Japanese encephalitis virus (JEV) is a mosquito-borne zoonotic flavivirus that causes encephalitis and reproductive disorders in mammalian species. However, the host factors critical for its entry, replication, and assembly are poorly understood. Here, we design a porcine genome-scale CRISPR/Cas9 knockout (PigGeCKO) library containing 85,674 single guide RNAs targeting 17,743 protein-coding genes, 11,053 long ncRNAs, and 551 microRNAs. Subsequently, we use the PigGeCKO library to identify key host factors facilitating JEV infection in porcine cells. Several previously unreported genes required for JEV infection are highly enriched post-JEV selection. We conduct follow-up studies to verify the dependency of JEV on these genes, and identify functional contributions for six of the many candidate JEV-related host genes, including EMC3 and CALR. Additionally, we identify that four genes associated with heparan sulfate proteoglycans (HSPGs) metabolism, specifically those responsible for HSPGs sulfurylation, facilitate JEV entry into porcine cells. Thus, beyond our development of the largest CRISPR-based functional genomic screening platform for pig research to date, this study identifies multiple potentially vulnerable targets for the development of medical and breeding technologies to treat and prevent diseases caused by JEV.
Numerous viruses in nature are capable of infecting both humans and domestic animals. Among these is Japanese encephalitis virus (JEV), a flavivirus which is closely related to Dengue Virus (DENV), Zika Virus (ZIKV), Yellow Fever Virus (YFV), West Nile Virus (WNV), and Hepatitis C Virus (HCV)\(^2\). JEV is the leading cause of viral encephalitis in humans in some Asian countries, with an estimated 60,000 to 6,000,000 clinical cases and ~15,000–20,400 deaths annually\(^3\), despite the widespread use of vaccine. While several inactivated and live vaccines are under development to prevent JEV infection, no antiviral drugs are available for the treatment of JEV-related diseases\(^4,5\). Despite achievements in control and prevention of JEV infection, this disease remains a major public health concern in Northern Europe and in South, East, and Southeast Asia, and it’s viewed as an emerging global pathogen\(^6,7\).

JEV is a mosquito-borne virus that can be greatly amplified in pigs, causing encephalitis and reproductive complications in swine species. Pigs are readily infected with JEV and can develop high levels of viremia\(^8\). JEV infection is usually asymptomatic in adult pigs, but manifestations of abortion, stillbirth, and birth defects, including central nervous system defects, are not unusual following infection in pregnant swine, which can result in substantial economic losses to pork producers\(^9,10\). Accordingly, JEV outbreaks represent a major threat to both public health and the agricultural economy, especially in areas with low vaccine coverage and/or limited diagnostic capacity\(^11\). Currently, JEV is only endemic in the Asia-Pacific region; however, it was previously shown that domesticated pigs could potentially become amplification hosts upon the introduction of JEV in other countries\(^12\).

The JEV infection cycle starts with binding to unknown cellular receptors and attachment factors\(^13\), followed by viral entry to enable replication. Subsequently, the JEV RNA genome is replicated, viral particles are matured, packaged, and released from cells. JEV infects a variety of cell types from diverse species (including mammals, birds, amphibians, and insects), suggesting that JEV can likely access multiple cell types using multiple host receptors\(^14\). In recent years, tremendous progress has been made in understanding the viral components required for the various steps of JEV entry and replication, but little is known about the host cell components involved in this process\(^15-18\). Understanding virus–host interactions by elucidating the molecular mechanisms of viral transmission can help identify potential antiviral targets for developing both prophylactic and therapeutic medicines.

Efforts to treat and prevent viral infections have traditionally been aided by genetic screening research to improve our understanding of viral dependencies and to identify potential antiviral strategies. The emergence of CRISPR genetic screening tools has spurred a new era of efficient, versatile, and large-scale screening efforts, with notable examples for flaviviridae family viruses\(^19-21\). Indeed, work in human cell lines based on CRISPR-based screening strategies (with virus-induced cell death readout phenotypes) have successfully identified required host genes for infection by DENV, ZIKV, WNV, YFV, and HCV\(^20-24\). These studies have repeatedly illustrated that genome-scale CRISPR screening represents a powerful tool for both basic biology and medical research. However, we are unaware of any genome-scale efforts to examine JEV infection; moreover, there are no reports of genome-scale CRISPR/Cas9 libraries for screening studies in pigs.

Aiming to develop such a resource, and specifically seeking to study the genetic basis of resistance against JEV infection, we develop a resource which we term PigGeCKO (for porcine genome-scale CRISPR knockout), which is comprised of a library of ~85,000 sgRNA constructs (both as plasmids and as prepared lentiviruses). After developing this genome-scale sgRNA library, we infect JEV-susceptible porcine kidney-15 (PK-15) cells\(^25\) stably expressing the Cas9 protein with the pooled lentiviral sgRNA library, and use Fluorescence-Activated Cell Sorting (FACS) to isolate and enrich cells harboring sgRNA constructs. We then perform positive selection screening by exposing the PigGeCKO cell collection to repeat rounds of JEV challenge, retaining the viable (i.e., JEV-resistant) cells from each round. PCR amplification and deep sequencing enable us to detect enrichment among the candidate JEV-infection-associated genes for annotations relating to HSPGs and endoplasmic reticulum-associated protein degradation (ERAD) pathways. We then generate gene knockout (KO) and knockdown cell lines for six of the candidate genes, and successfully confirm their requirement for JEV infection in porcine cells. These newly discovered host genes are potential targets for the development of therapies for the treatment of Japanese encephalitis and porcine diseases caused by JEV, and can also be used in the construction of genetically edited disease-resistant animal models. We anticipate that our benchmark-setting CRISPR/Cas9 screening resources will greatly facilitate basic and applied functional genomics research in pigs.

**Results**

**Strategy for identifying genes essential for JEV-induced cell death in pigs.** The overall development process of the PigGeCKO resources is depicted in the schematic diagram in Fig. 1a, and proceeded as follows. We initially used CRISPR-offinder (v1.2)\(^26\) to design 85,674 specific and predicted high-efficiency single guide RNAs (sgRNAs), that collectively targeted 17,743 protein-coding genes, 11,053 long ncRNAs (lncRNAs), and 551 micro-RNAs (miRNAs) in the porcine genome, as well as 1000 negative control sgRNA constructs predicted not to target any porcine genome loci (Fig. 1b, Supplementary Data 1). Three sgRNA constructs were designed for each targeted locus, all loci which met the selection criteria detailed in the Methods were targeted. These designed sgRNA constructs were synthesized as an oligo array, which was employed as the template for PCR amplification of the sgRNA oligos that were subsequently cloned into lentiviral vectors using Gibson assembly.

To test the quality of the PigGeCKO plasmid library, we amplified the cloned sgRNA constructs using PCR, performed deep sequencing, and found that 96.2% (82,426/85,674) of the initially designed and synthesized sgRNA sequences were present in the plasmid library (Fig. 1c, Supplementary Data 2). Although a small fraction of sgRNAs were under- or over-represented, ~90% of the sgRNAs were within a range covering a tenfold difference in frequency (Fig. 1c). In parallel with our sgRNA library preparation, we developed a PK-15 cell line that stably expressed high levels of Cas9 (PK-15-Cas9, Clone-#14, Supplementary Fig. 1). We used an sgRNA lentivirus targeting the randomly selected ANPEP gene to assess the capacity of this cell line for gene editing (Supplementary Fig. 2a), and found that gene-editing activity tended to be stable ~6–10 days post-infection of the sgRNA-harboring lentivirus in PK-15-Cas9 cells (Supplementary Fig. 2b). We then generated the PigGeCKO lentivirus library by transfecting HEK293T cells with the lentiviral sgRNA plasmids together with helper plasmids. To minimize the chance of inserting multiple sgRNAs into the same PK-15-Cas9 cells, we employed a low multiplicity of infection (MOI) to obtain a transduction rate of around 30% according to a previous study\(^27\). The lentivirus sgRNA library was subsequently transduced into PK-15-Cas9 cells. We performed FACS-based sorting on the signal from the green fluorescent protein (GFP) reporter, which was included in all PigGeCKO constructs. Then, infected cells were screened for the presence of sgRNA construct sequences by PCR analysis and deep sequencing.
Streptococcus pyogenes Cas9; Puro, puromycin; LncRNA, Long non-coding RNA; Designed, sgRNA designed by CRISPR-of promoter; EGFP, Enhanced green pools. sgRNA, small guide RNA; PCR, Polymerase chain reaction; hU6, human U6 promoter; Ubi, Ubiquitin promoter; CMV, Human cytomegalovirus cell populations (the distribution of sgRNAs.

To determine the optimal selection to identify required host genes JEV-induced cell death screening of the PigGeCKO cell collection (Fig. 1e). Finally, one of the originally designed sgRNA sequences (targeting the IZUMO3 gene) was randomly selected to evaluate potential off-target effects (Supplementary Fig. 3a). A T7EN I cleavage assay revealed no off-target cleavage for any of the predicted potential off-target sites (Supplementary Fig. 3b). Collectively, this work demonstrates the development of a highly active and specific PigGeCKO resource with high utility for functional genomics research in pigs.

**JEV-induced cell death screening of the PigGeCKO cell collection to identify required host genes.** We then developed a screening strategy, illustrated in Fig. 2a, to identify host genes required for successful JEV infection. To determine the optimal virus level for JEV-induced cell death in PK-15 cells for CRISPR screening, we examined JEV-induced cell death following infection at MOIs of 0, 0.01, 0.05, and 0.1. As the infection dose of JEV was increased, we observed cytopathic effects (CPE) at approximately days 4 post virus infection; phenotypes included the rounding up and enlargement of cells, the formation of syncytia, and the detachment of cells into the medium (Supplementary Fig. 4).

Having established that PK-15 cells are a suitable model for identifying host genes with functions relating to JEV infection, we undertook our screening to identify host genes that modulate susceptibility to JEV-induced cell death. The PigGeCKO cell collection was infected with JEV and incubated for 11 days to enable selection of cells resistant to JEV-induced killing. We conducted four rounds of JEV challenge, employing untreated PK-15-Cas9 cells as a negative control to confirm the cell death caused by JEV infection in each round (Fig. 2a). At an MOI of 0.03, all untreated JEV-infected PK-15-Cas9 cells died, whereas a small number of viable cells from the JEV-infected PigGeCKO...
cell collection were detected. Surviving cells were collected and used for subsequent JEV challenge rounds, and the sgRNA constructs in surviving cells were PCR amplified and deep sequenced to identify candidate genes.

Focusing on protein-coding genes, our screen found that a total of 2181 unique sgRNA sequences were present in at least ten cells in the third and fourth rounds of JEV challenge. Among the originally designed 52,928 sgRNAs targeting 17,743 protein-coding genes (Supplementary Data 3), after JEV challenge, only 280 of the sgRNA constructs were present in at least 1000 of the total population of analyzed cells (~0.5% of the total analyzed cells) (Supplementary Data 3). The top ten most enriched genes included SLC35B2, PRKCSH, SMOX, RNF145, DMGDH, SEC63, EXT2, CALR, B3GAT3, and GSTO2.
candidate genes after the third JEV challenge round were (highest-to-lowest) SEC63, PRKCSH, SLC35B2, RNF145, SMOX, CALR, DMGDH, EXT2, B3GAT3, and GSTO2 (Fig. 2b): the fourth challenge round identified the same enriched genes with the exception of EXT1 and GSTO2 (Fig. 2c). When taking into consideration the design of three sgRNAs constructs for each targeted locus of the porcine genome, we found that two sgRNAs for each of the SEC63 and B3GAT3 genes were among the most highly enriched sequences after the third rounds of JEV challenge (Fig. 2b, c).

After the third JEV challenge round, there were 57 sgRNA constructs present in at least 10,000 cells, and 219 sgRNA constructs present in at least 1000 cells; after the fourth JEV challenge round, these numbers were 57 and 239 respectively (Supplementary Data 3). Comparison of enriched sgRNAs from the positive selection CRISPR screening revealed that 86.3% of the very highly enriched (i.e., ≥10,000) sequences were common to both the third and fourth challenge rounds, and that 89.9% of the highly enriched (≥1000) sequences were common to both rounds (Fig. 2d, e). These results highlight the capacity for CRISPR-based positive selection screening to consistently identify strong candidate genes. To explore the predicted biological functions of the candidate JEV-resistance genes, we performed KEGG pathway enrichment analyses for top 0.5% ranked sgRNA targets from the third and fourth JEV challenge rounds. These analyses revealed that the candidate JEV-resistance genes were significantly enriched for HSPGs metabolism and for Golgi and endoplasmic reticulum (ER) functions (Fig. 2f). Among these genes, B3GAT3 and EXT1 have known roles in JEV replication.28,29 We also found a large difference in the abundance of metabolic pathway hits between the third and fourth rounds of viral challenge. We speculate that cell growth may be adversely affected, and cell death may occur more frequently following knockout of genes involved in metabolism, although further study is warranted to verify this possibility. These results indicate that key host factors involved in JEV replication can be identified through multiple rounds of CRISPR screening.

Knockout of HSPGs pathway-related genes significantly inhibits JEV entry. There are conflicting findings from previous studies of possible functional roles for heparan sulfate pathway proteins as cellular attachment factors during initiation of JEV infection.29,30 HSPGs encompass a diverse class of proteins defined by the substitution with heparan sulfate glycosaminoglycan (GAG) polysaccharide chains.31,32 Our genome-scale CRISPR screen for JEV-infection related genes indicated that 10 genes associated with HSPGs metabolism were among the most highly enriched sgRNA targeted genes: EXT1, EXT2, GLCE, HS6ST1, B3GAT3, B4GALT7, XYLTL7, EXT3, SLC35B2, and GAA (Fig. 3a). Among these genes, SLC35B2, EXT1, and EXT2 were ranked in top 10 from both the third and fourth JEV challenge rounds. Notably, the EXT1 and HS6ST1 genes were each targeted by three separate sgRNA constructs, all of which were highly enriched, clearly indicating potential JEV-infection-related functions (Fig. 3b). HSPGs synthesis and sulfation is driven by >20 different genes;28 as shown in Fig. 3c, the significant enrichment of specific sgRNAs identified seven genes potentially involved in HSPGs synthesis and metabolic pathways, and two genes potentially involved in sulfuration modifications of HSPGs in porcine cells (Fig. 3d).

Building from these initial candidate hits, we first generated KO cells of SLC35B2, HS6ST1, B3GAT3, and GLCE using the CRISPR/Cas9 editing system. Before validating the specific hits, initial evaluations showed that the integrated expression of a randomly selected scrambled sequence negative control sgRNA did not affect the normal replication of JEV (Supplementary Fig. 5). Subsequently, we found that individual knockouts of SLC35B2, HS6ST1, B3GAT3, and GLCE exhibited inhibition of cell death induced by JEV infection at an MOI of 0.03, although the suppression of cell death was not complete at high-dose JEV challenges (MOI = 1) (Supplementary Fig. 6a and b). Sanger sequencing confirmed that each of these KO cell lines had one or more nucleotide deletions predicted to cause a frameshift mutation in the coding regions of the targeted gene (a non-integer multiple of 3) (Fig. 4a, Supplementary Fig. 7a). Moreover, the results of Edu fluorescence assays showed no difference in the rates of cell proliferation between corresponding KO and wild-type (WT) PK-15 cells (Supplementary Fig. 7b).

Then, viral loads in JEV-infected SLC35B2, HS6ST1, B3GAT3, and GLCE KO cells and in WT PK-15 cells were measured at 18 hpi (hours post-infection) by plaque assay, which only using JEV at an MOI of 0.03 or 0.1 (Fig. 4b). In agreement with the reduced viral loads observed in KO cells, the results from the immunofluorescence assays showed that the expression of the JEV-encoded NS3 protein in all four KO cell lines was modestly reduced or undetectable following JEV infection at both MOIs of 0.03 and 0.1 (Supplementary Fig. 8). Next, cell cultures were sampled at 18 hpi for quantification of JEV genome copy number based on absolute quantitative PCR analysis targeting the C gene of JEV. These analyses revealed that knockout of these four HSPGs-related genes significantly inhibited JEV replication (12 hpi) (Fig. 4c). Importantly, use of an antibody against Heparin/Heparan Sulfate antibody (10E4) to conduct immunofluorescence assays revealed that knockout of the SLC35B2 and HS6ST1 genes resulted in a significant reduction in the HSPGs sulfuration level compared to WT cells (Fig. 4d). This observation clearly suggests that sulfuration modifications of HSPGs can significantly and functionally impact the interaction between JEV and HSPGs in PK-15 cells, potentially during viral entry.

To exclude the possibility of other defects that may result in the inhibition of JEV infection in these KO cells, we performed rescue experiments to demonstrate complementation by the intact genes. Surprisingly, we found that knockout of SLC35B3 or B3GAT3 resulted in complete inhibition plasmid delivery using lipofection transfection reagent, thus preventing ectopic expression of SLC35B3 and B3GAT3 in corresponding KO cells (Supplementary Fig. 9). Thus, the other two genes, HS6ST1 and GLCE were selected for further study. We found that the ectopic expression of HS6ST1 or GLCE in corresponding KO cells by rescue assays resulted in partial recovery of JEV replication (Fig. 4e–h,
Supplementary Fig. 10). As shown in Fig. 4e, the proportion of NS3-positive cells was significantly increased during restoration of HS6ST1 expression in HS6ST1-deficient cells. And the proportion of NS3-positive cells was also significantly increased during restoration of GLCE expression in GLCE-deficient cells (Fig. 4g). Further analysis revealed that the knockout of the EMC3 gene significantly inhibited JEV replication in PK-15 or ST cells (Supplementary Figs. 12–14). These results were consistent with CRISPR-mediated knockout of HSPGs pathway candidate genes, thus further confirming that JEV replication requires a complete HSPGs pathway. Collectively, these results indicate that HSPGs can only act as a cellular adhesion factor or cofactor that mediates JEV entry.

**EMC3 is required for JEV replication.** The endoplasmic reticulum membrane complex (EMC) is known to be required for infection by flaviviruses, which have RNA genomes. However, it is unclear whether EMC family genes are involved in JEV replication. Interestingly, our genome-scale CRISPR JEV-infection screen showed that EMC3 and EMC6 genes, both of which encode ER membrane protein complex subunits, were ranked 44 and 34 among the candidate hits in the fourth JEV challenge round, respectively (Supplementary Data 3). A previous study identified that knockout of the EMC6 gene led to significant inhibition of JEV replication in human HEK293T cells, which was consistent with our findings in pig. As such, we only generated two independent EMC3 null cell lines using CRISPR/Cas9. Sanger sequencing confirmed the presence of 1 or 2 bp deletions or insertions (a non-integer multiple of 3) in both EMC3 KO cell lines (Fig. 5a, Supplementary Fig. 15a), and immunoblotting confirmed that the EMC3 protein was not expressed in cells of either KO line (Fig. 5b). Moreover, the results of EdU fluorescence assays showed no difference in the rates of cell proliferation between EMC3 KO and WT PK-15 cells (Supplementary Fig. 15b).

Subsequently, viral concentrations in JEV-infected EMC3 null and WT PK-15 cells were determined at 12, 24, and 36 hpi by both plaque assay and quantification of JEV genome copy number based on absolute quantitative PCR analysis using a pair of primers targeting the C gene of JEV. Together, these analyses revealed that knockout of the EMC3 gene significantly inhibited JEV replication (12 hpi) at an MOI of 1 (Fig. 5c, d). Both JEV-infected EMC3 KO cell lines possessed substantially reduced levels of viral NS3 protein expression as determined via immunofluorescence analysis (Fig. 5e) and immunoblotting (Fig. 5f). Next, the ability of EMC3-deficient cells to resist

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**Fig. 3 Significant enrichment of specific sgRNAs targeting 10 genes involved in HSPGs synthesis and metabolic pathways.** a Venn diagram showing the overlapping enrichment of specific sgRNA targeted genes identified in the JEV screen of the cell knockout collection, HSPGs biosynthesis and metabolism, and GAG metabolism pathways. b Enrichment of specific sgRNAs targeting 10 genes involved in HSPGs synthesis and metabolic pathways. Only the top 0.5% of sgRNA was counted. c Schematic diagram, adapted from Tanaka et al., showing the various classes of chemical components comprising HSPGs with known enzymes for their biosynthesis. Red indicates targeted genes identified in this study. d Schematic diagram, adapted from Blondel et al., showing the known components and localization information for the sulfurylation modifications known to occur for some HSPGs. Red indicates targeted genes identified in this study. JEV, Japanese encephalitis virus; HS, Heparan sulfate; GAG, Glycosaminoglycan; HSPGs, heparan sulfate proteoglycans; PAPS, 3’-Phosphoadenosine-5’-phosphosulfate; JEV3rd, the third challenge round of JEV screening; JEV4th, the fourth challenge round of JEV screening. Source data are provided as a Supplementary Data file.
Fig. 4 Knockout of genes coding for the HSPGs pathway proteins SLC35B2, HS6ST1, B3GAT3, and GLCE significantly inhibits JEV replication in PK-15 cells. a Alignment of the nucleic acid sequences of clonal KO cells of SLC35B2, HS6ST1, B3GAT3, and GLCE with WT cells. sgRNA targeting sites are highlighted in red. The red characters ‘‒’ indicate the deleted bases in the KO cells. PAM sites are indicated in blue letters. b Virus plaque assays for determination of viral concentration in clonal SLC35B2, HS6ST1, B3GAT3, and GLCE KO cell lines following infection with JEV at an MOI of 0.03 or 0.1. c Absolute quantitative real-time PCR for determination of JEV copy number in clonal SLC35B2, HS6ST1, B3GAT3, and GLCE KO cell lines following infection with JEV at an MOI of 0.03 or 0.1. d Detection of HSPGs sulfurylation level in clonal SLC35B2 and HS6ST1 KO cell lines by immunofluorescence. Scale bar, 200 μm. e, f Rescue assays for ectopic expression of HS6ST1 in HS6ST1-deficient cells. HS6ST1-KO-rescue: Transfection of pcDNA3.1-HS6ST1 vector in HS6ST1-deficient cells; HS6ST1-KO-NTC: Transfection of pcDNA3.1 empty vector in HS6ST1-deficient cells. g, h Rescue assays for ectopic expression of GLCE in GLCE-deficient cells. GLCE-KO-rescue: Transfection of pcDNA3.1-GLCE vector in GLCE-deficient cells; GLCE-KO-NTC: Transfection of pcDNA3.1 empty vector in GLCE-deficient cells. Detection of N3 by immunofluorescence microscopy, the original data came from Supplementary Fig. 10a (e) and Supplementary Fig. 10c (g), respectively. RT-qPCR assay (f, h) for determination of relative mRNA level of JEV C gene in rescue assays of HS6ST1 and GLCE, respectively. PAM, protospacer adjacent motif; JEV, Japanese encephalitis virus; MOI, multiplicities of infection; hpi, hours post-infection; KO, knockout; WT, wild-type; DAPI, 4′,6-diamidino-2-phenylindole. Data are represented as means ± S.D.; n = 3 (b, c, e, f, g, h). *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significant. P values were determined by two-sided Student’s t-test. Source data are provided as a Source Data file.
JEV-induced death at an MOI of 1 was evaluated. The result from cultures grown using a Real-Time Cell Analyzer assay verified that EMC3-deficient cells were able to completely resist JEV-induced PK-15 cell death (Fig. 5g). Subsequently, we found that rescue assays for ectopic expression of EMC3 in EMC3-deficient cells resulted in partial recovery of JEV replication (Fig. 5h–j, Supplementary Fig. 16a). As shown in Fig. 5j, the proportion of NS3-positive cells was significantly increased during restoration of EMC3 expression in EMC3-deficient cells. Further analysis revealed that knockdown of EMC3 significantly inhibited JEV replication in both PK-15 and ST cells (Supplementary Fig. 17, 18). In a final step, we further investigated the effects of EMC3 knockout on virus particle assembly in EMC3-deficient cells by negative-staining electron microscopy. In WT cells infected with JEV, virus particles were observed in the ER, but were not observed in the ER of EMC3-deficient cells after JEV infection (Fig. 5k). Compared with the WT cells, at 24 h (hrs) post-JEV infection, EMC3-deficient cells displayed dramatic changes in membrane morphology, potentially related to the observed suppression of JEV transcription and/or translation (Fig. 5k, Supplementary Fig. 19). Collectively, these results demonstrate that the EMC3 is required for JEV-induced PK-15 cell death.
CALR is required for JEV replication. Among the top 10 ranked genes in the genome-scale CRISPR screen for JEV-infection screening candidates was a gene known to function in intracellular calcium homeostasis: CALR. To explore the potential function of CALR in mediating JEV replication, CALR KO cells was generated by CRISPR/Cas9 technology. Sanger sequencing showed that the selected CALR-deficient cells have a 1 bp insertion (Fig. 6a, Supplementary Fig. 15a), and immunofluorescence confirmed that the CALR protein was not expressed in the KO cells (Fig. 6b). Moreover, the results of Edu fluorescence assays showed no difference in the rates of cell proliferation between CALR KO and WT PK-15 cells (Supplementary Fig. 15b).

Next, plaque assays and absolute quantitative real-time PCR were used to measure viral concentrations in JEV-infected CALR null and WT PK-15 cells at 12 and 24 hpi. Concurrently, JEV-infected cell cultures were harvested at 12, 24, 36, and 48 hpi, and viral RNAs were extracted from cell suspensions and cDNAs were synthesized as absolute quantitative PCR template. As shown in Fig. 6c, d, knockout of the CALR gene significantly inhibited JEV replication at an MOI of 1. Moreover, immunofluorescence results showed that the NS3 protein was only weakly expressed at 12 and 24 hpi in JEV-infected CALR null cell lines (Fig. 6c). Next, the ability of CALR-deficient cells to resist JEV-induced death at an MOI of 1 was evaluated. Results of the cell proliferation assay and Real-Time Cell Analyzer assay showed that, compared to WT cells, knockout of CALR can also confer resistance to JEV-induced death (Fig. 6f). Subsequently, we found that rescue assays for ectopic expression of CALR in CALR-deficient cells resulted in partial recovery of JEV replication (Fig. 6g-i, Supplementary Fig. 16b). As shown in Fig. 6h, the proportion of NS3-positive cells was significantly increased during restoration of CALR expression in CALR-deficient cells. Further analysis revealed that knockdown of CALR significantly inhibited JEV replication in PK-15 or ST cells (Supplementary Figs. 17, 18). We also further investigated the effects of CALR knockout on virus particle assembly in CALR null cells by negative-staining electron microscopy. In WT PK-15 cells infected with JEV, virus particles were observed in the mitochondria, but not in the mitochondria of CALR-deficient cells after JEV infection (Fig. 6i). Furthermore, these results showed that JEV infection leads to diminished mitochondrial cristae in WT cells, but which are preserved by CALR-deficient cells (Fig. 6j, Supplementary Fig. 20). These results indicate that CALR is required for JEV replication.

Discussion

Our results highlight the power of CRISPR/Cas9-based screening for functional analyses in pigs, and we present in Fig. 7 a preliminary proposed model for JEV entry and replication in porcine cells based on our findings. The CRISPR screens recovered JEV entry cofactor HSPGs-related host factors (SLC35B2, HS6ST1, EXT1, EXT2, GLCE, B3GAT3, B4GALT7, XLYT7, and EXT13), as well as multiple host factors involved in calcium homeostasis (CALR), and transmembrane protein processing and maturation (EMC3 and EMC6). In addition, a large number of other candidate host factors involved in JEV infection of host cells were identified with our CRISPR screen, warranting further investigation. Furthermore, to determine which genes were essential for cell growth and survival, we felt the best strategy to achieve this was to apply negative selection to CRISPR screening. In this study, we aimed to determine host factors that participate in JEV replication, which is a positive selection. Therefore, it was difficult to determine which of these genes are essential for cell growth. The functions of candidate genes were finally tested through knockout or knockdown assays, confirming that these genes identified by CRISPR screening were reliable.

While genome-scale CRISPR/Cas9 mutagenesis methods obviously facilitate gene functional studies in both cellular and animal models19–24, and the ability to programmably target the entire coding or regulatory genome represents a significant advance over spontaneous or random mutagenesis. Genome-scale CRISPR/Cas9 approaches share with classical forward genetic screens the requirement for an assay to enrich for cells exhibiting the phenotype of interest38–40. JEV causes encephalitis in humans and reproductive disorders in pigs, the latter leading to substantial economic losses10. In the present study, we developed a series of porcine genome-scale CRISPR/Cas9 knockout library resources that facilitate the pooled screening of genes that prevent JEV invasion or replication, thereby inhibiting JEV-induced cell death in porcine cells. Furthermore, we demonstrated how lentiviral delivery of a PigGeCKO library targeting 17,743 protein-coding genes enables positive selection screening for JEV-replication-associated host genes.

The first candidate genes examined from this screen are known to function in HSPGs metabolism, specifically in the synthesis and modification of heparan sulfate chains in normal cells. The murine homolog of EXT1 protein is known to be localized to the Golgi apparatus41, where it binds with EXT2 to form a complex known to modify heparan sulfate41. EXT1, EXT2, and EXT1L3 together contribute to heparan sulfate chain elongation42. In mice, GLCE is an epimerase enzyme required for the biosynthesis of HSPGs, which are composed of a core protein and one or more heparan sulfate glycosaminoglycans13. The B3GAT3 enzyme catalyzes the formation of glycosaminoglycan-protein linkages by glucuronic acid transfer, which is the final step in the biosynthesis of proteoglycan-linked regions28. A previous study identified that knocking out the B3GAT3 gene can significantly inhibit JEV replication28, a conclusion that was further confirmed by our genome-scale...
CRISPR screening and subsequent hypothesis-driven functional analyses. A different study showed that the reduced activity of the B4GALT7 enzyme is associated with a reduced substitution of the proteoglycans decorin and biglycan (both of which have glycosaminoglycan carbohydrate chains) and alterations in heparan sulfate biosynthesis. Our work strongly supports that HSPGs pathway genes can mediate JEV replication in porcine cells. Previous studies have shown that the SLC35B2 gene product is located in the microsomal membrane and functions to transport 3'-phosphoadenosine-5'-phosphosulfate (PAPS) from the cytosol (where it is synthesized) into the Golgi lumen, whereas the...
HS6ST1 sulfotransferase enzyme uses PAPS as a substrate for heparan sulfate biosynthesis. 45, 46 Accordingly, our immunofluorescence assays showed that SLC35B2 or HS6ST1 gene knockout in PK-15 cells significantly reduced the extent of sulfurylation modification to heparan sulfate. Therefore, we believe that sulfurylated HSPG serves as an adjunct or adhesion factor during JEV invasion of host cells. It should be noted that our initial screening efforts were based on relatively low MOI JEV challenges of PK-15 mutant cells. In CRISPR screening assays, we selected a relatively low JEV-infection dose in order to screen for as many candidate host factors for participation in JEV replication as possible. In some cases, knockout of genes from the HSPGs pathway only led to inhibition of relatively low dose JEV infections, but did not prevent replication of relatively high doses of JEV (Supplementary Fig. 6a and b). A previous study showed that some spatially adjacent residues within the JEV domain III are involved in heparin binding. 47 These results indicate that JEV and HSPG may interact with each other on the surface of host cells.

Fig. 7 A proposed model for JEV entry and replication in porcine cells. The JEV-infection cycle starts with binding to co-factors HSPGs, and/or unknown cellular receptors, followed by viral entry to enable replication. The EMC complex protein (EMC3 and EMC6) and CALR calcium-binding protein of the ER lumen are involved in JEV replication of host cells. Subsequently, the JEV RNA genome is replicated, viral particles are matured and packaged, and are released from cells. JEV, Japanese encephalitis virus; HSPG, heparan sulfate proteoglycan; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PAP, 3′-phosphoadenosine-5′-phosphate; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; APS, Adenosine 5′-phosphosulfate; PPI, pyrophosphate; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; EMC, endoplasmic reticulum membrane protein complex.
cells. In addition, it has already been known that highly sulfated forms of heparin sulfate can bind to the envelope protein and are involved in initial flavivirus attachment. Therefore, in combination with the results of our Real Time Cell Analysis (RTCA) assays (Supplementary Fig. 6a and b), we speculate that HS PGs may only serve as an adjunct or attachment factor for JEV entry. Thus, knocking out HSPGs pathway genes can only effectively inhibit cell death induced by low dose of JEV infection. However, for high-dose JEV infection, it is possible that JEV can directly enter host cells through other unknown pathways. Although these experiments succeeded in identifying many candidate host factors involved in JEV replication at a relative low dose, future CRISPR screening efforts employing a higher dose viral challenge would further confirm the key host factors that participate in JEV high-dose infections.

The second host cellular process implicated in JEV infection involves the EMC3 subunit of the ER membrane protein complex, which is involved in ER-mitochondrial membrane tethering and required to facilitate lipid transfer from the ER to the mitochondrial membrane, impacting nearly all aspects of cell physiology. Previous research has found that the ER membrane protein complex is a transmembrane domain insertase, thus, loss of EMC complex proteins cause ER stress and altered protein trafficking. For ZIKV, this complex was required for viral protein accumulation in a cell line harboring a ZIKV replicon. Our CRISPR screen showed that knockout of EMC3 and EMC6 can inhibit JEV-induced cell death. In particular, knockout or knockdown of the EMC3 gene significantly inhibited the replication of JEV in PK-15 or ST cells. EMC3 and EMC6 are associated with the ERAD pathway, which may participate in the secretory protein quality control processes that guide the removal of aberrantly folded proteins from the ER. Thus, whether the EMC subunits participate in JEV protein biogenesis, misfolding, or direct interaction with JEV particles or JEV-encoded proteins requires further study. For example, by using small molecule inhibitors of ER stress.

The final gene we examined via follow-up hypothesis-driven studies was CALR, which encodes a multifunctional soluble protein that can bind Ca\textsuperscript{2+} ions. Knockout of CALR resulted in a strong JEV-resistance phenotype. Proper folding in the ER is a prerequisite for the correct localization and function of most secreted transmembrane proteins. In addition, previous studies have found that CALR and calnexin (CAXX) chaperones mediate nascent glycoprotein folding in the ER. Thus, we hypothesize that CALR appears to be an essential gene that links JEV replication to downstream cell death pathways, possibly due to calcium homeostasis disequilibrium. For these reasons, CALR null cells represent a highly useful model for studying the relationship between calcium ion homeostasis and JEV infection. Additional studies in this area are currently ongoing.

While this study validated six genes selected with follow-up knockout studies and infection assays, our positive selection screening strategy yielded many candidates that may function in JEV infection and as such merit further investigation. We plan to extend the screening described herein to achieve full genome saturation to increase the search scope for host factor genes to further deepen our understanding of the multifaceted and spatiotemporally programmed interactions between JEV and host cells. To further verify that the required host factors we identified as necessary for JEV infection of PK-15 cells contribute the same roles in other cells, we also selected the JEV-susceptible ST cell line for RNAi-based knockdown assays. These experiments confirmed that individual knockdown of all six genes also led to significant inhibition of JEV replication in ST cells, thus suggesting that the functions of these genes are conserved across different types of porcine cells.

It is important to consider that some genes may be necessary for normal cell growth, so their knockout may inhibit cell proliferation, which would prevent detection of required genes. JEV is a RNA virus and therefore lacks an envelope, and previous studies in humans using CRISPR-based screening strategies have identified many required host genes for cell death induced by DENV, ZIKV, WNV, YFV, and HCV; it is notable that few of these genes appear to overlap. One on the other hand, the identified genes may differ owing to heterogeneities in the various cell types or species; on the other hand, perhaps the host factors for JEV infection simply differ greatly from other flavivirus family viruses. Our results highlight the complexity of JEV entry, replication, packaging, and release from host cells, yet also lead the way for a variety of hypothesis-driven basic biological and medical studies to deepen our understanding of this complex process.

**Methods**

**Plasmids construction.** The lenti-Cas9-Puro and lenti-sgRNA-EGFP vectors were kindly provided by Professor Xingfu Huang at ShanghaiTech University. The lenti-Cas9-Puro vector was used for generation of the Cas9-expression cell line (PK-15-Cas9). To construct the lentiviral sgRNA vector, paired oligonucleotides of sgRNA (50 μM per oligo) were annealed and cloned into lenti-sgRNA-EGFP which was linearized with BsiI (Supplementary Data 4). To perform rescue experiments, the encoding region of SLC35B2, HS6ST1, B3GAT3, GLCE, EMC3, and CALR were PCR amplified using cdNA from PK-15 cells as a template, with forward primer flanked by NheI restriction sites and reverse primer flanked by XbaI restriction sites of pcDNA3.1(+)(Thermo Fisher Scientific), respectively. Then, PCR products were digested and cloned into the NheI and XbaI sites to generate pcDNA3.1-SLC35B2, pcDNA3.1-HS6ST1, pcDNA3.1-B3GAT3, pcDNA3.1-GLCE, pcDNA3.1-EMC3, and pcDNA3.1-CALR vectors, respectively. All plasmids were confirmed by Sanger sequencing (Tsingke). All primer sequences are listed in Supplementary Data 4.

**Genome-wide porcine sgRNA library design.** Three sgRNAs were designed against each protein-coding gene, lncRNA, and miRNA using software of CRISPR-ofigler (version 1.2, http://www.biooools.com), Sequences of protein-coding genes, lncRNA, and miRNA, were found from databases of Ensemble (version 10.2, www.ensembl.org/index.html), ALDB (http://202.200.112.245/aldb54), and miRBase (www.mirbase.org), respectively. Briefly, the selected sgRNAs were weighted based on targeting the first 50% of the open reading frames and minimizing potential off-target sites. The maximum number of mismatches allowed up to three nucleotides to the DNA target in the 20-mer targeting region of selected sgRNAs targeting the miRNA hairpin region, and to avoid overlap between sgRNAs in the same given targets.

**Construction of a genome-wide sgRNA library plasmid.** The sgRNA library was synthesized using CustomArray 90 K arrays (CustomArray Inc.), and amplified by PCR using Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB) to produce sub-pools for Gibson assembly (NEB). The PCR reaction was performed in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific) with 16 cycles. In total, 40 PCR reactions were performed using 50 ng of oligo pool per 50 μL of reaction volume. The PCR products were mixed and purified using a MinElute PCR purification Kit (QIAGEN), and then ligated into the linearized lenti-sgRNA-EGFP vector using Gibson assembly. The ligation mixtures were transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Transgen). To achieve sufficient coverage, parallel transformations were performed, counting the number of colonies to reach 200-times total sgRNAs in the library. The sgRNA library plasmids were extracted with a Plasmid Plus Maxi Kit (QIAGEN). The library plasmids were amplified using PolynU+ (tetralon GXX DNA) with 16 reaction cycles. PCR products were purified using a QIAxquick Gel Extraction Kit (QIAGEN) and then analyzed by high-throughput sequencing to examine the sgRNA coverage in the library plasmids. All trimmers for constructing sgRNA expression vector are listed in Supplementary Data 4.

**Cell culture and transfection.** PK-15, HEK293T, and BHK-21 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and ST (CRL-1746) cell line was purchased from ATCC(USA). The cell lines were then subjected to mycoplasma detection. For all experiments, cells were maintained in Dulbecco’s Modified Eagle Media (DMEM) supplementing with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and incubated at 37 °C with 5% CO\textsubscript{2}. For transfection assays, PK-15 cells were seeded into 6-well plates and transfected (approximately 80% confluent) with 2 μg plasmid DNA using JetPRIME (PolyPlus) according to the manufacturer’s instructions. At 48 h post-transfection, cells were incubated with JEV-PR9 strain at 37 °C with 5%
Briefly, PCR mixtures (10 µL) contained 5 µL RealUniversal Premix, 0.3 µL forward primer (0.3 µM), 0.3 µL reverse primer (0.3 µM), and 1 µL cDNA template. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The pCAGGS plasmid was used as a positive control. The band intensity was quantified using the Image Lab software (Bio-Rad). All primers are listed in Supplemental Data 4.

**Real-time reverse transcription PCR (qRT-PCR) analysis.** Total RNA from cells was extracted with TransZol Up (TransGen), and viral RNAs were extracted from cell suspensions using a Viral RNA Extraction Kit (TaKaRa) following the manufacturer's instructions. Total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) for assessing RNA quantity and quality. cDNAs were synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). qPCR reactions were prepared with RealTime SYBR Green Premix (Tiangen) following the manufacturer's instructions. The qPCR reactions were performed with a LightCycler® 96 System (Roche) programmed for one cycle of 15 min at 95 °C, followed by 39 cycles of 10 s at 95 °C, 30 s at 60 °C. Relative expression levels were calculated using the \(2^{-\Delta\Delta C_{T}}\) method. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a normalization control. For absolute quantitative real-time PCR, about 1 µL of viral RNAs were used as template to synthesize cDNAs. Absolute quantitative real-time PCR assay was performed with SYBR green I (TOYOBO) and primers binding to C of JEV in a final reaction volume of 20 µL. PCR mixtures contained 10 µL SYBR® Green Real-time PCR Master Mix, 1 µL forward primer (0.5 µM), 1 µL reverse primer (0.5 µM), and 2 µL cDNA template. The results were monitored using a LightCycler® 96 System (Roche) programmed for one cycle of 15 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C, 10 s at 72°C. The JEV C protein encoding cDNA sequence from GenBank (accession number: AF014161.1) was cloned into PMD19-T vector and used as a standard for the quantification of JEV copy numbers. All primers used in quantitative PCR are listed in Supplementary Data 4.

**T7 endonuclease I cleavage detection assay and Sanger sequencing.** All potential off-target sites with high homology in the sgRNAs were predicted using software CRISPR-offinder²⁶. Genomic DNAs were extracted using the Tiangen Genomic DNA Kit (TIANGEN) from mutated clonal cells for PCR amplification, and 7 T7 endonuclease I (T7EI) cleavage detection assay was employed to determine off-target effects. CRISPR/Cas9-induced lesions at the endogenous target site were quantified using the T7 endonuclease I cleavage detection assay to investigate the insertions/deletions (indels) generated by nuclease-mediated non-homologous end joining (NHEJ). The gene fragments of off-target sites were amplified with primers specific to each locus by 35 cycles of PCR with TaKaRa LA Taq (TaKaRa). The PCR products were purified, denatured, and precipitated using a Thermosol. The hybridized DNA were digested with T7EI (NEB) for 15 min and separated with a 2% agarose gel. The agarose gels were stained with GelRed and the signal of DNA in the gel was quantitated by densitometry using Image Lab software (Bio-Rad). All primers are listed in Supplementary Data 4.

**Illumina sequencing of sgRNAs in the genome-wide library and enriched mutants.** The genomic DNA of each sample was extracted using a Blood & Cell Culture DNA Midi Kit (QIAGEN). The sgRNA-coding region was amplified by PCR using O5® Hot Start High-Fidelity DNA Polymerase (NEB) in a reaction volume of 50 µL. PCR products were mixed and purified with a MinElute PCR purification Kit (QIAGEN). The purified PCR products were amplified by PCR using different barcoded primers. All PCR products were pooled and purified with a MinElute PCR purification Kit (QIAGEN), followed by Illumina HiSeq 3000. Next-generation sequencing. Mapped read counts were subsbtantly used as input for the MAGeCK analysis software package (version 0.5)⁴⁶. Then, the top 0.5% ranked sgRNAs from the third and fourth JEV challenge rounds were used to identify enriched targeting protein-coding genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov)⁷⁷. All primers are listed in Supplementary Data 4.

**Virus plaque assay.** Plaque assays were performed on BHK-21 cells. Briefly, BHK-21 cell monolayers at 50% confluence were infected with serially diluted virus at 37 °C with 5% CO₂. At 2 hrs after infection, inoculum was removed, and the cells were overlaid with 30% DMEM, 50% Agarose LMP (Genview), 2% FBS and 1% penicillin–streptomycin for 3 days. Cells were fixed with 10% formaldehyde neutral solution overnight at room temperature, then stained with 0.5% crystal violet for 2 h at room temperature. Plaques were counted manually and plaque-forming units were calculated. Three independent experiments were performed, with results presented as means ± S.D.

**Immunofluorescence assay.** The expression level of JEV NS3 protein in WT and gene KO PK-15 cells as indicated in figures was determined by immunofluorescence assay. Briefly, cells grown on the glass coverslip in 6-well cell culture plates were infected with JEV at an MOI of 0.5. After different setting times, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with precooled PBS, and then permeabilized for 10 min with 0.5% Triton-X100 in PBS. Cells were reacted with NS3 (antibody, #ANT030, 1:400) or Alexa Fluor 488-conjugated secondary antibodies for 1 h at room temperature, then stained with 0.5% crystal violet for 2 h at room temperature. Plaques were counted manually and plaque-forming units were calculated. Three independent experiments were performed, with results presented as means ± S.D.
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