate containing ampicillin. Plasmid DNA was purified using the Nucleobond Xtra Midi EF purification system (Macherey-Nagel).

3. Cell culture and transfection conditions.

HeLa (ATCC, CCL-2) and RD (ATCC, CCL-136) cells were maintained in DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids, and 10% fetal bovine serum (FBS). 2 x 10^6 HeLa cells were plated one day before transfection. The Cas9-expression plasmid (15 μg) and sgRNA library plasmids (15 μg) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Genomic DNA was isolated 72 h post-transfection. Single cell derived knockout cell lines were obtained by limiting dilution. HAP1 cells were purchased from Haplogen and maintained in IMDM supplemented with 10% FBS.

4. Viruses.

Poliovirus type 1 (chat; ATCC VR-1562) was expanded by growth in HeLa cells and titered using HeLa cells. Enterovirus 68 (Fermon; ATCC VR-561) was expanded and titered in RD cells. For the virus binding assay, EV-D68 was concentrated by ultracentrifugation.

5. Virus infection and screening.

Cells that had been transfected 6 days previously with the Cas9 plasmid and sgRNA library were reseeded and infected with poliovirus 1 at an MOI of 0.01 at 37ºC for 1 h. Cells were infected with enterovirus D68 in the same manner except that the incubation was at 33ºC. Infected cells were washed and resuspended in

complete growth medium. The culture medium was changed every 2 or 3 days. After 12 days, surviving colonies were fixed and stained with crystal violet (0.05%) in phosphate buffered saline (PBS) solution containing 1% formaldehyde and 25% methanol or isolated and expanded for further analysis.

6. Whole genome sequencing.

Genomic DNA was purified with a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. 1 μg of genomic DNA was fragmented using the Covaris system (Life Technologies) and polished to generate blunt ends using End Repair Mix. Fragmented DNA was ligated with adaptors to produce libraries and subjected to WGS using an Illumina HiSeq X Ten Sequencer at Macrogen. Mapping programs and parameters were as described previously(Kim et al., 2015a).

7. Re-infection test.

Surviving cells were plated in 96-well plates at 2 x 10^4 cells per well to test whether surviving cells were resistant to viral infection. For the reinfection assay, ten-fold diluted poliovirus 1 (MOI 0.1–0.0001) was added to each well and the cultures were incubated at 37ºC for 2 days. For enterovirus D68, three- or ten-fold diluted virus was added to each well and the cultures were incubated at 33ºC for 3 days. To measure cell viability after virus infection, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay was

performed as previously described(Kim et al., 2002). The viability of infected cells was normalized to the viability of mock-infected cells (expressed as 100%).

8. Lectin staining.

For measuring the level of 2,3-linked sialic acid on the cell surface, cells were detached from plates using trypsin and stained with fluorescein-labeled MALI (Vector Laboratories). As a control, HeLa cells were treated with 100 mU/ml neuraminidase from Clostridium perfringens (Sigma-Aldrich) as described previously(Liu et al., 2015). Flow cytometry was performed using a BD Accuri flow cytometer (BD Biosciences) and the data were analyzed using the FlowJo program.

9. Virus binding assay.

3 x 10^4 cells were plated on an 8 chamber slide (SPL Lifesciences) and incubated overnight. The next day, cells were treated with neuraminidase for 1 hour at 37 ºC. Cells were then incubated on ice for 15 min, after which EV-D68 (MOI 50) was added to the cells. After incubation on ice for 1 hour, the cells were washed three times with cold PBS and then fixed with 4% paraformaldehyde. EV-D68 was stained with anti-enterovirus D68 VP1 antibody (Genetex, 132313) and Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (Life technologies, A11008). After counterstaining with Vectashield mounting medium with DAPI (Vector Laboratories), cells were visualized using confocal microscopy (Zeiss LSM 710;

Carl Zeiss) and ZEN software.

Results

1. Gene knockout screens using pooled sgRNA libraries

A total of 30,840 pairs of oligonucleotides that encoded sgRNAs, collectively targeting 10,280 protein-coding genes, were individually synthesized and cloned in a sgRNA expression plasmid. Using Cas-OFFinder and Cas-Designer (available at www.rgenome.net), I carefully chose three target sites per gene to avoid off-target effects and in-frame mutations as much as possible (Bae et al., 2014; Park et al., 2015). All the sgRNAs in the library contained two extra guanine nucleotides to produce ggX_{20} sgRNAs, further reducing off-target effects (Cho et al., 2014; Kim et al., 2015b).

To test whether cells resistant to viral infection or drug treatment could be selected by transient transfection of pooled sgRNA plasmids (**Fig. 1A**), I mixed 3 sgRNA plasmids specific to the hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) gene with the library of 30,840 sgRNA plasmids and co-transfected this mixture into HeLa cells with the Cas9 plasmid. Transfected cells were treated with 6-thioguanine (6-TG), which is cytotoxic to cells that express the *HPRT1* gene. Several colonies survived 6-TG treatment (**Fig. 1B**), showing that the *HPRT1* gene was disrupted efficiently in HeLa cells. Sanger sequencing showed that each resistant colony had mutations at sgRNA target sites in the *HPRT1* gene, leading to the gene knockout. As expected, no colonies were obtained from cells transfected with the library alone (**Fig. 1B**). These results show that a sgRNA plasmid in a pool of tens of thousands of sgRNAs can still direct Cas9 to induce complete knockout of

a target gene in human cell lines.

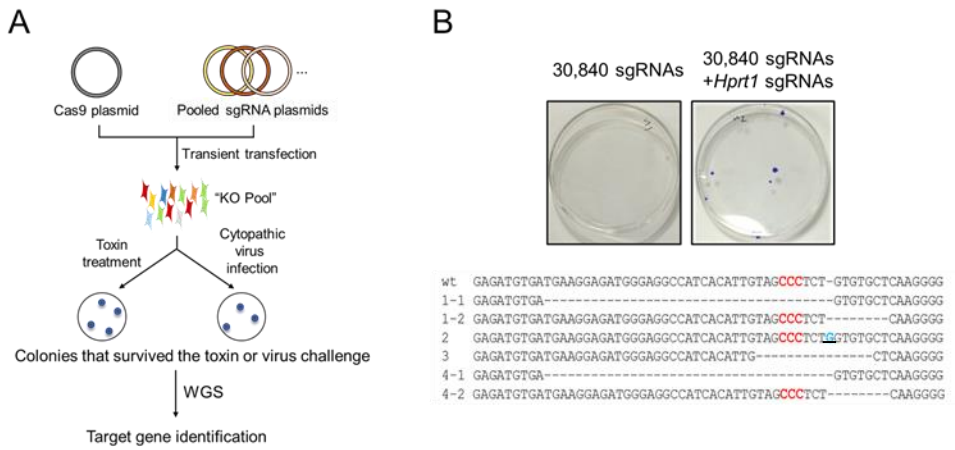


Figure 1. Gene knockout screens using pooled sgRNA libraries. (*A*) Schematic illustration of forward genetic screens in human cells using pooled sgRNA libraries. (*B*) Crystal violet staining of 6-TG resistant cells, previously transfected with the sgRNA library. 6-TG resistant cells appeared when sgRNAs targeting *HPRT1* were diluted in the library containing 30,840 sgRNAs. The mutated sequences from surviving clones are shown below. Bold characters indicate the PAM and the underlined character indicates an insertion.

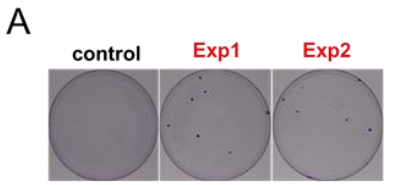
2. Knockout screens for viral resistance

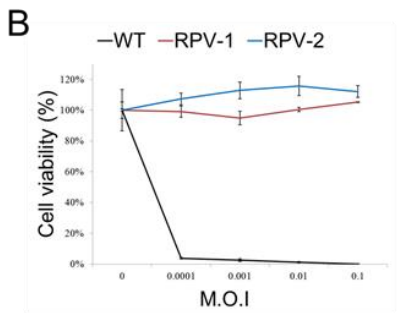
a. Screen for poliovirus resistance

Encouraged by this proof-of-principle experiment, I performed pooled sgRNA screens to identify genes essential for viral infection or replication. I chose two cytopathic viruses, poliovirus (PV1) and enterovirus D68 (EV-D68) for analysis in this study. HeLa cells were first transfected with the library of 30,840 plasmids and then subjected to PV1 infection. A few colonies survived among ~1 million PV1-infected cells (**Fig. 2A**). I expanded two clones (termed RPV-1 and RPV-2) and confirmed that they were resistant to PV1 regardless of the viral titer using a cell viability assay (**Fig. 2B**).

To identify target genes disrupted in these two clones, I carried out WGS using genomic DNA isolated from these clones and investigated whether Cas9-induced mutations were present at any of the 30,840 target sites. By using the Integrative Genomics Viewer (IGV) (Robinson et al., 2011), which aligns paired-end sequence reads to the human reference genome, I was able to determine computationally whether mutations were induced at each of the 30,840 target sites (**Fig. 2C**). The two clones harbored small insertions or deletions (indels) in 7 and 15 genes, respectively (**Fig. 2C** and **Fig. 3**). All of these mutations occurred at on-target sites of each sgRNA. Their mutation patterns analyzed by WGS also revealed that Cas9-mediated cleavage occurred ~3bp upstream of the PAM, as previously known (Cho et al., 2013). These multiple on-target mutation effects were derived from co-transfected sgRNA expression plasmids, implying that the phenotypes that can occur

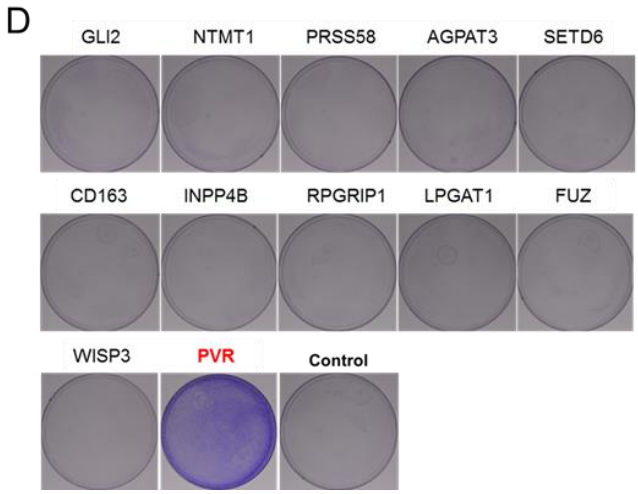
with combinatorial gene knockout events can be identified using this plasmid library system. Among these mutated genes, only one, which encoded poliovirus receptor (PVR), a well-known receptor of PV1 infection, was completely disrupted in both of the clones (**Fig. 2C** and **Fig. 4**). To confirm that disruption of this gene was alone responsible for the resistance to PV1 infection, I separately transfected HeLa cells with sgRNAs specific to each of the 12 completely mutated genes and infected the cells with PV1. As expected, transfection of the PVR-targeted sgRNAs into HeLa cells gave rise to viral resistance. No virus-resistant colonies were obtained from cells transfected using sgRNAs targeted to the other genes (**Fig. 2D**). These results suggested that possible false-positive genes could be eliminated by screens using separate sgRNAs.





C

Gene	RPV-1	Gene	RPV-2
PVR	ins1	**PVR**	Δ2 / Δ14 / Δ17
GLI2	Δ11 /Δ27 / Δ35	**CD163**	Δ3 / Δ5
NTMT1	Δ1 / Δ3 / ins1	**INPP4B**	ins1 / ins1
PRSS58	Δ2 / Δ686	**RPGRIP1**	Δ8
AGPAT3	ins1	**LPGAT1**	Δ4 / Δ5
SETD6	ins1 / Large del	**FUZ**	Δ3 / ins1
SCARA5	+/ Δ9 / Δ20	**WISP3**	ins1 / Δ1384
		SIRT3	+ / Δ43
		KLF16	+ / Δ1
		HPSE	+ / ins1
		ULK2	+ / Δ26
		SMARCA5	+ / Δ9
		PARM1	+ / Δ3
		THOC1	+ / Δ3 / Δ14
		DHRS12	+ / Δ3



(With Chonsaeng Kim in Korea Research Institute of Chemical Technology (KRICT))

Figure 2. Pooled sgRNA screens for poliovirus 1 resistance. (*A*) Crystal violet staining of PV1-resistant cells after 12 days of infection. (*B*) Viability of wild-type (WT) HeLa cells and two colonies obtained from cells transfected with the library after re-infection with PV1. (n=3, ± s.d.) (*C*) List of genes in which mutations were observed at target sites in the two colonies that survived PV1 challenge. The gene encoding PVR is marked in red. Genes completely disrupted are denoted in bold. (*D*) Crystal violet staining of PV1-resistant cells after transfection of sgRNAs targeting the indicated genes. PV1-resistant colonies formed only after transfection of sgRNAs targeting the *PVR* gene. Other genes were also mutated in clones RPV-1 and -2, but targeting these genes did not make HeLa cells resistant to PV1.

RPV-1

PVR

hg19	CACCAAACGCAGGG**CCC**CAG-CTATTCGGAGTCCAAACGGCTGGA	
1	CACCAAACGCAGGG**CCC**CAG**T**CTATTCGGAGTCCAAACGGCTGGA	ins 1

GLI2

hg19	ATGGGCCGGGCCGACTCCACACACG**CGG**AACACCAAGCTGCCTCC	
1	ATGGGC----------CAAGCTGCCTCC	27del
2	ATGGG----------CCTCC	35del
3	ATGGGCCGGGCCGAC----------**CAAGCTGCC**AGCTGCCTCC	11del

NTMT1

hg19	TGGACTGTGGAGCTGGCATTGGGAGGATCA-CCAAG**CGG**C	
1	TGGACTGTGGAGCTGGCATTGGGAGGAT----CAAG**CGG**C	3del
2	TGGACTGTGGAGCTGGCATTGGGAGGATCA--CAAG**CGG**C	1del
3	TGGACTGTGGAGCTGGCATTGGGAGGATCA**C**CCAAG**CGG**C	ins 1

PRSS58

hg19	TTAGAGTCTGCTGGGATTGTAA**CCC**CCAATATCACCCGAAGCTTTCT	
1	TTAGAGTCTGCTGGGATTGTAA**CCC**CC--TATCACCCGAAGTTTTCT	2del
2	TTAGAGTCTGCTGG----------	686del

AGPAT3

hg19	AAGTGGGAGGAGGACCGGGACA-CCG**TGG**TCGAAGGGCTGAGGCGC	
1	AAGTGGGAGGAGGACCGGGACA**A**CCG**TGG**TCGAAGGGCTGAGGCGC	ins 1

SETD6

hg19	CTCCTGTCGCAGCACACCTGCTCCAT-CGG**CGG**CCTGCTGGAGCGAGG	
1	CTCCTGTCGCAGCACACCTGCTCCAT**T**CGG**CGG**CCTGCTGGAGCGAGG	ins 1
2	----------CCTGCTGGAGCGAGG	large de

SCARA5

hg19	ACGTCGGCCAGCTCCTCGCCC**AGG**ATGCCCACCCTGCGCGCCAG	
1	ACGTCGGCCAGCTCCTCGCCC**AGG**ATGCCCACCCTGCGCGCCAG	+
2	ACGTCGGCCA---------CC**AGG**ATGCCCACCCTGCGCGCCAG	9del
3	ACGTCGGCCAGCTCCTCGC----------GCCAG	20del

RPV-2

PVR

hg19	CACCAAACGCAGGG**CCC**CAGCTATTCGGAGTCCAAACGGCTGG	
1	CACCAAACGCAGGG**CCC**CAG--ATTCGGAGTCCAAACGGCTGG	2del
2	CACCAAACGCAGGG----------AGTCCAAACGGCTGG	14del
3	CACCAAACG----------GGAGTCCAAACGGCTGG	17del

CD163

hg19	GCGTCCAGAACCTGCAC**TGG**AATTAGCCCATCC	
1	GCGTCCAGAACCTG-----**G**AATTAGCCCATCC	5del
2	GCGTCCAGAACCTG---**TGG**AATTAGCCCATCC	3del

INPP4B

hg19	GTAGGCACACAGCATCTCACCT**CCA**CAA-TTTCGGTGCTGGAGTAT	
1	GTAGGCACACAGCATCTCACCT**CCA**CAA**T**TTTCGGTGCTGGAGTAT	ins 1
2	GTAGGCACACAGCATCTCACCT**CCA**CAA**A**TTTCGGTGCTGGAGTAT	ins 1

RPGRIP1

hg19	CACAGACAGCTCCACACAG**CCG**GTGCACCGGTGCCGGAGAAACCC	
1	CACAGACAGCTCCACACAG**CCG**G--------TGCCGGAGAAACCC	8del

LPGAT1

hg19	TATCCAGCATACCATCCCC**AGG**AAGCTACCATTCCTAAAA	
1	TATCCAGCATA-----CCC**AGG**AAGCTACCATTCCTAAAA	5del
2	TATCCAGCATACCA----C**AGG**AAGCTACCATTCCTAAAA	4del

FUZ

hg19	TCTCT**CCA**CGTT-GCGGATATTGGTCAGTTCTTCAAG	
1	TCTCT**CCA**CGT----GGATATTGGTCAGTTCTTCAAG	3del
2	TCTCT**CCA**CGTT**T**GCGGATATTGGTCAGTTCTTCAAG	ins 1

WISP3

hg19	AAATGCCCTCAGCAGAAG**CCC**CG-TTGCCCTCCTGGAGTGAGCCTGGT	
1	AAATGCCCTCAGCAGAAG**CCC**CG**T**TTGCCCTCCTGGAGTGAGCCTGGT	+
2	AAATG----------	1384del

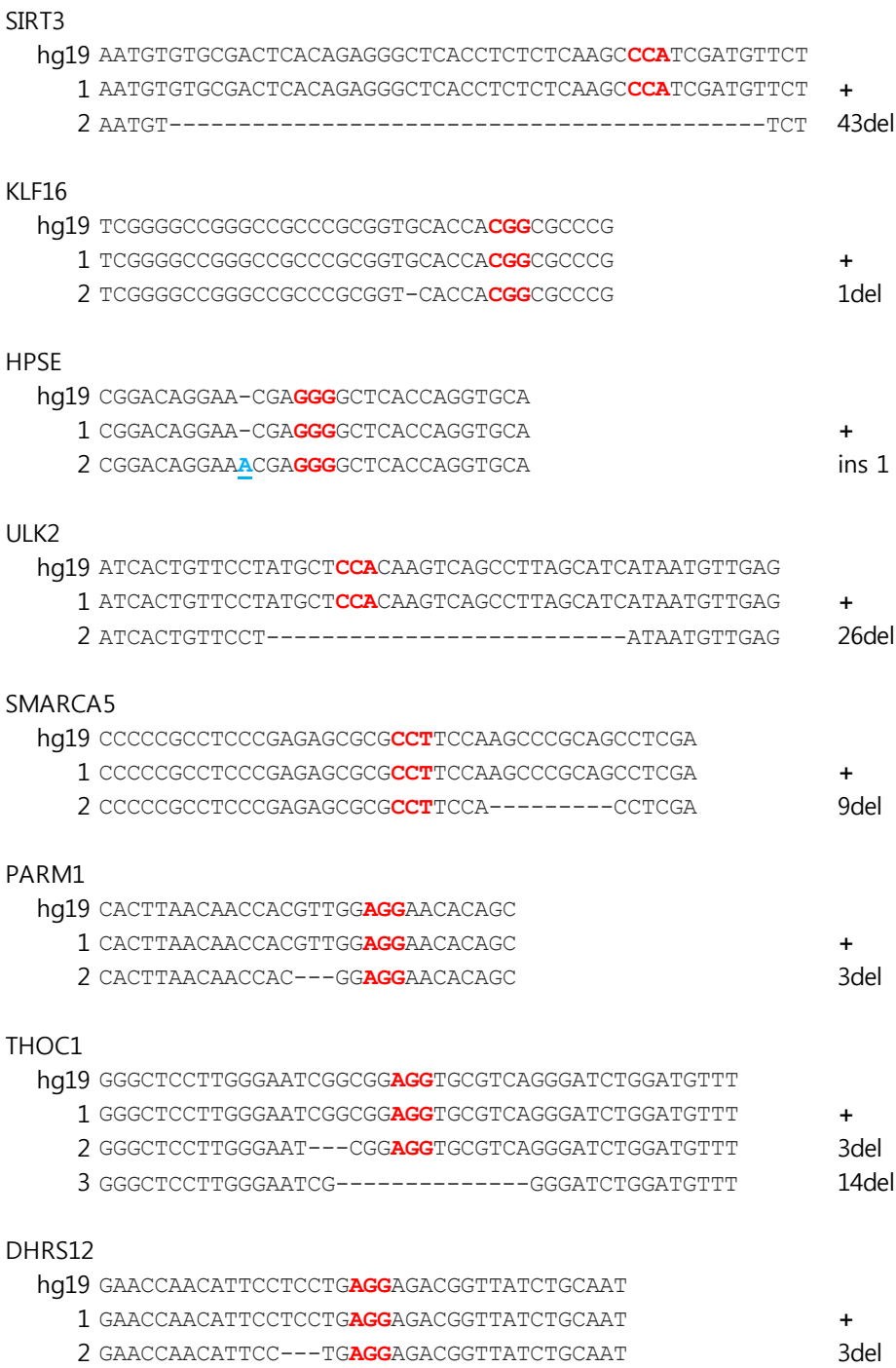


Figure 3. Sequence of RPV-1, 2 clones. The PAM sequences are indicated in bold, and the insertion sequences are underlined. + indicates wild type.

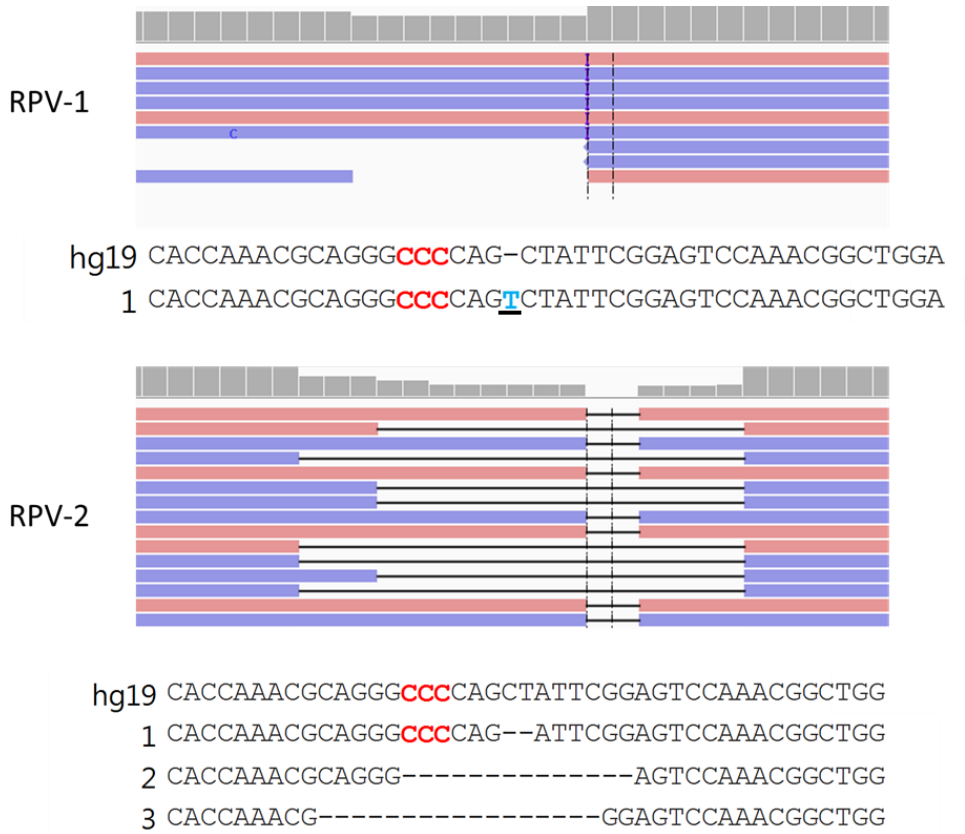


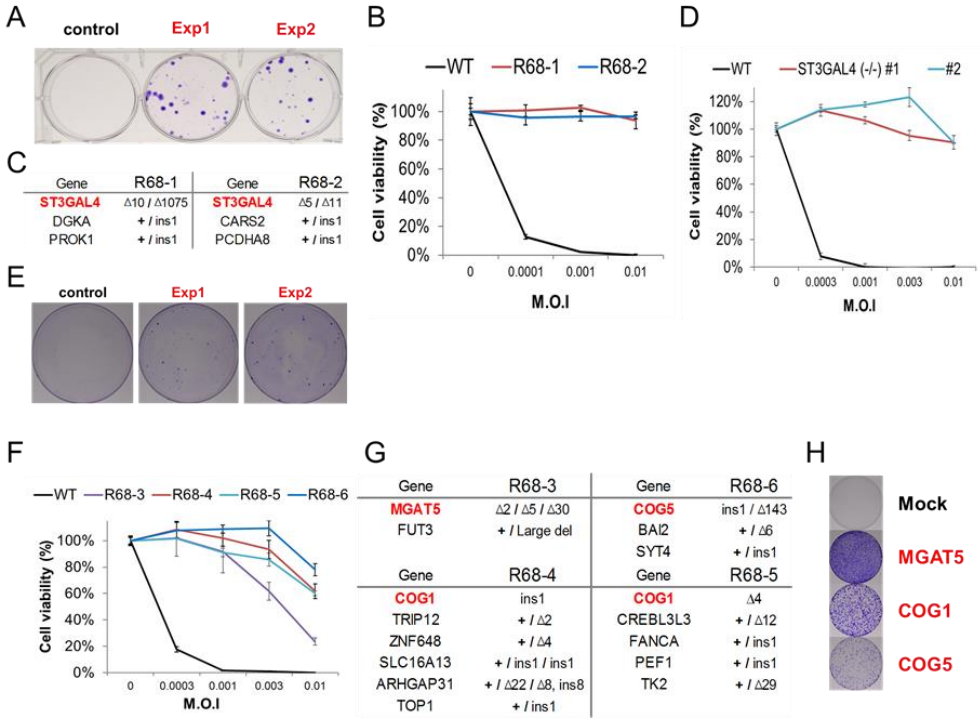
Figure 4. IGV view of the *PVR* sgRNA target site in the RPV-1 and RPV-2 clones. The WGS data shows that both clones have mutations at the *PVR* target site: wild-type and mutant sequences are presented. Small indels were verified using IGV. The genomic location is chr19:45,150,636-45,150,704. The PAM sequences are indicated in bold, and an insertion is underlined.

b. Screen for enterovirus D68 resistance

For the next screen, I infected HeLa cells transfected with the library with EV-D68 and obtained many resistant clones (**Fig. 5A**). I expanded two clones (R68-1, R68-2) and confirmed that they were resistant to EV-D68 infection (**Fig. 5B**). WGS showed that one gene encoding ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (*ST3GAL4*) was completely disrupted in the two clones (**Fig. 5C** and **Figs. 6 and 7**). Transfection of each sgRNA targeted to the *ST3GAL4* gene into HeLa cells gave rise to numerous virus-resistant colonies (**Fig. 8**). Furthermore, *ST3GAL4* knockout cells created by using two sgRNAs targeted to different sites in the gene were also resistant to EV-D68 infection (**Fig. 5D**). Taken together, these data showed that the gene identified by WGS of resistant clones is an essential factor for EV-D68 infection or replication.

To identify other genes essential for EV-D68 infection, I first picked several additional resistant colonies and checked whether these clones also had mutations at the *ST3GAL4* target sites. All of the clones I analyzed had indels in the *ST3GAL4* gene. I hypothesized that *ST3GAL4*-disrupted cells had a selective advantage over other gene-disrupted cells in the presence of the enterovirus and that other genes essential for the viral infection could be identified using a dropout library in which the three sgRNAs specific to *ST3GAL4* were excluded. Indeed, I was able to obtain many resistant colonies from additional screening using the *ST3GAL4* dropout library (**Fig. 5E**). Interestingly, unlike the *ST3GAL4* KO clones, four clones (R68-3 to R68-6) isolated from cells transfected with the dropout library were partially resistant to EV-D68 (**Fig. 5F**). WGS showed that these clones did not harbor

mutations in the *ST3GAL4* gene. Instead, 2-6 other genes were mutated in each of these clones. Only a single gene was completely disrupted in each clone. The *COG1* gene, which encodes a component of oligomeric Golgi complex 1, was disrupted in two clones. The other two clones harbored tri-allelic mutations in the *MGAT5* gene, which encodes mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase, or bi-allelic mutations in the *COG5* gene, which encodes a component of oligomeric Golgi complex 5 (**Fig. 5G** and **Figs. 9 and 10**). All of the other mutated genes were accompanied by a wild-type allele. Transfection of sgRNAs specific to each of the three completely-disrupted genes into HeLa cells gave rise to EV-D68 resistant colonies (**Fig. 5H**).



(With Chonsaeng Kim in KRICT)

Figure 5. Pooled sgRNA screens for enterovirus D68 resistance. (*A*) Crystal violet staining of EV-D68-resistant cells after 12 days of infection. (*B*) Viability of wild-type (WT) HeLa cells and two colonies obtained from cells transfected with the library after re-infection with EV-D68. (n=3, ± s.d.) (*C*) List of genes that were mutated in clones R68-1, 2. (*D*) Viability of wild-type (WT) and *ST3GAL4* knockout cells after EV-D68 infection. (n=3, ± s.d.) (*E*) Colony formation after EV-D68 selection in cells transfected with the *ST3GAL4* dropout library. (*F*) Viability of EV-D68 resistant clones obtained from cells transfected with the *ST3GAL4* sgRNA dropout library. (n=3, ± s.d.) (*G*) List of genes that were mutated in each clone. (*H*) Crystal violet staining of EV-D68 resistant cells after transfection with the indicated sgRNAs.

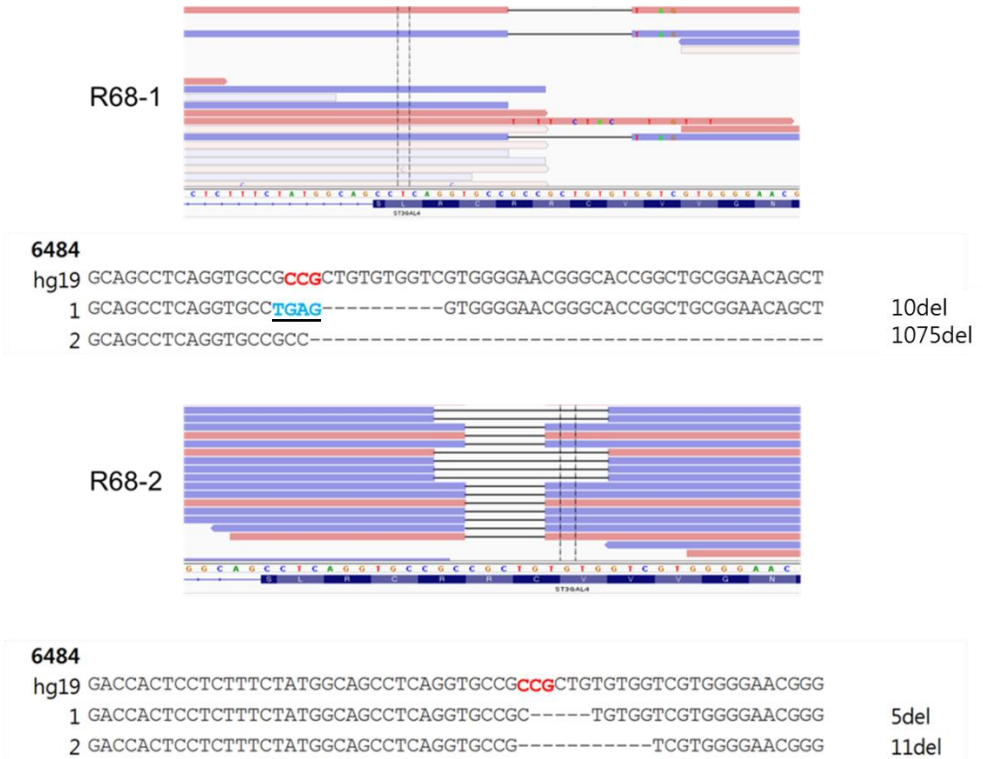


Figure 6. IGV view of the *ST3GAL4* sgRNA target site in the R68-1 and R68-2 clones. The WGS data reveal that both clones have mutations at the *ST3GAL4* target site. The genomic location is chr11:126,277,962-126,278,040. The PAM sequences are indicated in bold, and an insertion is underlined.

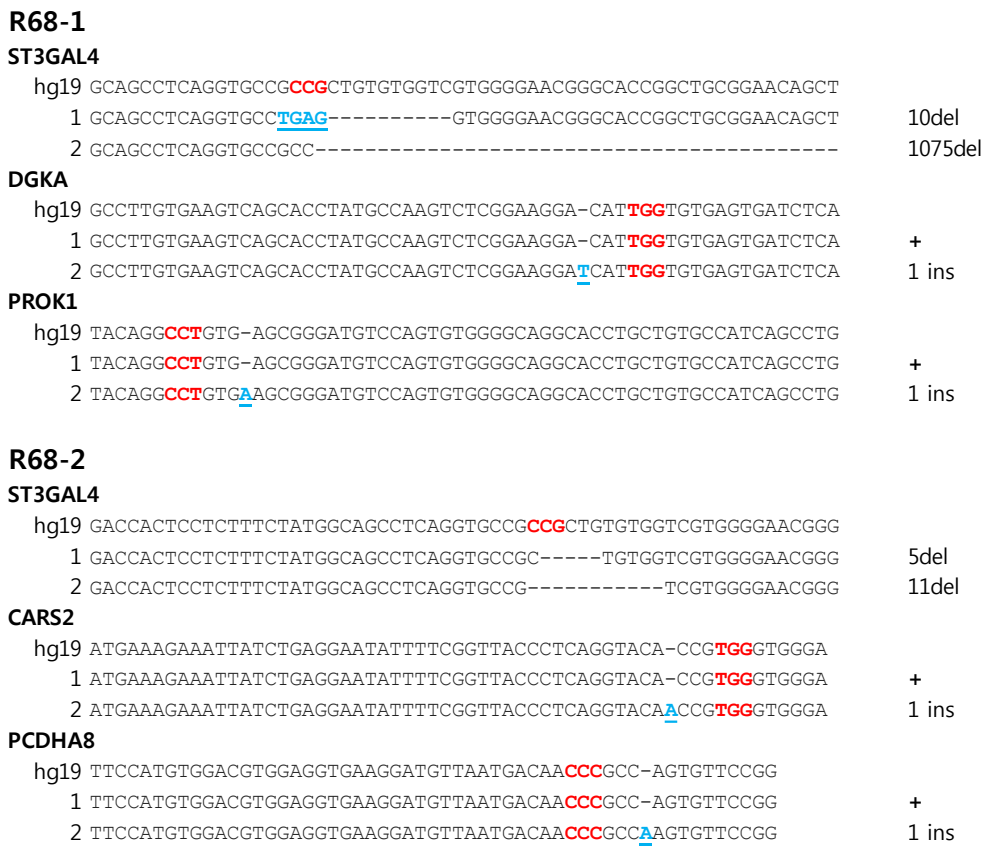


Figure 7. **Sequences of the sites containing sgRNA library-induced mutations in the R68-1 and R68-2 clones.** IGV was used for the analysis. The PAM sequences are indicated in bold, and the insertion sequences are underlined. + indicates wild type.

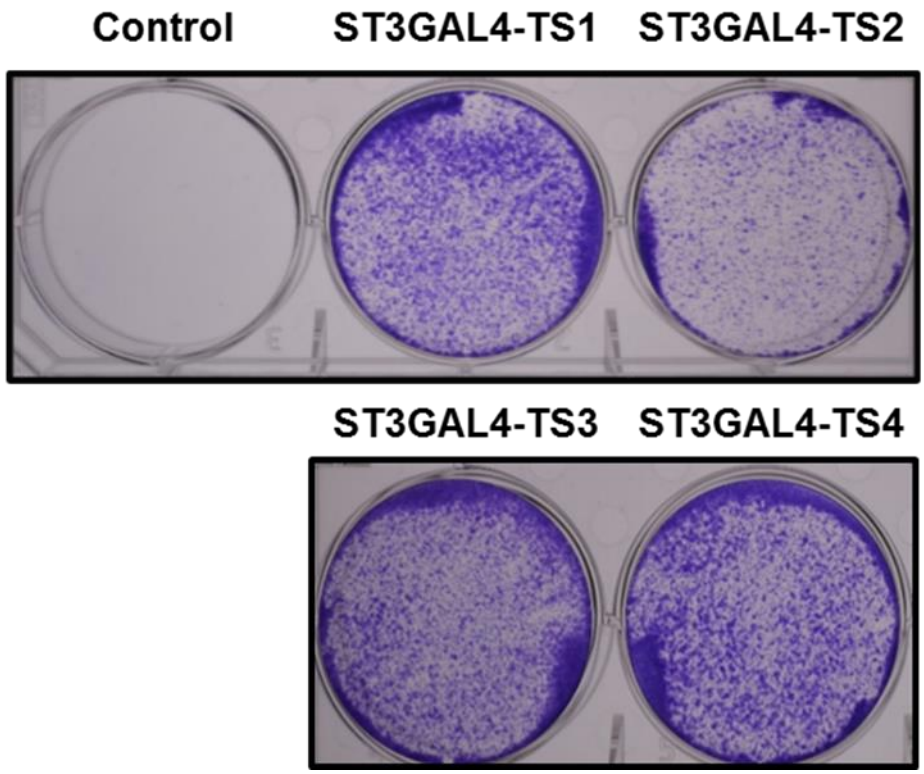


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Figure 8. EV-D68-resistant colonies formed after transfection of sgRNAs targeting the *ST3GAL4* gene.

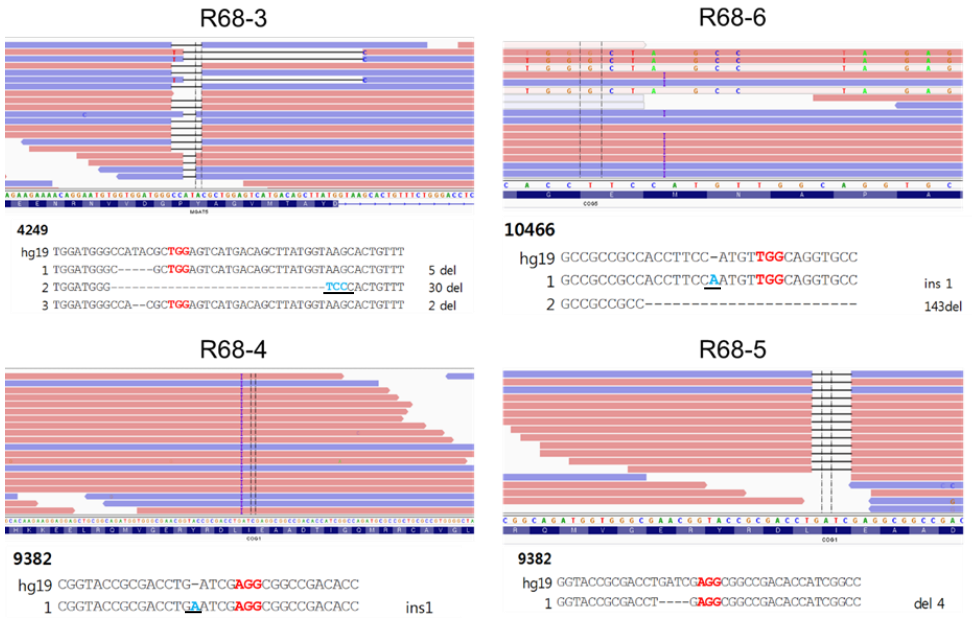


Figure 9. IGV view of the genes that were knocked-out in clones R68-3, 4, 5, and R68-6. R68-3; MGAT5, chr2:135,012,121-135,012,263. R66-4, 5; COG1, chr17:71,189,364-71,189,404. R68-6; COG5, chr7:107,204,316-107,204,356. The PAM sequences are indicated in red, and the insertion sequences are indicated in blue.

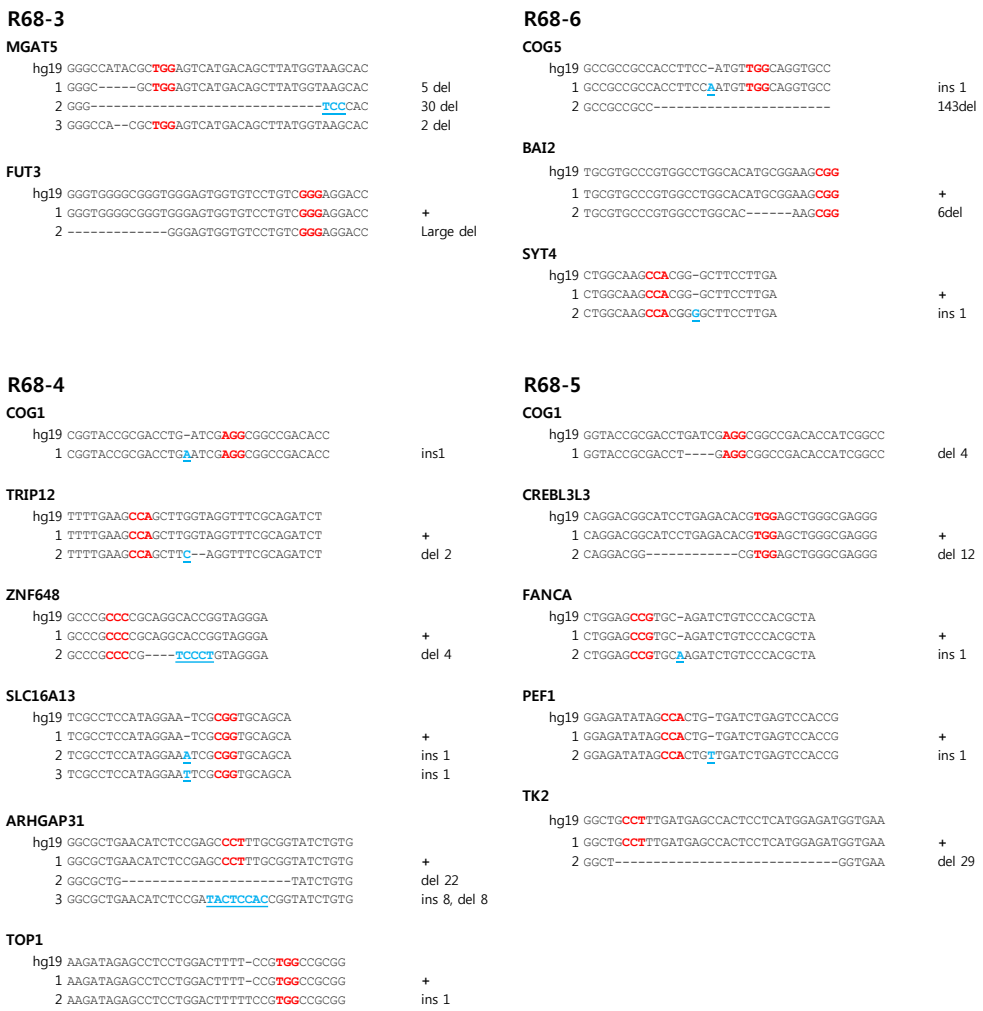


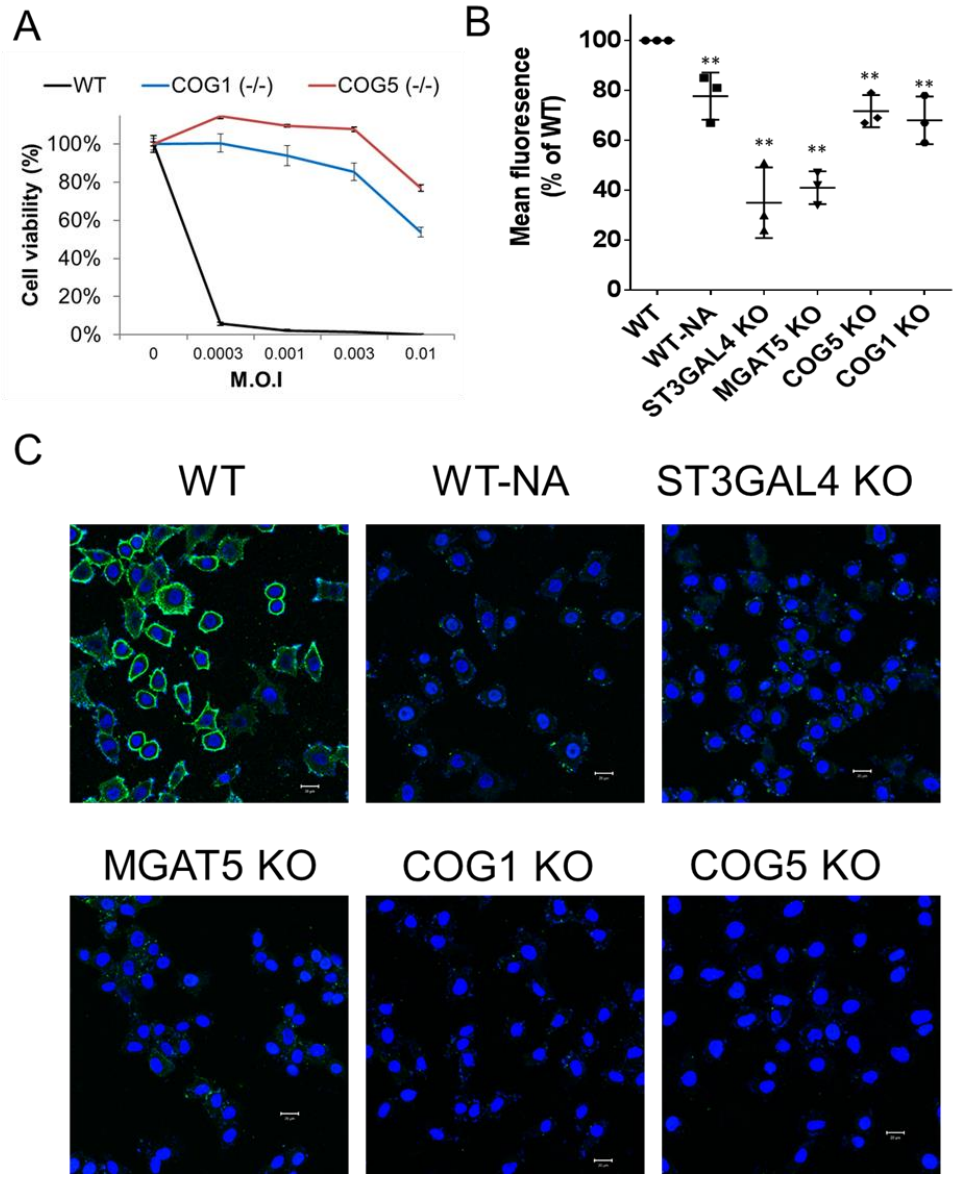
Figure 10. Sequences of the sites containing sgRNA library-induced mutations in the R68-3, 4, 5, and R68-6 clones. IGV was used for the analysis. The PAM sequences are indicated in bold, and the insertion sequences are underlined. + indicates wild type.

3. Enterovirus-D68 requires sialic acid of host cells for infection

a. Genes related to sialic acid presentation are essential for enterovirus D68 infection

To understand the mechanism behind the viral resistance, I then characterized these genes using KO cells. Recently, Baggen et al. showed that EV-D68 entry into cells is dependent on cell surface sialic acid and identified the *ST3GAL4* and *MGAT5* genes as essential for EV-D68 infection via their role in sialic acid conjugation in the Golgi (Baggen et al., 2016). I hypothesized that the *COG1* and *COG5* genes, the other two genes I identified in this study, might also be associated with sialic acid conjugation, because the conserved oligomeric Golgi (COG) complex has been proposed to act in the glycosylation process in cells (Ungar et al., 2002). Indeed, *COG1* and *COG5* KO clones were resistant to EV-D68 infection (**Fig. 11A**). To analyze surface sialic acid presentation by flow cytometry, those KO cells were stained with fluorescein-labeled *Maackia amurensis* lectin I (MALI), which binds selectively to 2,3-linked sialic acid. HeLa cells treated with neuraminidase (WT-NA in **Fig. 11**), which removes sialic acid residues from glycoproteins on the cell surface, were used as a control. The *ST3GAL4*, *MGAT5*, *COG1*, and *COG5* KO cells all showed a reduction in the level of 2,3-linked sialic acid on their surfaces (**Fig. 11B**). I then incubated these KO cells with EV-D68 virus particles at 4 ºC and stained with anti-EV-D68 VP1. Unlike HeLa cells to which virus particles were well attached, all of the KO clones were poorly associated with

EV-D68 on their cell surfaces (**Fig. 11C**). These results show that *COG1* and *COG5* play an important role in presenting sialic acid on the cell surface and that disruption of these genes leads to resistance to EV-D68 entry into cells.



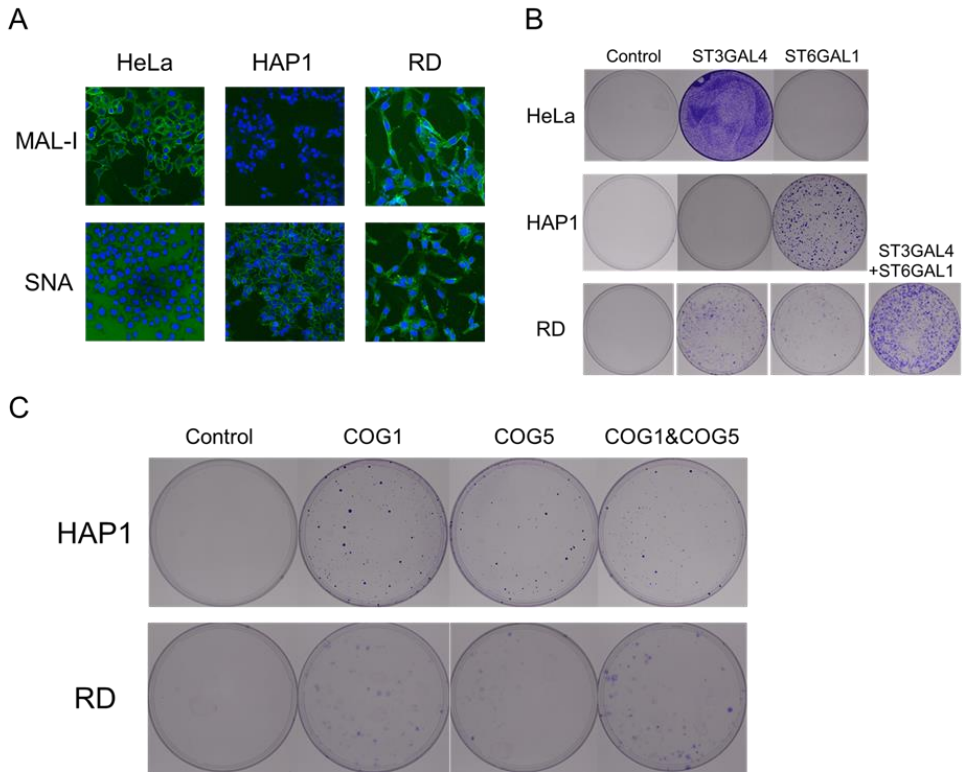
(With Chonsaeng Kim in KRICT)

Figure 11. Genes related to sialic acid presentation play an important role in enterovirus D68 infection. (*A*) Viability of wild-type (WT) and *COG1* and *COG5* knockout cells after EV-D68 infection. (n=3, ± s.d.) (*B*) Quantification of 2,3-linked sialic acid presentation by flow cytometry. Cells were analyzed after incubation with fluorescein labeled MALI. ** $P < 0.01$ (n=3, ± s.d.) (*C*) Confocal microscopy of knockout cells and WT cells (including WT-NA cells treated with neuraminidase) immunostained with anti-VP1 antibody after incubation with EV-D68 on ice for 60 min. EV-D68 was visualized with Alexa Fluor 488 conjugated secondary antibody (green) and cell nuclei were stained with DAPI (blue). Scale bar, 20 μm.

b. Sialic acid transferases, which mainly present sialic acids, are essential for EV-D68 infection

Though sialic acid presentation is important in EV-D68 infections, a previous gene trap showed that *ST6GAL1*, which mainly catalyzes 2,6-linked sialic acid formation, is considerably more important than *ST3GAL4* in HAP-1 cells for viral infection (Baggen et al., 2016). However, I could only identify *ST3GAL4*, not *ST6GAL1*, as an essential factor for viral infection in HeLa cells. To investigate this, I stained HAP-1 and HeLa cells with MALI and *Sambucus nigra* Lectin (SNA), which bind to 2,3-linked and 2,6-linked sialic acid, respectively. The HeLa cells contained many 2,3-linked sialic acid molecules on their surface but almost no 2,6-linked sialic acid was observed. However, the HAP-1 cells showed an opposite pattern (**Fig. 12A**). From these contrasting results, I hypothesized that the sialic acid transferase, which mainly presents sialic acid, would be a key factor for viral infection. To confirm this, I transfected sgRNAs targeting *ST3GAL4* and *ST6GAL1* into HeLa cells and then infected the cells with EV-D68. Only the sgRNAs targeting *ST3GAL4* conferred resistance to the HeLa cells. I was unable to isolate any resistant colonies from the *ST6GAL1* sgRNA-transfected cells (**Fig. 12B**). In contrast, when I transfected the same sgRNAs to HAP-1 cells, *ST6GAL1* targeting sgRNAs conferred resistance as previously shown (**Fig. 12B**). In RD cells, which have been most frequently used for EV-D68 infections, both sialic acids were presented well (**Fig. 12A**). Unlike in HeLa and HAP-1 cells, sgRNAs targeting both *ST3GAL4* and *ST6GAL1* induced EV-D68 resistance in RD cells. Furthermore, a co-transfection of *ST3GAL4* and *ST6GAL1* targeting sgRNAs showed more resistant clones, suggesting

that both sialyltransferases are important (**Fig. 12B**). From these results, I could infer that sialic acid transferases, which mainly present sialic acid, are essential host factors for EV-D68 infection. As each cell type had different dependence on sialic acid transferases, I tested whether *COG1* and *COG5* were required for EV-D68 infection in various cells. HAP-1 and RD cells were transfected with the Cas9-expression plasmid and plasmids expressing sgRNAs targeting *COG1* and *COG5*. These cells were then infected with EV-D68. Both sgRNAs targeting *COG1* and *COG5* induced EV-D68 resistance in HAP1 and RD cells **(Fig. 12C)**. These results, combined with resistance results obtained with HeLa cells presented in Fig. 3H, suggest that *COG1* and *COG5* are host factors that act on broad cell types.



(With Chonsaeng Kim in KRICT)

Figure 12. Sialic acid transferase, essential for viral infection, is dependent on the sialic acid presentation pattern of each cell line. (*A*) Lectin staining of HAP-1, HeLa, and RD cells. HAP-1 cells displayed strong 2,6-linked sialic acid (SNA binds to) presentation, whereas HeLa cells exhibited 2,3-linked sialic acid (MALI binds to). RD cells showed strong presentation of both sialic acids. (*B, C*) Each cell line was transfected with the indicated gene-targeting sgRNA and infected by EV-D68. Surviving cells are shown stained.

Discussion

In this study, I provided proof-of-principle cases of genome-scale gene knockout screens implemented with WGS to identify causal mutations. I showed that genes essential for a drug cytotoxicity or viral infection could be identified by isolating cells that survived the drug or virus challenge, respectively. Unlike siRNA or shRNA, sgRNA coupled with Cas9 leaves footprints at the target sites. WGS of cells that survived a viral challenge led to the identification of causative mutations in a target gene. Many genetic screens have been performed using arrayed RNAi libraries. However, these have recently been criticized for their lack of reproducibility derived from incomplete knockdown and high off-target effects of RNAi. In contrast, CRISPR/Cas9 completely disrupts genes, and the Cas9/sgRNA complex can discriminate 1–2 bp mismatches in the genome against RNAi in which partial matching of the seed region is sufficient for knockdown. Moreover, I tried to reduce off-target events even further by selecting target sites that were different from any other sites in the human genome by at least three nucleotides and by adding extra guanines (Bae et al., 2014; Cho et al., 2014). These aspects render this CRISPR screening approach more reproducible. In my results, as shown in Fig. 2, WGS showed that 20 genes, in addition to *PVR*, were mutated. In sequence analysis, these mutations had occurred at on-target sites of each sgRNA and not from off-target events. These might be introduced by transfection of multiple sgRNA plasmids into individual cells. Among the 20 genes mutated, 12 genes which were completely disrupted were tested using separate sgRNAs and none was related to viral resistance except for PVR, as shown in Fig. 2D. Although 11 genes other than PVR among the

completely disrupted 12 genes could be false positives in this screen, possible false positives were efficiently eliminated by using this separate sgRNA test.

In general, antiviral drugs target the virus itself. However, drug-resistant viruses have been reported that do not respond to drugs by mutating the target site. To overcome this limitation, key host factors could be good targets because of the low possibility to developing resistant mutations. It is also important that host factors are selectively essential for viral infection but not cell growth. This screening system is highly suitable to identify such novel targets for therapeutics, as I isolated the resistant cells after a gene knockout and virus challenge. Cells could grow and survive even after host factors are completely lacking. I show here that four host factors for EV-D68 infection, namely *ST3GAL4*, *MGAT5*, *COG1* and *COG5*, are potential antiviral targets. These 4 proteins were involved in sialic acid presentation on the cell surface (**Fig. 11B**) and EV-D68 binding (**Fig. 11C**). *ST3GAL4* and *MGAT5* were previously reported to be essential factors for EV-D68 by haploid genetics (Baggen et al., 2016). *COG1* and *COG5* are novel host factors for EV-D68, to the best of my knowledge. These two proteins are components of the conserved oligomeric Golgi (COG) complex required for normal Golgi morphology and function (Foulquier et al., 2006; Ungar et al., 2002). CHO cells with mutations in the COG complex showed defects in the Golgi-associated glycosylation reaction, similar to that observed in COG1 and COG5 knockout cells. I also found that these proteins were required in various cells, including HeLa, HAP-1, and RD cells. Interestingly, defects in COG genes were identified in patients with a mild form of congenital disorder involving glycosylation type II (CDG-II) (Foulquier et al., 2006). Sadat and

colleagues reported that CDG-IIb patients showed impaired viral replication and cellular entry (Sadat et al., 2014). These data imply that an acute viral infection using the sialic acid receptor could be inhibited by lowering the activity of the COG complex transiently, since the COG complex is a valuable target for antiviral agents against the EV-D68 or influenza virus.

Many studies have shown that lentiviral-based libraries encoding sgRNAs enable forward genetic screens in human and other mammalian cells. Unlike these approaches, which count the number of sgRNA sequences before and after selection by PCR and deep sequencing to identify target genes indirectly, my method relies on WGS to identify causal mutations directly in cells after selection. WGS might be considered cumbersome in terms of cost and technical accessibility. However, as sequencing technology further developed recently, WGS became quite achievable through HiSeq X Ten, with prices similar to those of the MiSeq or HiSeq platforms normally used for lentivirus-based library screening approaches.

Transient transfection of sgRNA and Cas9 plasmids into human cells gives rise to high-level expression of these components, resulting in efficient disruption of the target genes. In particular, for cells, which are non-immortalized or pluripotent stem cells that are easily differentiated, 2–3 weeks required to disrupt genes by lentiviral sgRNA expression could be cumbersome in screening in contrast to ~3 days required with transient plasmid expression. Although there are cell lines in which transfection with cationic lipids such as Lipofectamine is not efficient, alternative methods including electroporation could be a solution. In addition, small populations resulting from low transfection efficiency could be selected over non-

transfected cells owing to stringent selection process. Furthermore, as shown in Fig. 2C, multiple gene knockouts can be generated in individual cells, demonstrating new possibilities for discovery of novel genes that function in combination. As shown in Fig. 5E, the dropout library was easily constructed by using individually synthesized libraries, rather than employing the *in situ*-synthesized libraries on microarrays used for lentivirus-based screening. Dominant sgRNA targeting *ST3GAL4*, as presented in Fig. 5A, was excluded and successful screens to find other host factors for virus were achieved.

Despite the potential utility of this method, there is a limitation that genes exerting mild phenotypes could be missed due to the stringent selection performed in this experiment, in comparison to lentivirus-based screening. This method is unlikely to replace current methods using lentiviral sgRNA libraries in many settings, but will still provide a new option to researchers, especially when lentiviral production and infection is inefficient or not feasible. I propose that this pooled sgRNA screening method and resources will be broadly useful for forward genetic studies and drug target discovery.

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Chapter 2. Arrayed CRISPR Screens with Image-based Assay for Viral Infection

Introduction

Functional genetic screens are generally performed using two different methods: pooled or arrayed. Genetic screens using the clustered regularly interspaced repeat (CRISPR)-CRISPR-associated (Cas) system have been widely applied to various systems to reveal relationships between phenotypes and genes (Koike-Yusa et al., 2013; Shalem et al., 2014; Wang et al., 2014). However, most screens are conducted in a pooled format, which uses lentiviruses or plasmids to deliver a genome-wide library of single guide RNAs (sgRNAs). Pooled screens have some advantages, including easy preparation and reduced labor. However, they also have limitations in that phenotypes that are mild or have growth disadvantages can be overlooked. On the other hand, arrayed screens that are conducted by singular reagents arranged in a multi-well format could overcome these limitations, as each well has individual genetic perturbations. However, small interference RNA (siRNA) library screens, which are the most representative arrayed screen method, are recently being criticized for low reproducibility induced by high off-target effects and incomplete gene knockdown (Buehler et al., 2012; Kaelin, 2012). In contrast, genome editing based on CRISPR-Cas9 results in complete knockout of the target gene. Furthermore, off-target effects can be greatly reduced by selecting unique target sites and modifying guide RNAs (Bae et al., 2014b; Cho et al., 2014; Fu et al., 2014). These comparative advantages of the CRISPR system could help overcome the limitations of siRNA library screening. Arrayed CRISPR screens could be a very useful platform to fill the caveats of pooled CRISPR screens and siRNA library screens.

Some groups have previously attempted to develop arrayed CRISPR screens (Datlinger et al., 2017; Hultquist et al., 2016; McCleland et al., 2016; Strezoska et al., 2017; Tan and Martin, 2016). These studies were based on guide RNA libraries consisting of individual lentiviral sgRNAs for single genes or synthesized CRISPR RNA (crRNA), in a manner similar to siRNA screens. Although these prior studies have their strengths, they also have some weaknesses. Arrayed lentiviral particles are cumbersome to prepare because each lentiviral vector should be delivered to packaging cell lines individually. Moreover, challenges such as cross-contamination and variable lentiviral titers in the multi-well plate make it difficult to apply lentiviral particles to arrayed screens in a large scale. Furthermore, because of the low expression of sgRNAs and the additional time to select out the un-transduced cells with selection marker, it will take more time for screening. This could also be another drawback of the lentiviral method in arrayed screens because a long culture period is not feasible in multi-well plates. Although synthetic crRNA is easier to prepare compared to lentivirus particles, RNA synthesis is cost-inefficient.

In this study, I present a new CRISPR screen method using an arrayed library of sgRNAs to identify essential host genes for Coxsackievirus infection. Delivery of Cas9 and sgRNA expression plasmids is the most frequently applied method because of simplicity and high expression levels of both components, which result in high efficiency of genome editing in eukaryotic cells over 2-3 days. With respect to rapid and highly efficient genome editing, this method would reduce the signal-to-noise levels in high-throughput assays, thus improving the reliability and sensitivity of genetic screening.

Enteroviruses from the *Picornaviridae* family are human pathogens that cause a wide range of illnesses, including aseptic meningitis, encephalitis, and myocarditis(Fields et al., 2007). These viruses depend on host proteins for their life cycle. Determining the role of host factors in viral infection would help understand the basic aspects of virus-host interaction and explore novel therapeutic targets. Host factors that are indispensable for viruses and affect host cells minimally would be ideal targets. I chose coxsackievirus B3 (CVB3), which is a member of human enteroviruses. Some groups have reported that CVB3 exploits kinases, proteins in endocytic pathways, and Golgi-localized proteins for its infection (Coyne and Bergelson, 2006; Lanke et al., 2009; Patel et al., 2009). To further investigate the host factors required for CVB3 infection, I selected up to 1,514 encoding kinases, proteins related to endocytosis, and Golgi-localized proteins. I constructed a plasmid library expressing sgRNA targeting these 1,514 genes (3 sgRNAs per gene, 4,542 sgRNAs) and performed a high-throughput screen to identify the genes required for CVB3 infection.

Materials and Methods

1. sgRNA array construction.

An empty sgRNA expression vector cleaved using *Bsa*I (New England Biolabs) was ligated with annealed oligonucleotide mixtures (3 oligonucleotide pairs for each gene). Ligation products were transformed into DH5α competent cells. Transformed E.coli cells were plated on a 10-cm LB plate with ampicillin and plasmids are purified from them.

2. Pooled libray screen and analysis of target genes.

Human GeCKOv2 CRISPR knockout pooled library was a gift from Feng Zhang (Addgene # 1000000048) (Sanjana et al., 2014). Oligo pool for focused library was purchased from CustomArray Inc. The single-stranded oligos were amplified to double-stranded DNA by PCR and assembled to lentiviral sgRNA plasmid using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). 3 x 10^7 cells were transduced with a lentiviral sgRNA library at a multiplicity of infection (MOI) of 0.3. After 24 h, cells were selected with puromycin. Those cells were cultured for approximately 2 weeks and 3×10^7 cells were infected with CVB3 (MOI 0.00001). Genomic DNA was extracted from the initial cell population and surviving cells from CVB3 infected populations. The region containing the gRNA was amplified with primers F-TCTTGTGGAAAGGACGAAACACCG and R-TCTACTATTCTTTCCCCTGCACTGT using 10 μg of genomic DNA and

sequenced using an Illumina HiSeq 4000 for 100× coverage. After sequencing, reads were aligned to the sgRNA library and counted. Those sgRNA counts are analyzed by MAGeCK (Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout) version 0.5.5 according to the instructions(Li et al., 2014).

3. Cell culture and transfection conditions.

HeLa (ATCC, CCL-2) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Using 96-well plates (PerkinElmer, Product number 6005550), 25ng of arrayed sgRNAs and 25ng of Cas9 expression plasmids were reversely transfected to 5 x 10^3 HeLa cells using Lipofectamine 2000 in each well. At 5 days post-transfection, cells were used for CVB3 infection. Single cell derived knock out cell-lines were obtained by limiting dilution.

4. Virus and plasmids.

Coxsackievirus B3 (Nancy; ATCC VR-30) were expanded by growth in HeLa cells and titered using HeLa cells. The pLuCBV3 plasmid, which contains a coxsackievirus B3 subgenomic replicon carrying the firefly luciferase gene, was provided by Eun-Seok Jeon (Samsung Medical Center, Seoul, Korea)(Lim et al., 2012). CSDE1-expressing plasmid was purchased form Vigene Bioscience (CH842097) and plasmid encoding eGFP-CSDE1 was constructed using eGFP-N1

plasmid (Clontech Laboratories). pCI-FLAG–ACBD3, which contains a Flag-tagged *ACBD3* gene, was obtained from Jun Sasaki (Fujita Health University, Aichi, Japan)(Sasaki et al., 2012). Checkmate mammalian two-hybrid system was purchased from Promega Corporation. Dual luciferase reporter plasmids containing viral IRES were gift from Sung Key Jang (POSTECH, Pohang, Korea).

5. Antibodies and chemicals.

Anti-CSDE1 rabbit antibody was obtained from Bethyl Laboratories (catalog no. A303-159A). ACBD3 specific rabbit antibody (catalog no. HPA015594), murine anti-FLAG antibody (catalog no. F3165), and beta actin murine monoclonal antibody (catalog no. A1978) were purchased from Sigma-Aldrich. Murine monoclonal anti-VP1 antibody was obtained from Leica (NCL-ENTERO). Anti-RACK1 mouse antibody was purchased from BD Biosciences (catalog no. 610177). Rabbit polyclonal anti-3C antibody was generated by immunization with recombinant 3C protein. Secondary antibodies conjugated to horseradish peroxidase were purchased from Thermo Fisher Scientific. Alexa Fluor 488-conjugated goat anti-rabbit antibody was obtained from Life Technologies. Chemical inhibitor Dynasore was purchased from Merck Millipore and C75, itraconazole were purchased from Sigma-Aldrich.

6. Virus infection and screening.

Cells transfected with the Cas9 plasmid and sgRNA library in 96-well plate were infected with Coxsackievirus B3 at an MOI of 5 at 37ºC for 8 h. Infected cells were fixed and permeabilized with a 3:1 mixture of ice-cold methanol-acetone. Cells were incubated with anti-3C antibody and anti-rabbit secondary antibody conjugated to Alexa Fluor 488 to detect infected cells, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Product # 62248, Thermo Fisher Scientific). Images were captured with the Operetta system (Perkin Elmer). The number of infected cells and the total number of nuclei were quantified using Harmony software installed on the Operetta system. The Z score for CVB3 infection was calculated as previously described (Yasunaga et al., 2014). Candidates genes were selected which are $Z < -1.8$ in duplicate screens.

7. Re-infection test and replicon assay.

Cells were plated to 96-well plates at 2×10^4 cells per well to test whether knock out cells were resistant to viral infection and whether replication of the CVB subgenomic replicon was inhibited in those cells. For the reinfection assay, ten-fold diluted CVB (MOI 0.1 ~ 0.0001) was added to each well and the cultures were incubated at 37ºC for 2 days. To measure cell viability after virus infection, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay was performed as previously described (Kim et al., 2002). The viability of infected cells was normalized to the viability of mock-infected cells (expressed as 100%). For the replicon assay, the pLuCBV3 plasmid was linearized

with ClaI (New England Biolabs) and used as a template for in vitro RNA transcription performed with a MEGAscript T7 kit (Ambion) as suggested by the manufacturer's protocol. The in vitro transcribed replicon RNA was purified using the TRIzol LS reagent (Invitrogen). Transfection was performed with 50 ng of replicon RNA per well with the Lipofectamine 2000 (Thermo Fisher Scientific) reagent. At 4 h post-transfection, cells were washed, resuspended in complete growth medium, and incubated at 37ºC or lysed in 20 µl of lysis buffer (0 h). At indicated time points, cells were washed and lysed in lysis buffer. Lucifease activity was measured using a luciferase assay kit (Promega Corporation) according to the manufacturer's protocol and a luminometer (LB960 centro XS3, Berthold Technologies). Luciferase activity was expressed in fold increase as compared with the activity measured at 0 h.

8. RNA interference.

CSDE1 siRNA (L-015834-00) and *RACK1* siRNA (L-006876-00) were obtained from Dharmacon. Control and human *ACBD3* siRNAs have been described previously (Dorobantu et al., 2014). siRNAs were used at a concentration of 20 nM. Cells were transfected with siRNAs in 6-well plates using Lipofectamine 2000 (Thermo Fisher Scientific). After 24 h, cells were trypsinized and replated in 96-well or 12-well plates and virus infection or replicon RNA transfection was performed 2 days later. Depletion of the target proteins was confirmed by immunoblot analysis. The blots were developed using a LAS 4000 imager (Fujifilm).

9. IRES activity test.

IRES activity was measured as previously described with minor modification (Kang et al., 2015). Wild type (HeLa) and *CSDE1* knock out cells were plated into 96-well plate and transfected with reporter plasmids using Lipofectamine 2000. 48 hours post-transfection, Firefly and *Renilla* luciferase activities were measured.

10. Isolation of CVB3-resistant colonies.

HeLa cells transduced with GeCKO library or transfected with Cas9 plasmid and candidate sgRNAs were infected with CVB3. Infected cells were washed and changed to complete medium. The culture medium was changed every 2 or 3days. After 14 days, surviving colonies were fixed and stained by crystal violet (0.05%) in phosphate buffered saline (PBS) solution containing 1% formaldehyde and 25% methanol or isolated and expanded for further analysis.

Results

1. Genetic screens for host factors required for CVB3 infection

a. Pooled lentiviral CRISPR screen to identify host factors for CVB3 infection

To compare the commonly used pooled screen with the arrayed screen, a lentivirus-based pooled sgRNA screen was conducted to uncover the host genes essential for CVB3. The GeCKOv2 CRISPR knockout pooled library, which is the most widely used, was transduced into HeLa cells at 0.3 multiplicity of infection (MOI), and sgRNA-expressing cells were selected with puromycin. Two weeks later, these cells were subjected to CVB3 infection. I performed targeted deep sequencing for 100× coverage using genomic DNA isolated from hundreds of surviving cells and compared the abundance of sgRNAs from the initial population and the cells surviving after the CVB3 infection, using MAGeCK software (**Fig. 1A**) (Li et al., 2014). Only one gene, *CXADR* (Coxsackievirus and adenovirus receptor), which is well known as the receptor of CVB3, was shown as a strong candidate (**Fig. 1B**). Though the lentivirus-based CRISPR screen was easy to perform, I wondered if I had missed other important host factors using this pooled screen. Therefore, I tried to screen each gene one by one in an arrayed format.

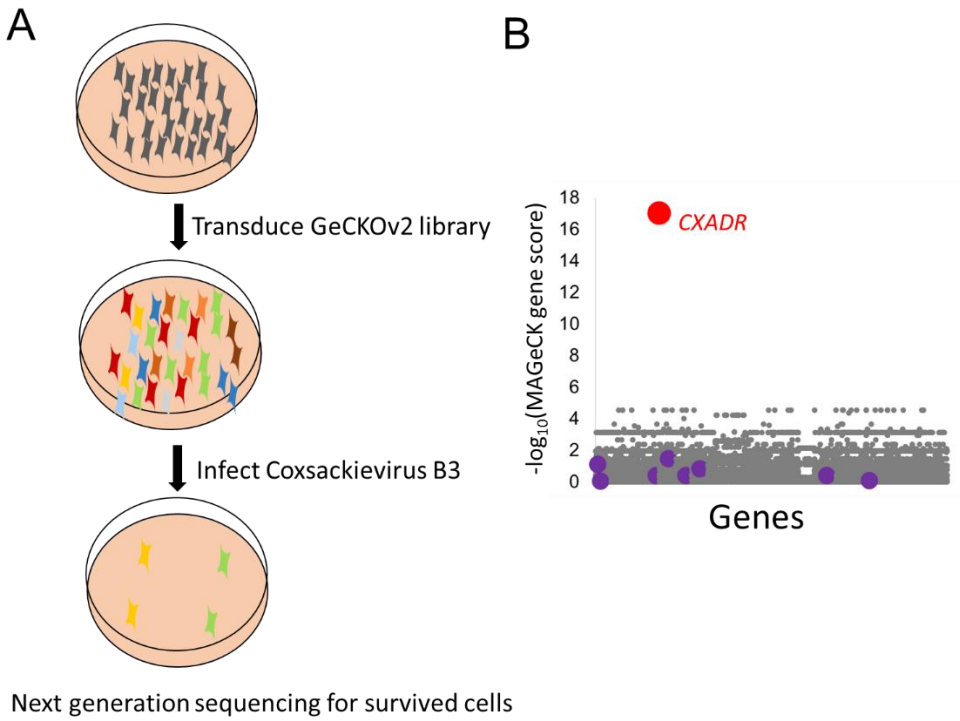

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Figure 1. GeCKO library screens for CVB3 resistance. (*A*) Schematic presentation of the GeCKO library screen. (*B*) Sequencing analysis of surviving cells. Each circle represents a gene. The y axis indicates the MAGeCK gene score. Genes were randomly distributed on the x-axis. Red colored circle indicates the enriched *CXADR* gene. Purple colored circles indicate eight gene hits in the arrayed screen in the order, *ACBD3, ADCY8, CSDE1, DNM2, FASN, RACK1, OSBP*, and *SACM1L*.

b. Arrayed CRISPR screens

To identify host factors for CVB3 infection in an arrayed format, I developed an image-based assay using sgRNA plasmids. As a proof-of-principle, I chose two genes: *CXADR* and *PI4KB*. *CXADR* is well-known as the receptor of CVB3, which was also identified from the pooled screen, and *PI4KB* is an essential factor for CVB3 replication (Bergelson et al., 1997; Hsu et al., 2010). I first transfected 3 sgRNA plasmids targeting *CXADR* and *PI4KB* into HeLa cells, along with the Cas9 expression plasmid to induce mutations in each gene. Mutations were induced very efficiently, and 71% and 80% of alleles were disrupted, respectively. As CRISPR knockout generally requires more time to show phenotypes compared to siRNA, experimental conditions like cell number and incubation time after transfection were optimized. To maximize Cas9-mediated mutation and minimize the effect of residual mRNA and proteins, I incubated the transfected cells for 5 days and then infected them with CVB3. The cells were then fixed and stained with the anti-3C rabbit polyclonal antibody and AF-488-conjugated anti-rabbit goat antibody. Images were acquired using the high-content imaging system, operetta. The CVB3 infection level was significantly reduced when the host factor-targeting sgRNAs were transfected, compared to that using the non-targeting sgRNA (**Fig. 2A, B**). This result implies that the arrayed sgRNA library based on plasmid expression could be useful for screening. To identify novel host factors for CVB3 infection, I chose three groups of genes encoding 469 kinases, 310 proteins related to endocytosis, and 735 Golgi-localized proteins, which are potential host factors for viral infection. Using a library of individually synthesized oligonucleotides, which encode sgRNAs targeting the

human genome that I have described previously (Kim et al., 2017), I prepared an arrayed sgRNA plasmid library in the 96-well format consisting of three target sites per gene for 1,514 genes. I carefully designed those target sites to be unique in the human genome and to have high microhomology score to avoid in-frame mutations as much as possible (Bae et al., 2014a; Bae et al., 2014b). To examine the activity of this library, I investigated the gene disruption rate after transfecting sgRNAs targeting 35 genes. Most of the genes were knocked out efficiently, implying that this library is useful and reliable. The average mutation rate for 35 genes was quite high at 72.8% (**Fig. 2C**).

I performed an arrayed CRISPR screen based on the plasmid sgRNA library for 1,514 genes in duplicate (**Fig. 3A**). I calculated the Z score for CVB3 infection rate reduction for each plate, and identified the candidate genes with $Z < -1.8$ in the duplicate screens (**Fig. 4A**, yellow box indicates the region for candidate genes). From the primary screens, I chose 10 genes as candidates (**Fig. 4B**). To confirm these results, the same sgRNAs used in the screen were transfected again into HeLa cells along with Cas9 and then the infection rate was measured (**Fig. 3B**). Most of the genes, except two from the primary screens, were confirmed by reduced infectivity. To further verify this result, I transfected newly designed sgRNA sets for each gene and found that CVB3 infection was significantly inhibited (**Fig. 3C**). Of the 10 genes identified in the primary screen, 8 were confirmed in the secondary screens. This result shows that the arrayed CRISPR screen is quite reliable and reproducible.

I next investigated whether these 8 genes could be identified using a pooled library

of 4,542 sgRNAs targeted to the same 1,514 genes. This focused, pooled library was transduced into HeLa cells at 0.3 MOI. Two weeks later, these cells were subjected to CVB3 infection. Targeted deep sequencing revealed that only one gene, *CXADR*, was identified as a strong candidate (**Fig. 5**). Note that I identified the same gene using the GeCKOv2 library (**Fig. 1**).

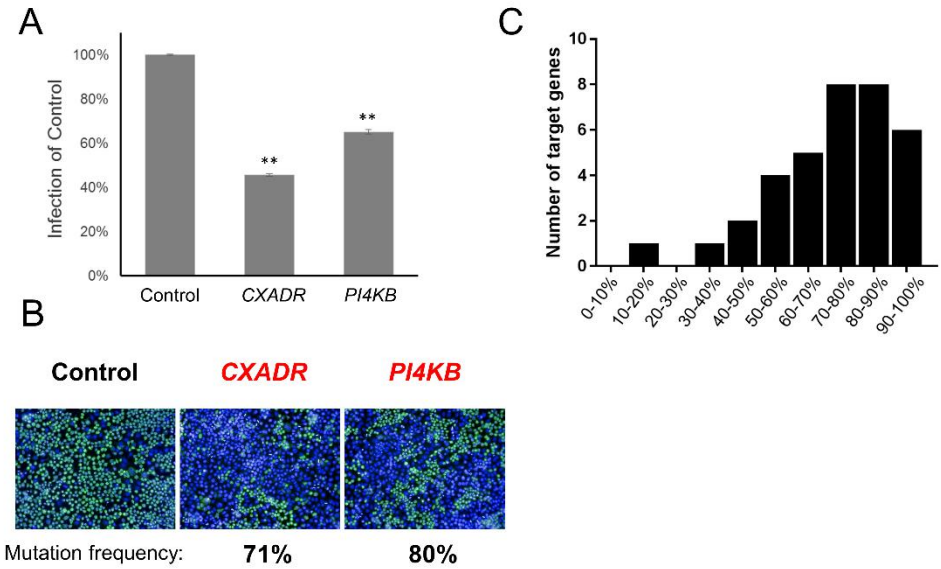

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Figure 2. Plasmid-based sgRNA constructs as a tool for a genetic perturbation screen in human cells. (*A, B*) HeLa cells were transfected with sgRNA-expressing plasmids (Control, *CXADR*, and *PI4KB*) and infected with CVB3 at an MOI of 5. At 8 hours post infection, cells were fixed and stained with anti-3C antibody (green) and DAPI (blue). (*A*) Quantification of infection. Mean ± s.e.m for quadruplicate experiments. ** $p < 0.01$. (*B*) Representative images of infection are shown. (*C*) Mutation frequencies determined after transient transfection of 35 sgRNAs and Cas9 plasmid.

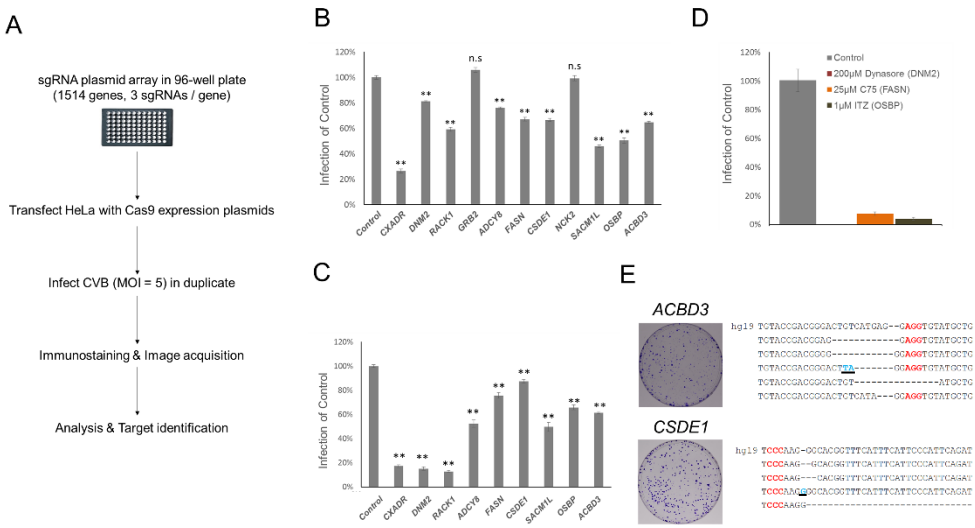

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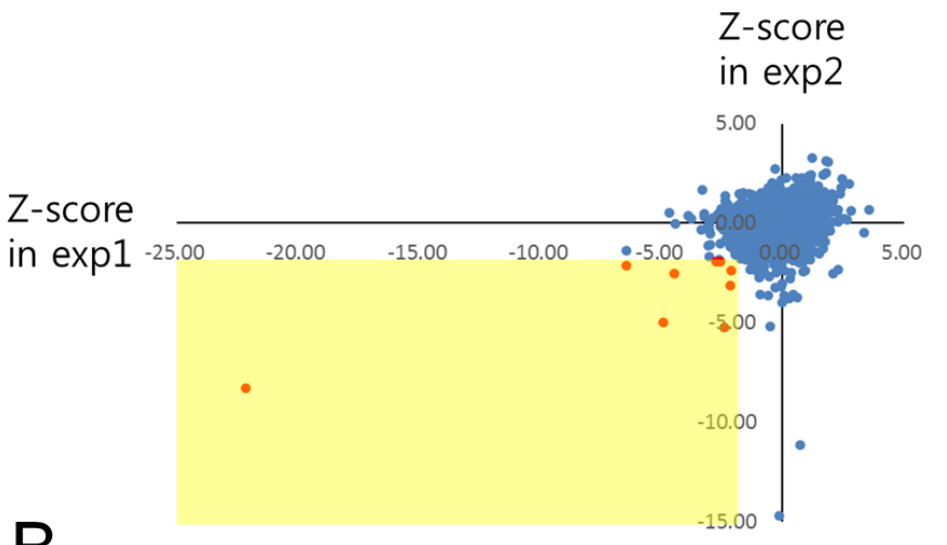
Figure 3. Candidate identification. (*A*) Schematic presentation of the arrayed CRISPR screen. (*B*) Quantification of CVB3 infection in HeLa cells transfected with sgRNA-expressing plasmids targeting 10 genes from primary screens. Mean ± s.e.m for quadruplicate experiment. ** $p < 0.01$, n.s; non-significant. (*C*) Similar quantification of virus infection in (*B*) using newly designed sgRNA. (*D*) The percentage of CVB3-infected cells (normalized to DMSO control) treated with 3 known inhibitors of the candidates. Mean ± s.d for triplicate experiments. (*E*) HeLa cells were transfected with *ACBD3* and *CSDE1* targeting sgRNAs and infected with CVB3. Surviving cells were stained and are shown. Surviving colonies were expanded and the target region was sequenced to confirm the mutations. Red characters indicate the PAM and blue characters indicate insertions. Hg19 is the wild type sequence of each gene.

A



B

List of 10 candidate genes	
Gene	Full Name
DNM2	dynamin 2
RACK1	receptor for activiated C kinase 1
GRB2	growth factor receptor bound protein 2
ADCY8	adenylate cyclase 8
FASN	fatty acid synthase
CSDE1	cold shock domain containing E1
NCK2	NCK adaptor protein 2
SACM1L	SAC1 suppressor of actin mutations 1 like
OSBP	oxysterol binding protein
ACBD3	acyl-CoA binding domain containing 3

Figure 4. Z-score for duplicate array screens and the list of candidate genes. (A) Z score for each gene was plotted on the x-axis (exp 1) and y-axis (exp 2). Yellow box denotes candidate genes (red circles) that showed decreased CVB3 infection after gene knockout. (B) List of 10 candidate genes of which 8 genes were confirmed by a secondary screen are indicated in grey background.

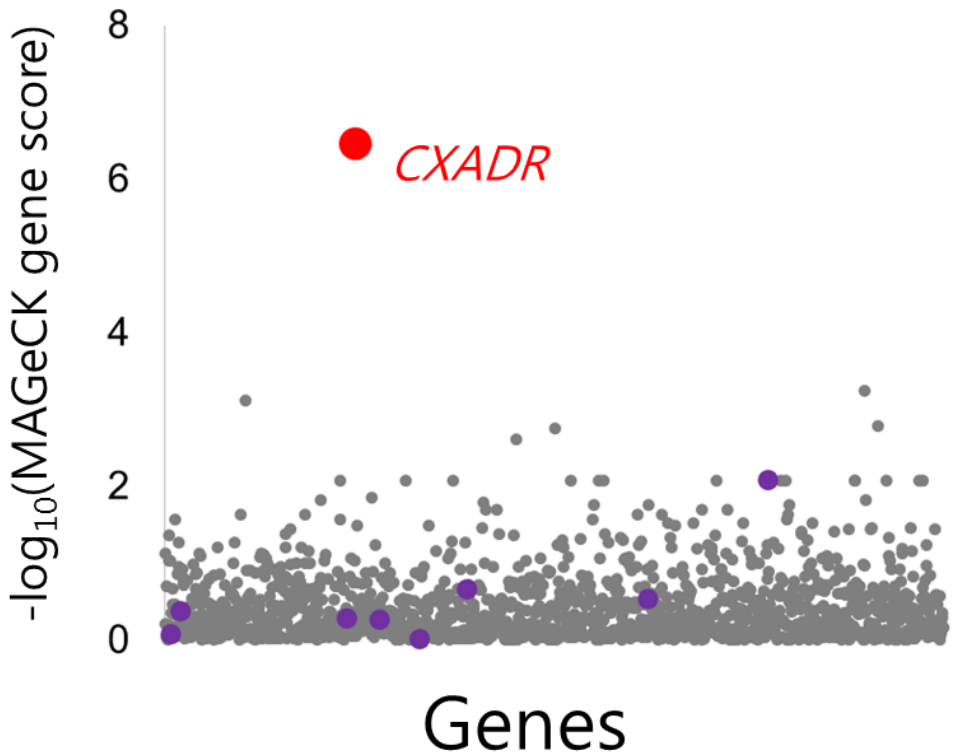


Figure 5. Pooled screen for CVB3 resistance using focused library. A lentivirus-based pooled focused sgRNA screen (pooling 4,542 sgRNAs) was conducted. Sequencing analysis of surviving cells. Each circle represents a gene. The y axis indicates the MAGeCK gene score. Genes were randomly distributed on the x-axis. Red colored circle indicates the enriched *CXADR* gene. Purple colored circles indicate eight gene hits in the arrayed screen in the order, *ACBD3, ADCY8, CSDE1, DNM2, FASN, RACK1, OSBP*, and *SACM1L*. *CXADR* was the only gene with false discovery rate (FDR) under 10%.

2. Validation of candidate genes from genetic screens

a. DNM2, FASN, OSBP, and SACM1L are known to be important for enterovirus infection

It has been previously reported that some candidates are required for coxsackievirus infection. Dynamin 2 protein encoded by *DNM2* is required for CVB3 entry (Patel et al., 2009). Fatty acid synthase encoded by *FASN* is required for CVB3 replication (Wilsky et al., 2012). OSBP and SACM1L are required for sterol/PI(4)P exchange at the ER-Golgi interface, and rhinovirus and enterovirus, which belong to *Picornaviridae* like CVB3, use these processes for viral replication (Mesmin et al., 2013; Roulin et al., 2014; Strating et al., 2015). My screening results are in agreement with these previous reports and give me confidence in this approach. The proteins encoded by *DNM2*, *FASN*, and *OSBP* have known chemical inhibitors: Dynasore, C75, and Itraconazole (ITZ), respectively. As expected, CVB3 infection was dramatically reduced by these chemicals (**Fig. 3D**). In contrast, cell viability was not affected at the same concentrations (**Fig. 6**).

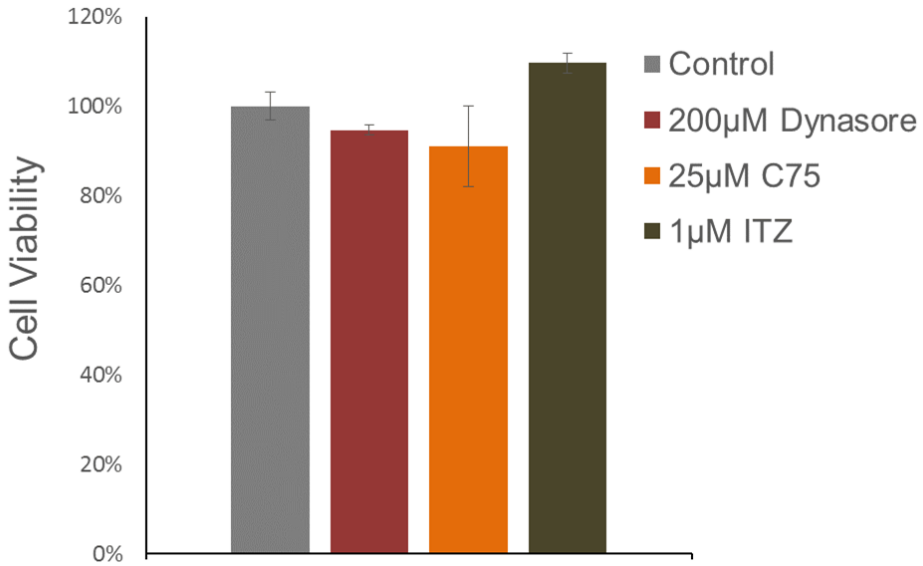


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Figure 6. Cell viability after treatment with three chemical inhibitors. Cells were treated with the indicated concentrations of three chemical inhibitors for 2 days and cell viability was measured by MTT assay.

b. sgRNAs against CSDE1 and ACBD3 make cells resistant to CVB3

Unlike siRNAs, sgRNAs coupled with Cas9 induce permanent gene knockout at the target sites. I examined whether knocking out the candidate genes could make cells completely resistant to CVB3 infection. Genes that make cells resistant to virus after being knocked out could be important anti-viral drug targets because these host factors are absolutely required for virus infection but are not essential for cell viability and proliferation. sgRNAs targeting the Cold Shock Domain-Containing Protein E1 (*CSDE1*) and the Acyl-CoA-Binding Domain-Containing Protein 3 (*ACBD3*) were found to induce CVB3-resistance without preventing cell proliferation (**Fig. 3E**). As expected, these resistant colonies had mutations at the *CSDE1* and *ACBD3* target sites (**Fig. 3E**). However, sgRNAs targeting other genes like *DNM2*, *FASN*, and *OSBP*, even though proved as important host factors by using chemical inhibitors (**Fig. 3D**), could not generate CVB3-resistant clones (**Fig. 7**). I also tried to isolate KO clones for these genes, but I could not obtain them. This suggests that they are essential factors for cell proliferation, as previously reported (Blomen et al., 2015). Accordingly, I chose *CSDE1* and *ACBD3* for further study among the candidates.

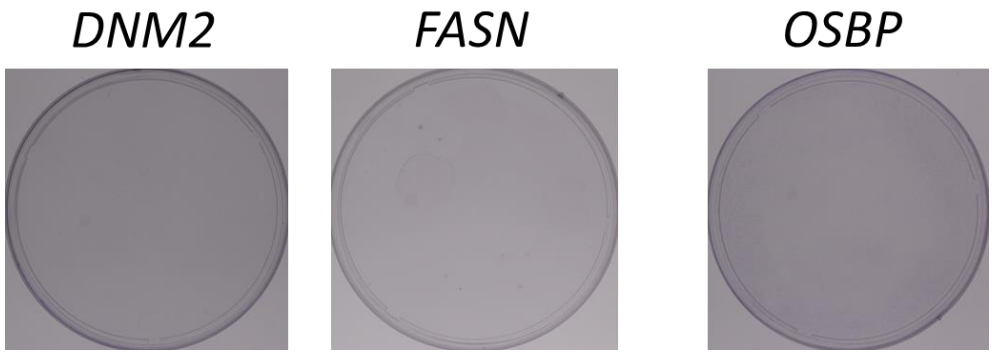

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Figure 7. No CVB3-resistant colonies were formed after transfection with sgRNAs targeting *DNM2*, *FASN*, and *OSBP* genes. CVB3 infection was performed after transfection with plasmids encoding Cas9 and sgRNAs targeting *DNM2*, *FASN*, and *OSBP*. No surviving colony was detected.

c. CSDE1 is required for CVB3 infection, especially in IRES dependent translation

I isolated a CSDE1 KO cell line for functional study and found that these cells were resistant to CVB3 infection at various M.O.I (**Fig. 8A**). Moreover, they did not permit viral replication, as shown by the CVB3 replicon assay expressing the luciferase reporter in place of the P1 structural region and an immunostaining assay (**Fig. 8B** and **Fig.9**). Western blot analysis also showed the absence of CSDE1 protein (**Fig. 8C**). CVB3 infection and replication was rescued when I transfected eGFP-CSDE1 plasmid into CSDE1 KO cells (**Fig. 8B, C**). These results suggest that CSDE1 is a key host factor for CVB3 infection. CSDE1 was previously known to be required for internal initiation of translation of the human rhinovirus internal ribosome entry site (IRES) (Anderson et al., 2007; Hunt et al., 1999). As human rhinovirus and CVB3 both belong to the enterovirus family and share similar genomic structure, I hypothesized that CSDE1 might be related to translation initiation directed by the CVB3 IRES as well. A dual luciferase reporter system, which encodes Firefly luciferase under the IRES sequence of CVB3 and constitutively expresses *Renilla* luciferase upstream of them, was used to confirm this hypothesis (Kang et al., 2015; Paek et al., 2008). CVB3 IRES sequences could not initiate translation in CSDE1-depleted cells as efficiently as in wild-type cells (**Fig. 8D**). To further investigate whether CSDE1 is required for IRES-dependent translation in other viruses, a similar dual luciferase reporter system for 4 different viruses, Poliovirus 1 (PV1), Enterovirus 71 (EV71), Hepatitis C virus (HCV), and Encephalomyocarditis virus (EMCV), was used (Kang et al., 2015; Paek et al., 2008).

Concerning the 4 IRES sequence origins, PV1 and EV71 belong to the same enterovirus family. IRES sequences from these could not initiate translation in CSDE1-depleted cells (**Fig. 8D**). In contrast, the other 2 IRES sequences (HCV and EMCV) could initiate high-level translation in both cells, indicating that CSDE1 is important factor for IRES-dependent translation especially in the human enterovirus family. These results suggest that CSDE1 could be a valuable target for development of broad-spectrum antivirals against diverse enteroviruses that cause numerous diseases in humans.

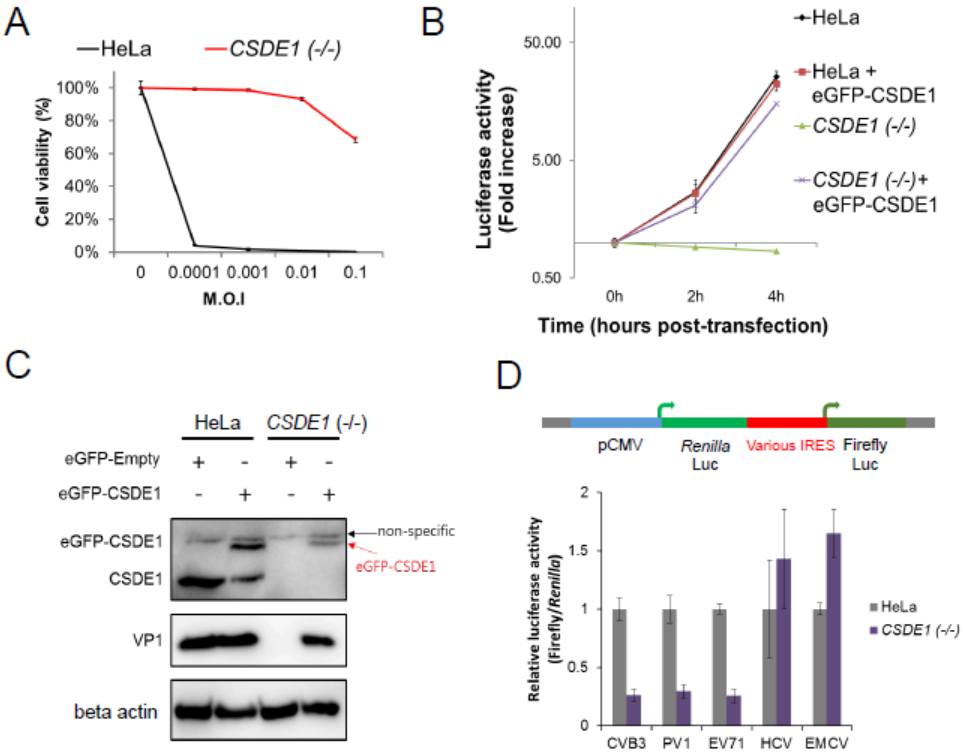

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Figure 8. CSDE1 is required for CVB3 infection. (*A*) Viability of wild-type (HeLa) and *CSDE1* Knockout (KO) cells after CVB3 infection. (*B*) Viral replication test of wild-type (HeLa) and *CSDE1* KO cells using the CVB3 replicon. Transfection with cDNA encoding the eGFP-CSDE1 protein into *CSDE1* KO cells rescued CVB3 replication. (*C*) Transfection with cDNA encoding the eGFP-CSDE1 protein into *CSDE1* KO cells rescued CVB3 infection. After 8 hours of CVB3 infection in wild-type (HeLa) and *CSDE1* KO cells transfected with the plasmid encoding the eGFP-CSDE1 protein, cells were harvested and lysed for western blot analysis using anti-CSDE1 antibody, anti-VP1 antibody, and anti-beta actin antibody. (*D*) CVB3 IRES-dependent translation assay of wild-type (HeLa) and *CSDE1* KO cells. Dual luciferase reporter plasmids containing the IRES sequence from 5 different viruses were transfected into cells and the activity of Firefly luciferase and *Renilla* luciferase was measured.

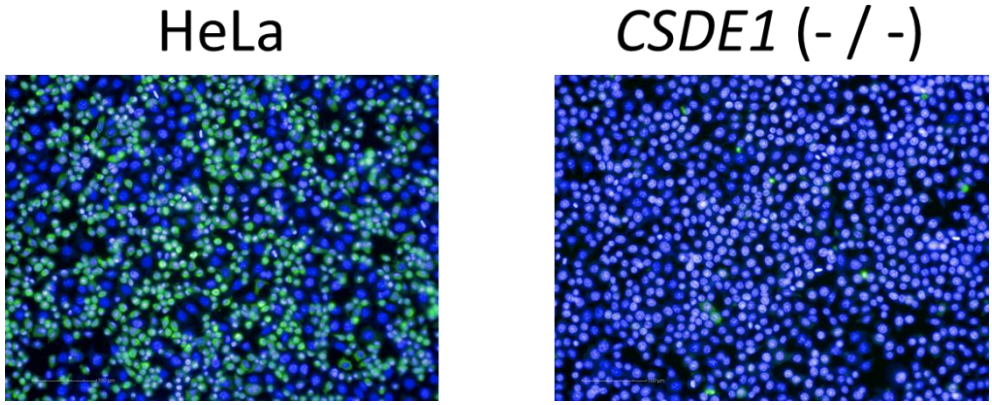

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Figure 9. Immunostaining of CVB3 infection in *CSDE1* KO cells. After CVB3 infection, cells were fixed and stained with an anti-3C antibody (green) and DAPI (blue).

d. ACBD3 is required for CVB3 infection but influences cell growth minimally

Another candidate, ACBD3, was also interesting because some groups have reported that ACBD3 is dispensable for PV and CVB3 replication through siRNA knockdown (Dorobantu et al., 2014; Teoule et al., 2013). In contrast, I could obtain CVB3-resistant cells after sgRNA transfection. To further investigate the role of ACBD3 in CVB3 infection, *ACBD3* KO clones were isolated. *ACBD3* knockout clones were resistant to CVB3 and blocked CVB3 replication completely, as shown by the CVB3 replicon assay (**Fig. 10A, B**). Western blotting and immunostaining assay also showed that viral protein expression was significantly inhibited in these cells (**Fig. 10C** and **Fig. 11**). Ectopic expression of FLAG-tagged ACBD3 protein rescued CVB infection, replication, and viral protein expression (**Fig. 10B, C** and **Fig. 12**). Sasaki et al. reported that the Aichi virus of the *Picornaviridae* family recruits ACBD3 and PI4KB to RNA replication sites and the viral protein 3A interacts with ACBD3 for recruitment(Sasaki et al., 2012). Lei et al. also reported that ACBD3 facilitates enterovirus 71 replication by interacting with the 3A protein(Lei et al., 2017). These reports, combined with my results, suggest that ACBD3 is broadly required for human enterovirus replication. To confirm the interaction between ACBD3 and the human enterovirus 3A protein, I conducted a simple luciferase assay with a mammalian two-hybrid system. Both 3A proteins from CVB3 and PV1 fused with the DNA-binding domain increased luciferase expression only in combination with the ACBD3 protein fused with the transcription activation domain (**Fig. 10D**). These results indicate that the CVB3 3A protein

interacts with ACBD3 and CVB3 uses ACBD3 as an essential host factor similar to that in Aichi virus or enterovirus 71. As ACBD3 is broadly required for enteroviruses infection, this protein might be valuable target for the development of broad spectrum anti-viral inhibitors. It is necessary that this host factor is essential for the virus but have a minimal effect on cell proliferation. To determine whether knocking out this protein could influence cell growth, I compared the growth of *ACBD3* KO clones with control HeLa cells (**Fig. 10E**). *ACBD3* KO clones showed growth patterns similar to those of HeLa cells. These results suggest that ACBD3 is required for CVB3 infection but minimally influences cellular proliferation.

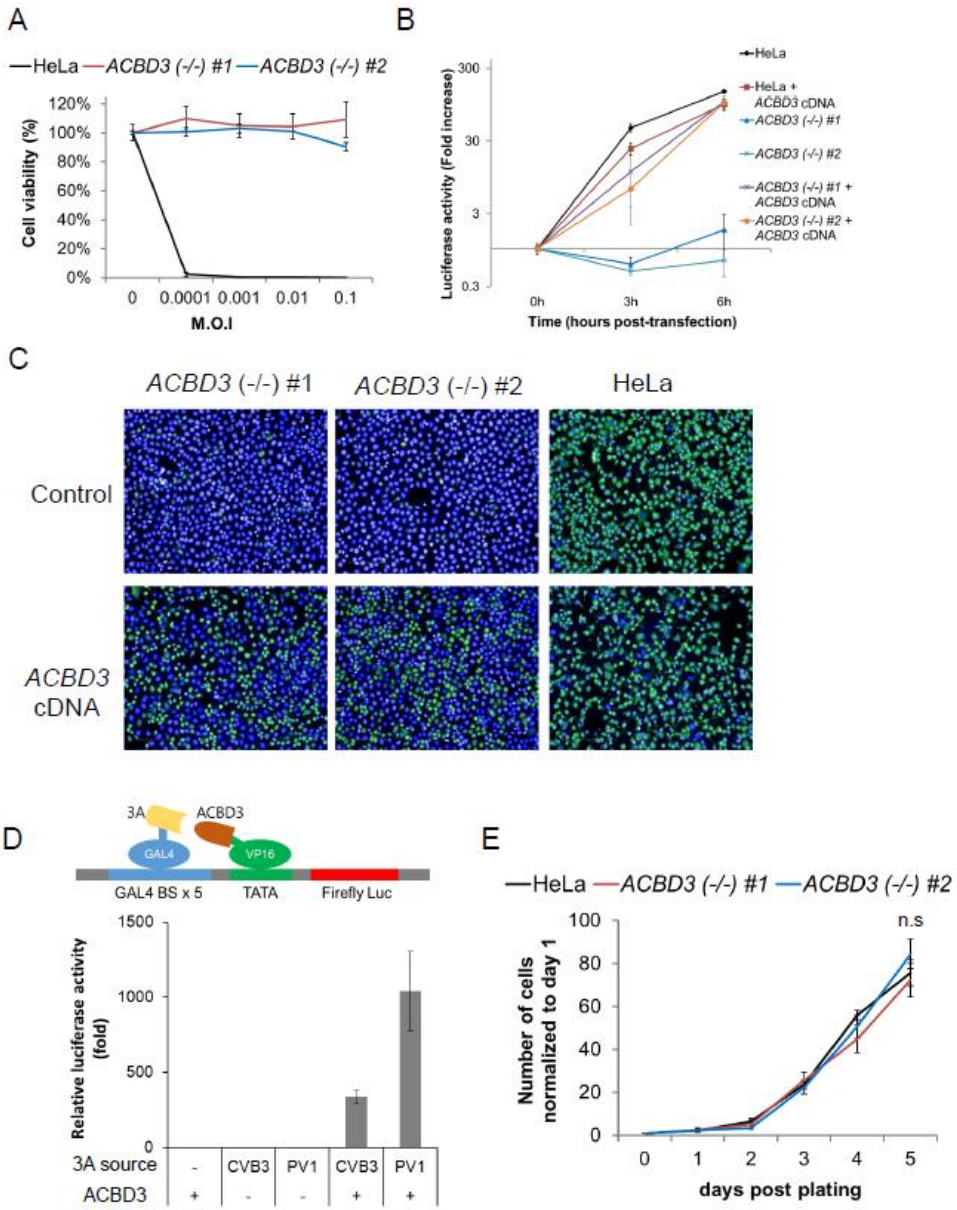

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Figure 10. ACBD3 is required for CVB3 replication. (*A*) Viability of wild-type (HeLa) and *ACBD3* Knockout (KO) cells after CVB3 infection. (*B*) Viral replication assay of wild-type (HeLa) and *ACBD3* KO cells using the CVB3 replicon. Transfection with cDNA encoding FLAG-tagged ACBD3 protein into *ACBD3* KO cells rescued CVB3 replication as shown by a viral replication assay using the CVB3 replicon. (*C*) Wild-type HeLa cells and *ACBD3* KO cells transfected with control and plasmid encoding FLAG-tagged ACBD3 protein were infected with CVB3 at an MOI of 5. At 8 hours post-infection, cells were fixed and stained with anti-3C antibody (green) and DAPI (blue). (*D*) Interaction between ACBD3 and the 3A protein of PV1 and CVB3 was confirmed using a mammalian two-hybrid system. (E) A fixed number of HeLa cells and KO cells were plated into 6-well plates and incubated. On each day, live cells were counted and normalized to the initial number of cells.

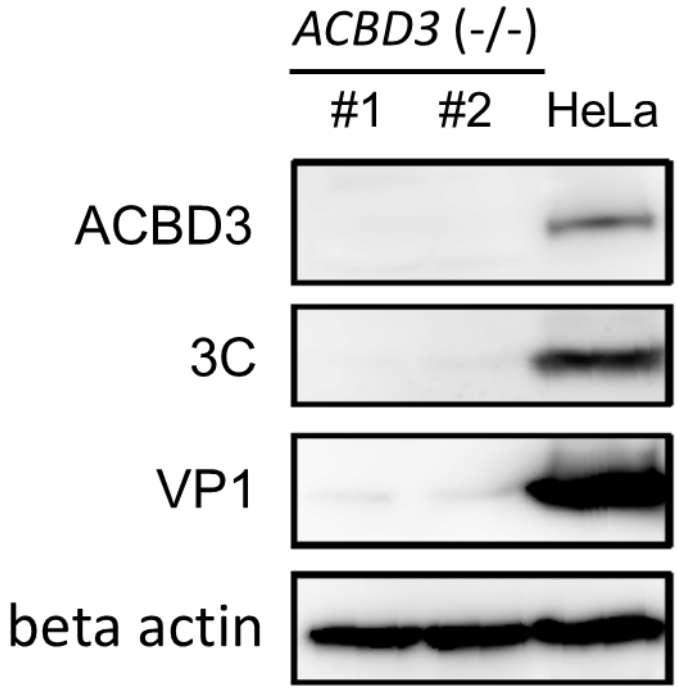

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Figure 11. Western blot analysis of the CVB3 infection in *ACBD3* knockout cells.

After CVB3 infection, cells were lysed for immunoblot analysis using an anti-ACBD3 antibody, anti-3C antibody, anti-VP1 antibody, and anti-beta actin antibody.

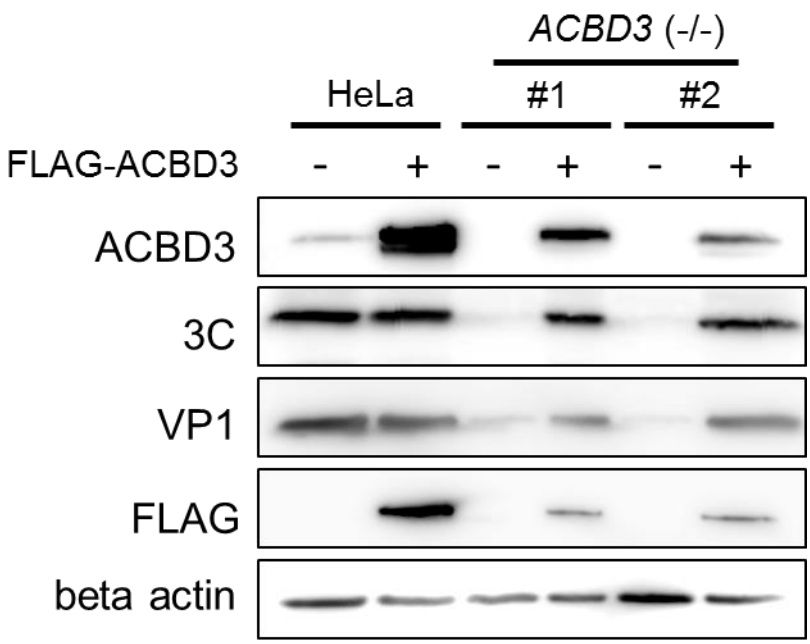

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Figure 12. Rescue of viral protein expression after transfection with a plasmid encoding FLAG-tagged ACBD3 protein in *ACBD3* knockout (KO) cells. Plasmid encoding FLAG-tagged ACBD3 protein was transfected into *ACBD3* KO cells and WT HeLa cells and the cells were then infected with CVB at 5 MOI. At 8 h post-infection, cells were harvested and lysed for immunoblot analysis using the anti-ACBD3 antibody, anti-3C antibody, anti-VP1 antibody, anti-FLAG antibody, and anti-beta-actin antibody. Expression of FLAG-tagged ACBD3 protein rescued the viral protein expression in the *ACBD3* KO cells.

3. Arrayed CRISPR screen reliably identifies host factors difficult to find using siRNAs

Since siRNA is the most widely used tool in arrayed screens, I tried to compare it with CRISPR. To do so, I performed CVB3 infection in cells transfected with siRNAs or sgRNAs targeting *CSDE1* and *ACBD3*. Both the knockdown by siRNAs and knockout by sgRNAs of CSDE1 showed reduction in CVB3 infection (**Fig. 13A**). However, knockdown of *ACBD3* could not restrict CVB3 infection in contrast to *ACBD3* knockout (**Fig. 13A**). Moreover, *ACBD3* knockdown failed to make HeLa cell resistant to infection and block viral replication (**Fig. 14**), even though knockdown efficiency of siRNA was high for both genes (**Fig. 13B, C**). This indicates that even a trace amount of ACBD3 protein could be sufficient for the virus, explaining why this gene was disputed in the previous studies using siRNAs. Another candidate, RACK1, also showed a pattern similar to ACBD3. Both siRNA and sgRNA against *RACK1* showed good reduction of protein levels (**Fig. 15**). CVB3 infection was significantly inhibited by sgRNA but was modestly inhibited by siRNA (**Fig. 15A**). This suggests that screening with sgRNAs could reduce the false-negatives induced by the incomplete knockdown events with siRNAs.

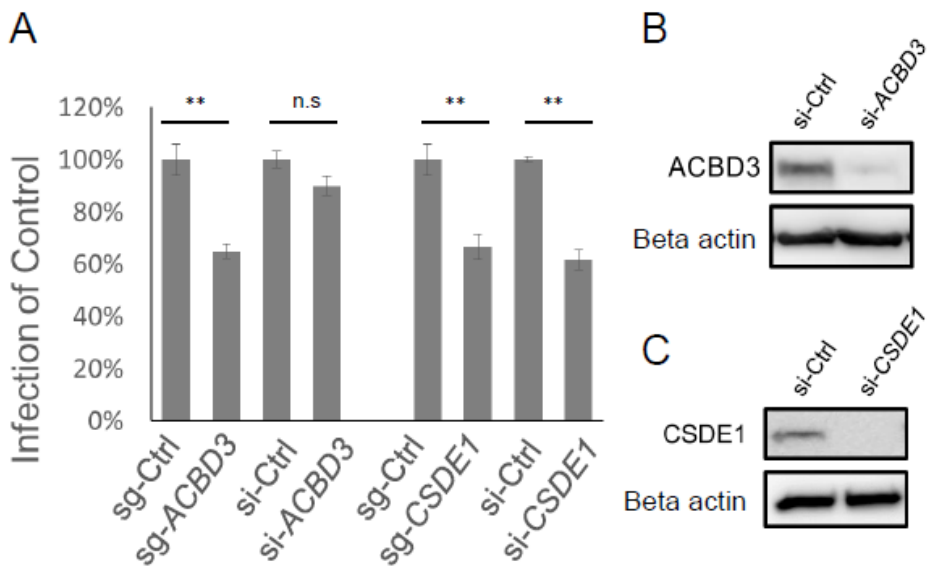

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Figure 13. HeLa cells were transiently transfected with siRNAs and sgRNAs targeting *ACBD3* or *CSDE1*. (*A*) Cells were infected with CVB3, fixed, and stained. The percentage of CVB3-infected cells (normalized to sgRNA or siRNA control). Mean ± s.e.m for triplicate experiments. ** $p < 0.001$, n.s; non-significant. (*B*) (*C*) Cells were transfected with control siRNA, *ACBD3* siRNA, and *CSDE1* siRNA. The expression level of each protein was measured by western blot using the anti-ACBD3 antibody (*B*) and anti-CSDE1 antibody (*C*).

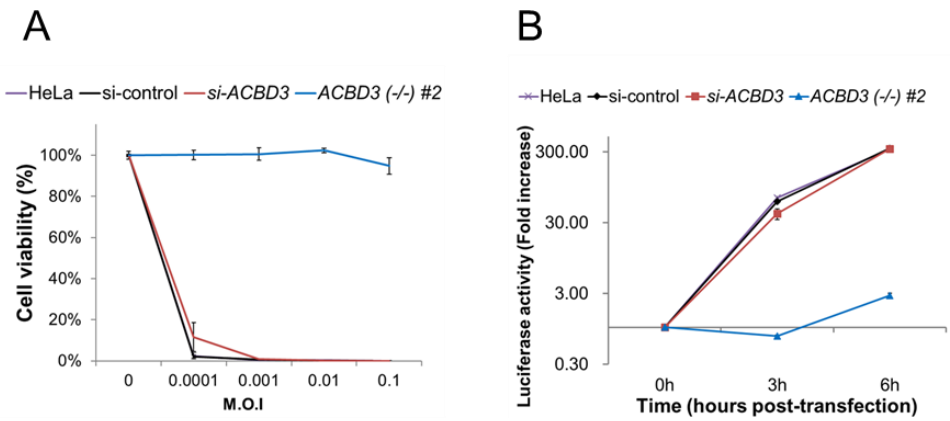

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Figure 14. siRNA targeting *ACBD3* does not inhibit host cell death and CVB3 replication. (A) Viability of cells transfected with control and *ACBD3* siRNAs after CVB3 infection. (B) Viral replication assay of cells transfected with control and *ACBD3* siRNAs using a CVB3 replicon.

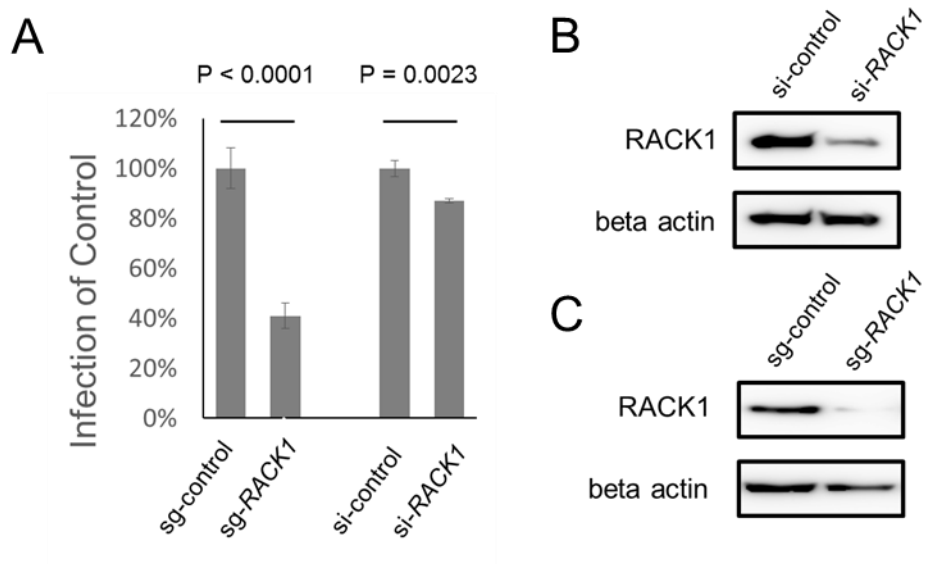

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Figure 15. *RACK1* sgRNA shows stronger inhibition of CVB3 infection. (A) HeLa cells were transiently transfected with siRNA and sgRNA targeting *RACK1*. Cells were infected with CVB3, fixed, and stained. Infection rate was shown as mean ± s.e.m for triplicate experiments. (B) (C) Cells were transiently transfected with *RACK1* siRNA or sgRNA and the expression level of RACK1 protein was measured by western blot analysis.

Discussion

In this study, I report a novel, high-throughput arrayed CRISPR screen method carried out with an image-based assay. Based on the arrayed plasmid library expressing sgRNA, I extended the library to cover 1,514 genes and successfully identified host factors required for CVB3 infection. Compared to pooled screens, arrayed screens can be more expensive and may require automation. However, with arrayed screens, higher hit rates and lower false positive/negative ratios are expected. Furthermore, arrayed screens can have broader screen phenotypes because each well has high individual perturbations compared to very low mixed perturbation in the pooled screen (< 0.01%). Notably, numerous arrayed siRNA screens have been performed in many labs and that automation facilities for siRNA screening have been developed in many research centers. This arrayed screens are compatible with these screening platforms. Currently, most CRIPSR screens are conducted in a pooled format, using the lentiviral system to identify viral host factors (Kim et al., 2017; Ma et al., 2015; Marceau et al., 2016; Park et al., 2017; Savidis et al., 2016; Zhang et al., 2016). Although these screens have been used successfully, only a few significant factors were selected, even though a genome-wide sgRNA library covering over 10,000 genes was used. In fact, in a previous pooled CRISPR screen that I performed, I was able to identify only a single host factor for enterovirus 68 in the initial screening (Kim et al., 2017). A strong effector in the pooled population could mask other genes that have milder effects. In contrast, I showed that genes with subtle effects or growth disadvantages, which make them difficult to be revealed in a pooled screen, can be identified using the present method when compared with the result of

pooled screen as shown in **Fig 1** and **Fig 5**. Viral infection is a complex process that involves diverse steps such as receptor binding, endocytosis, translation, replication, morphogenesis, and egress, and each step exploits a variety of host factors required for the process. In this screens, I could identify various host factors for CVB3, including entry-related factor, translational initiation factor of viral RNA, and several replication factors with different functions because many physically distinct cells are generated in each well, by the knockout of each gene. Furthermore, host factor genes such as *DNM2*, *FASN*, and *OSBP*, which are also important for cell proliferation, could be identified owing to the short assay time in contrast to pooled screens.

So far, arrayed screening formats have mostly relied on siRNA libraries. Arrayed siRNA screens and sgRNA screens were performed with some minor differences. siRNAs were spotted in a multi-well plate and reverse transfected into cells using liposomes. At 48 or 72 h post-transfection, cells were infected by virus and the viral infection was measured using various methods. For sgRNA screens, at 5 days after transfection, cells were infected by virus and the viral infection was measured using 3C-antibody staining and image acquisition with the Operetta system. Although those siRNA screens have advantages over pooled screens, disadvantages of siRNAs sometimes discourage their use in large scale screens. The biggest issue may be “low reproducibility” across screens, which is promoted by their off-target effects. Target mRNAs can repressed only by partial matching with the siRNA seed sequence, in contrast to the CRISPR system which requires a near perfect match, not only in the seed sequence. I analyzed off-target effects using sgRNAs specific to *ACBD3* and *CSDE1* with next generation sequencing: No off-

target mutations were detected at sites that differ by up to three nucleotides (**Fig. 16**). Here, this arrayed CRISPR screen identified 10 candidate genes of which 8 genes were confirmed by a secondary screens (**Fig. 3C**). This high rate (80%) of confirmation by secondary screens and only 2 false-positives among 1,514 genes suggests that this screen was reproducible and reliable and could be useful for large-scale screens. As shown in **Fig 13**, incomplete suppression of target genes can result in false-negatives during screening. ACBD3, which is essential even in a trace amount of expression, could not be revealed by the traditional siRNA screening, thus demonstrating the value of this screen.

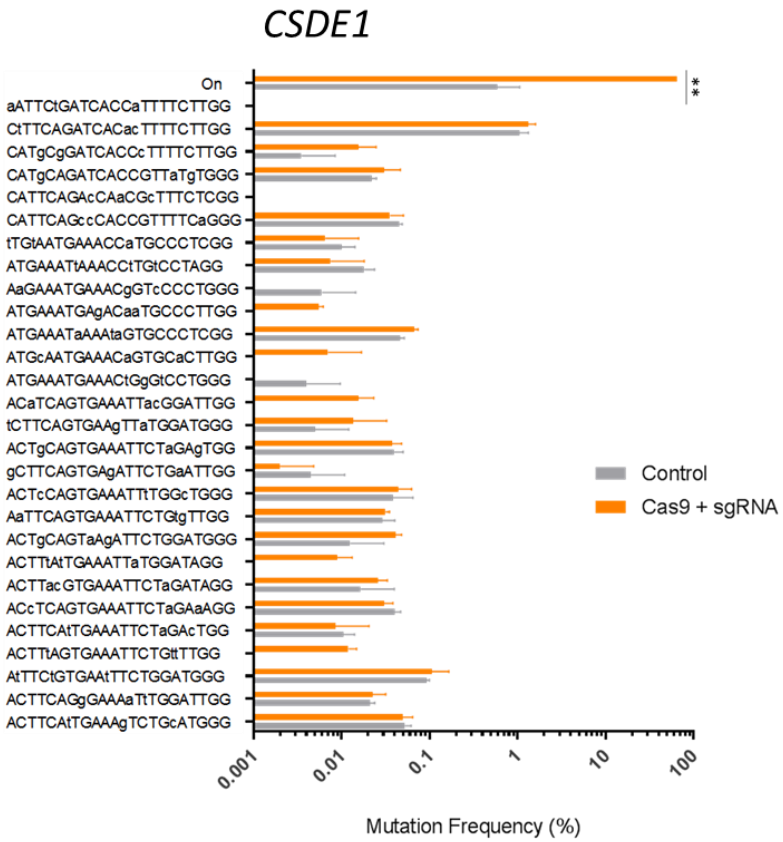
The arrayed CRISPR screen requires different considerations from those of pooled screens. I chose a plasmid-based library, expressing sgRNAs in multi-well plates. Compared with other libraries based on individual lentiviruses or synthetic crRNA (Metzakopian et al., 2017) including commercial sources, this plasmid-based library is scalable. This library was co-transfected with a plasmid expressing Cas9 using lipid-based reagents. As shown in **Fig 2C**, most genes were knocked out efficiently in HeLa cells within 5 days, demonstrating the strength of the plasmid based system. However, some cells are difficult to transfect using liposomes. These problems could be solved by electroporation, especially using electroporator equipped with multi-well modules, as reported by Hultquist et al (Hultquist et al., 2016). I validated the genes using original and newly designed sgRNAs. Moreover, I validated some candidate genes using known chemical inhibitors (**Fig. 3D**), analysis of protein expression levels by western blotting (**Fig. 8**), and rescue of the phenotype by cDNA expression (**Fig. 8** and **Fig.10**). Finally, I found clues to how

ACBD3 and CSDE1 affect CVB3 infection.

I identified two host factors, CSDE1 and ACBD3, which are crucial for viral infection. Unlike *DNM2*, *FASN*, and *OSBP*, which are also crucial for cell proliferation, these knocked out cell could proliferate well to produce resistant colonies (**Fig. 3**) and completely block viral infection (**Fig. 8** and **Fig.10**). I found that three viruses belonging to the human enteroviruses require CSDE1 for RNA translation. Other reports have described that human rhinovirus also uses CSDE1 for IRES-dependent translation (Boussadia et al., 2003; Hunt et al., 1999). Staring et al. reported that CSDE1 was selected as one of the host factors for poliovirus using haploid genetic screen (Staring et al., 2017). These results suggest that CSDE1 is a universal factor for translation initiation in human enteroviruses. Stone et al. reported that morpholino oligomers targeting IRES inhibited multiple species of picornaviruses (Stone et al., 2008). Using a similar approach targeting the IRES region, which interacts with CSDE1, potential broad therapeutic inhibitors against diverse human enteroviruses could be developed. Concerning ACBD3, several reports suggested that human enteroviruses use this factor for replication. In this study, I added that ACBD3 minimally affects cellular proliferation even after complete knockout. Seven acyl-CoA-binding protein domain-containing proteins (ACBD) including ACBD3 have been identified (Fan et al., 2010). Only ACBD3 possesses the GOLD domain at its C-terminus and this domain mediates interaction with the Aichi virus 3A protein (McPhail et al., 2017). The 3A proteins of diverse enteroviruses interact with ACBD3 and inhibitors that block this interaction could be potential broad antiviral therapeutics. In summary, I demonstrate that these two

proteins could be interesting targets to develop anti-enteroviral therapeutics.

The two remaining host factors identified in this screen, ADCY8 and RACK1, could also be interesting targets for further study. Coyne et al. reported that several ADCY family proteins, ADCY1, ADCY4, ADCY6, and ADCY7 were required for CVB3 replication (Coyne et al., 2011). In this screen, I identified ADCY8 as a host factor for CVB3. This may be due to the fact that different cells were used for the screens or due to the difference in the efficacy of siRNA or sgRNA targeting the *ADCY* family. This suggests that the two screening methods could be complementary to each other. It has been reported that RACK1 controls IRES-mediated translation of viruses, deactivates IRF3, and limits type I interferon signaling (Long et al., 2014; Majzoub et al., 2014). It is possible that these two functions could be required for CVB3 infection. Determining the molecular details of RACK1 in CVB3 infection could also be intriguing for further study.


CSDE1
On
aATTCtGATCACCaTTTTCTTGG
CtTTCAGATCACac TTTTCTTGG
CATgCgGATCACCc TTTTCTTGG
CATgCAGATCACCGTTaTgTGGG
CATTCAGAcCAaCGcTTTCTCGG
CATTCAGccCACCGTTTTCaGGG
tTGtAATGAAACCaTGCCCTCGG
ATGAAATtAAACCtTGtCCTAGG
AaGAAATGAAACgGTcCCCTGGG
ATGAAATGAgACaaTGCCCTTGG
ATGAAATaAAAtaGTGCCCTCGG
ATGcAATGAAACaGTGCaCTTGG
ATGAAATGAAACtGgGtCCTGGG
ACaTCAGTGAAATTacGGATTGG
tCTTCAGTGAAgTTaTGGATGGG
ACTgCAGTGAAATTCTaGAgTGG
gCTTCAGTGAgATTCTGaATTGG
ACTcCAGTGAAATTtTGGcTGGG
AaTTCAGTGAAATTCTGtgTTGG
ACTgCAGTaAgATTCTGGATGGG
ACTTtAtTGAAATTaTGGATAGG
ACTTacGTGAAATTCTaGATAGG
ACcTCAGTGAAATTCTaGAaAGG
ACTTCAtTGAAATTCTaGAcTGG
ACTTtAGTGAAATTCTGttTTGG
AtTTCtGTGAAtTTCTGGATGGG
ACTTCAGgGAAAaTtTGGATTGG
ACTTCAtTGAAAgTCTGcATGGG
**
Control
Cas9 + sgRNA
0.001
0.01
0.1
1
10
100
Mutation Frequency (%)

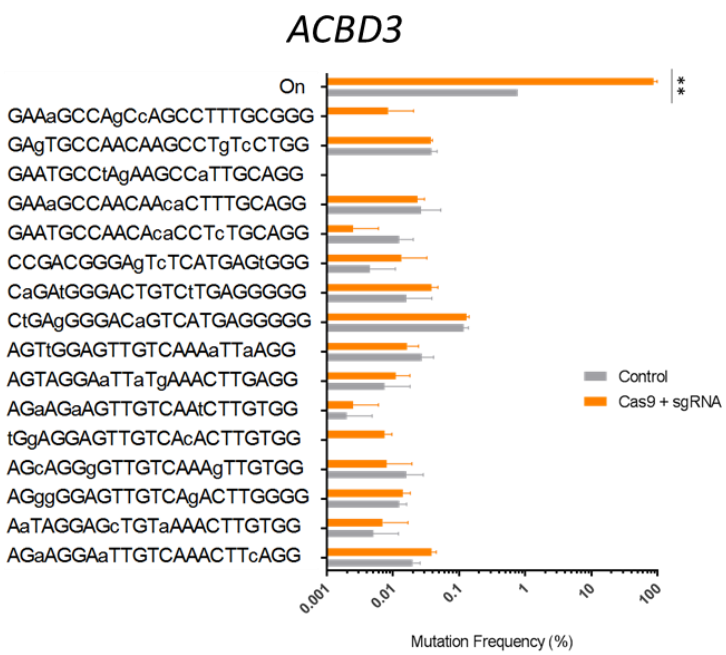

ACBD3
On
GAAaGCCAgCcAGCCTTTGCGGG
GAgTGCCAACAAGCCTgTcCTGG
GAATGCCtAgAAGCCaTTGCAGG
GAAaGCCAACAAcaCTTTGCAGG
GAATGCCAACAcaCCTcTGCAGG
CCGACGGGAgTcTCATGAGtGGG
CaGAtGGGACTGTCtTGAGGGGG
CtGAgGGGACaGTCATGAGGGGG
AGTtGGAGTTGTCAAAaTTaAGG
AGTAGGAaTTaTgAAACTTGAGG
AGaAGaAGTTGTCAAtCTTGTGG
tGgAGGAGTTGTCAcACTTGTGG
AGcAGGgGTTGTCAAAgTTGTGG
AGggGGAGTTGTCAgACTTGGGG
AaTAGGAGcTGTaAAACTTGTGG
AGaAGGAaTTGTCAAACTTcAGG
**
Control
Cas9 + sgRNA
0.001
0.01
0.1
1
10
100
Mutation Frequency (%)

Figure 16. Indel frequencies at *ACBD3* and *CSDE1* on-target and potential off-target sites in HeLa cells. Indel frequencies were detected by NGS. Value under 0.001% is not indicated on the graph. The values are the mean ± S.D for duplicate experiments. ** $P < 0.001$.

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국문 초록

인간 게놈 프로젝트가 완성된 이후, 인간의 유전체 정보에 대한 접근성이 매우 높아졌다. 그를 기반으로, 각 유전자의 기능을 밝히는 기능 유전체학의 중요성이 대두되었다. 기능 유전체학을 위해서는, 유전자의 발현을 조절할 수 있는 도구가 필수적이다. 특히 한번에 여러 개의 유전자의 발현을 조절하고, 원하는 표현형을 가진 세포의 유전자를 분석하는 순유전학의 경우, 다양한 조절을 쉽게 할 수 있는 유전자 조절 도구가 필수적이다. 결과적으로, CRISPR-Cas9 등의 유전자가위 기술이 기능 유전체학의 핵심적인 도구로 주목받기 시작하였다. RNA 간섭 기술 또한, 기능 유전체학에서 많이 사용되어 왔지만, 비특이적인 유전자 발현 억제 현상과, 완전한 유전자 발현 억제가 불가능하다는 점에서 많은 단점을 가지고 있다. 이런 이유로, CRISPR-Cas9 기술이 기능 유전체학에서, RNA 간섭의 훌륭한 대체제가 될 것이다.

이번 연구에서는, CRISPR-Cas9을 이용해 개발된 두가지의 순유전학 기술에 대해 소개하겠다. 1장에서는, 30,840개의 유전자 가위와 전장유전체분석 을 이용한 새로운 유전자 스크리닝 방식을 통해 바이러스 감염 관련 유전자를 발굴한 연구를 소개하겠다. 기존의 렌티바이러스 방법과 비교해서 위 방법은 빠르고, 바이러스가 포함되지 않으며, 실제로 도입된 돌연변이를 확인할 수 있다.

2장에서는, 1,514개의 바이러스 감염 관련 유전자를 표적하는, 개별로 배열된 4,542개의 유전자가위를 이용한 스크리닝을 소개하겠다. 한 홈 (well) 의 세포에 하나의 유전자를 조절하고, 바이러스 감염률을 이미지를 통해 관측함으로써, 새로운 바이러스 감염 관련 유전자를 발굴할 수 있었다. 또한 전체 스크리닝에서는 발굴할 수 없는 유전자를 찾아냄으로써, 개별 유전자 스크리닝의 높은 민감도를 보일 수 있었다.

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