New PARP gene with an anti-alphavirus function.

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Running title: new antiviral function of PARP12.

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ABSTRACT

Alphaviruses represent a highly important group of human and animal pathogens, which are transmitted by mosquito vectors between vertebrate hosts. The hallmark of alphavirus infection in vertebrates is the induction of a high-titer viremia, which is strongly dependent on the ability of the virus to interfere with host antiviral responses on both cellular and organismal levels. The identification of cellular factors, which are critical in orchestrating virus clearance without the development of cytopathic effect, may prove crucial in the design of new and highly effective antiviral treatments. To address this issue, we have developed a noncytopathic Venezuelan equine encephalitis virus (VEEV) mutant that can persistently replicate in cells defective in type I IFN production or signaling, but is cleared from IFN signaling-competent cells. Using this mutant, we analyzed i) the spectrum of cellular genes activated by virus replication in the persistently infected cells, and ii) the spectrum of genes activated during noncytopathic virus clearance. By applying microarray-based technology and bioinformatic analysis, we identified a number of ISGs specifically activated during VEEV clearance. One of these gene products, the long isoform of PARP12 (PARP12L), demonstrated an inhibitory effect on the replication of VEEV, as well as other alphaviruses and several different types of other RNA viruses. Additionally, overexpression of two other members of the PARP gene superfamily was also shown to be capable of inhibiting VEEV replication.
INTRODUCTION

The Alphavirus genus is a group of arthropod-borne viruses in the Togaviridae family, many of which are important human and animal pathogens (54). In nature, these viruses are transmitted by mosquito vectors between vertebrate hosts (45). While mosquitoes remain mostly unaffected by alphavirus infection, vertebrates develop diseases of varying severity. The hallmark of alphavirus replication in vertebrates is an induction of a high-titer viremia, which is required for further transmission of infection to feeding mosquitoes, thus perpetuating the cycle (24, 54). Alphaviruses are capable of reaching high titers due to highly efficient viral RNA replication and rapid production of viral nonstructural and structural proteins. However, the development of viremia is also strongly dependent on the virus’ ability to interfere with the development of antiviral responses at the cellular level, and in the organism as a whole (52, 53). Similar to many other viruses, alphaviruses have developed a complex system of countermeasures, which promote their replication in the presence of cellular molecular pattern recognition receptors (PRRs) (17-19). These viruses downregulate induction of the antiviral response and/or become at least partially resistant to its activation. The ability of the alphaviruses to interact with host responses is a multicomponent process, with some of the constituents being specific to each member of the genus. However, some mechanisms appear to be common for several different members of the genus.

Firstly, all characterized alphaviruses isolate their dsRNA replication intermediates from the cytoplasm into plasma and endosome membrane invaginations (spherules), in order to prevent these dsRNA molecules from being efficiently recognized by such known PRRs as RIG-I, MDA5 and PKR or TLR3 (14, 15, 23). These membrane
spherules are connected to the cytosol by very narrow openings, which are associated with viral nsPs. The dsRNAs are sufficiently isolated to prevent their recognition by dsRNA-specific antibodies unless the spherule membrane is permeabilized by nonionic detergents (14). However, such dsRNA compartmentalization is likely to be incomplete, because cells release cytokines in response to replication of alphavirus mutants that are incapable of inhibiting cellular transcription (5, 13, 21).

Secondly, in cells of vertebrate origin, alphavirus replication induces transcriptional and translational shutoff (10, 20, 22). Previously, it was thought that inhibition of translation downregulates virus replication and represents a mechanism of host defense. However, an accumulating amount of data demonstrates that, at least in the case of Sindbis (SINV) and Semliki Forest (SFV) viruses, translational shutoff is not only mostly independent of PKR (22, 50), but is also highly beneficial for translation of viral structural proteins (11, 12, 44). These viruses have developed translational enhancers in the 5' termini of their subgenomic RNAs (G-C-rich RNA sequences folded into stem-loops), which function only during virus-induced translational shutoff. The presence of these enhancers strongly increases expression of structural proteins by modified cellular translational machinery.

Inhibition of cellular transcription has been unambiguously demonstrated in cells of vertebrate origin, infected by evolutionarily distinct alphaviruses, such as SINV, Venezuelan (VEEV) and eastern (EEEV) equine encephalitis viruses (2-4, 18). Each of these viruses uses different virus-specific proteins to achieve the same goal: to downregulate activation of antiviral genes. Cellular transcription inhibition is an efficient means of promoting virus replication, but has some limitations. Both the SINV-specific
nsP2 and the VEEV- and EEEV-specific capsid proteins, which demonstrate transcription inhibitory activities, function in stoichiometric rather than catalytic, protease-dependent modes. This is likely to make them less efficient inhibitors during virus replication in vivo, in less permissive cells, in which viral structural and nonstructural proteins are synthesized to lower levels. Previously described noncytopathic clearance of SINV from neuronal cells strongly supports the possibility that cells with incomplete transcription and translation inhibition might downregulate virus replication and ultimately clear replicating virus (6, 25).

Identification of such noncytopathic clearance mechanisms is critical for further understanding of alphavirus pathogenesis and development of new means of therapeutic intervention. However, the dissection and study of these processes in the in vitro experiments are problematic. Most of the commonly used vertebrate cell lines are highly permissive for alphavirus replication and develop profound cytopathic effect (CPE) within a few hours post infection. This phenomenon is a result of numerous, profound changes in cellular metabolism manifested primarily by transcription and translation inhibition. This rapid CPE development could mask activation of the signaling pathways, which are better detectable in cells supporting lower levels of virus replication.

Therefore, in our recent studies, we have developed variants of VEEV, which exhibit a dramatically less cytopathic phenotype in all of the tested cell types, but retain RNA replication at the wt level (5). These viruses produced the same amounts of virus-specific nonstructural and structural proteins and, in the in vitro experiments, the infected cells released the same amounts of infectious virions as during the wt virus infection. Therefore, it was concluded that the capsid-specific mutations introduced, do not directly
affect virus replication. The unique feature of these designed VEEV mutants was their noncytopathic, persistent phenotype in cells defective in type I IFN signaling, such as IFN-α/βR−/− and STAT1−/− MEFs. However, cells competent in IFN production and signaling were able to clear these modified viruses. This clearance was a result of the virus’ inability to effectively inhibit induction of the antiviral response, particularly the release of type I IFN into the media and its autocrine signaling.

In this study, we used one of the noncytopathic VEEV variants to compare i) the spectrum of cellular genes activated by virus replication in cells defective in IFN-α/β signaling and incapable of virus clearance to ii) the spectrum of genes activated in cells with intact IFN-α/β signaling, which include the genes induced by both virus replication and the released type I IFN. The latter combined response was sufficient for virus clearance. Next, we identified a number of mouse ISGs, which were differentially expressed during virus clearance and demonstrated that the product of one of them, a long isoform of PARP12, exhibits a strong negative effect on replication of VEEV, other alphaviruses and viruses with both negative- and positive-strand RNA genomes.

MATERIALS AND METHODS

Cell cultures. The BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, Mo). The NIH 3T3 cells were obtained from the American Type Culture Collection (Manassas, Va). These cell lines were maintained at 37°C in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. The IFN-α/βR−/− mouse embryonic fibroblasts (MEFs) were kindly provided by Michael Diamond (Washington University, St. Louis, Mo). They were
propagated in Dulbecco’s MEM supplemented with 10% FBS and nonessential amino acids.

Plasmid constructs. Plasmids encoding VEEV genomes, pVEEV/GFP and pVEEV/GFP/C1, (see Fig. 1 for details) were described elsewhere (5). All other genes were synthesized by RT-PCR using specific primers and RNA isolated from NIH 3T3 cells. They were cloned into the plasmids, sequenced and re-cloned into pVEEV/GFP, pVEEV/GFP/C1 to replace the green fluorescent protein (GFP)-coding sequence. VEEV replicon-coding plasmids were described elsewhere (36). They were used to place heterologous genes of interest under control of the subgenomic promoter encoded by VEEV replicons. The schemes of the replicons’ and viral genomes are presented in the relevant figures. VEEV helper RNA-encoding plasmids were described elsewhere (51). All of the sequences and details of the cloning procedures can be provided upon request.

RNA transcriptions. Plasmids were purified by centrifugation in CsCl gradients. They were linearized using the MluI restriction site located downstream of the poly(A) sequence of the viral replicons’ and helper genomes. RNAs were synthesized by SP6 RNA polymerase in the presence of a cap analog using previously described conditions (37). The yield and integrity of transcripts were analyzed by gel electrophoresis under non-denaturing conditions, and transcription reactions were used for electroporation without additional purification (32). Released viruses were harvested at 24 h post electroporation, and titers were determined by plaque assay on BHK-21 cells (29).

Replicon RNAs were co-electroporated into the cells together with helper RNAs and
released viral particles were harvested at 24-30 h post electroporation. Titers were
determined by infecting BHK-21 cells with different dilutions of harvested particles and
staining them with VEEV nsP2-specific monoclonal antibody and secondary
AlexaFluor555-labeled antibody. Numbers of infected cells were assessed by
fluorescence microscopy.

Viral replication analysis. Cells were seeded into 35-mm dishes. After 4-h-long
incubation at 37°C, monolayers were infected at MOIs indicated in the figures or figure
legends, washed with PBS, and overlaid with 1 ml of complete medium. In some
experiments, cells were initially infected with packaged replicons expressing
heterologous genes of interest and at 1 h post infection, were superinfected with viruses.
At the times indicated in the figures, media were replaced by fresh media, and virus titers
were determined by a plaque assay on BHK-21 cells as previously described (29). VEEV
TC-83 derivatives were found to be equally efficient in plaque formation in both BHK-21
and NIH 3T3 cells used in this study (data not shown). Therefore, the applied MOIs are
equally applicable to the experiment performed in both cell lines. All of the virus-related
experiments were performed in BSL2 conditions.

Microarray analysis. NIH 3T3 cells and IFN-α/βR−/− MEFs were seeded at a
concentration of 2×10⁶ cells per 100-mm dish. After 4-h-long incubation at 37°C, cells
were infected with VEEV/GFP/C1 at an MOI of 20 PFU/cell or treated with 1000 IU/ml
of IFN-β. At the indicated time points, total RNA was isolated using TRIzol according to
the manufacturer’s instructions (Invitrogen). RNA was additionally purified with the
RNeasy minikit (Qiagen). RNA quality was tested using Agilent 2100 Bioanalyzer. The
cDNA synthesis, labeling, hybridization on Mouse Gene 1.0 ST Array GeneChips
(Affymetrix) and image processing were performed at Heflin Center Genomics Core
facility (UAB). Two independent RNA samples were prepared for each indicated time
point for VEEV/GFP/C1-infected cells and three RNA samples for IFN-β-treated cells.
The robust multichip average (RMA) algorithm was used to normalize the raw intensity
values using the GeneSpring software program, version GX 11.5 (Agilent). To find
differentially expressed genes, a one-way ANOVA statistical test with Benjamini-
Hochberg correction was applied to the normalized intensity data. Entries with more than
two-fold change in expression and p-value<0.05 were chosen for further analysis.

RT-qPCR. RNA samples, which were used for microarray analysis, were also analyzed
to compare concentration of specific cellular RNAs by qPCR. cDNA was synthesized
using 1 μg of total isolated RNA with a QuantiTect reverse transcription kit (Qiagen).
qPCR primers were designed for the following genes: Bst2 (NM_198095), Trim30
(NM_009099), USP18 (NM_115783), Ifi27 (NM_029803), PARP12 (NM_172893),
ISG20 (NM_020583) and MOV10 (NM_008619). qPCR was performed using SsoFast
EvaGreen Supermix (Bio-Rad) in a CFX96 real-time PCR detection system (Bio-Rad)
for 40 cycles with two steps per cycle, each step for 5 s (a denaturing step at 98°C, an
annealing/extension step at 60°C). Results of the quantification were normalized to the
amount of β-actin mRNA present in the same RNA samples. The RNA fold change was
determined by comparing data of VEEV/GFP/C1-infected NIH 3T3 cells (24 h PI) to
mock-infected sample by ΔΔCt method. Each qPCR was performed in triplicate, and means and standard deviation (SD) were calculated.

Western blotting. Equal amounts of proteins were separated on a 4-12% gradient NuPAGE gel. After protein transfer, the membranes were treated with VEEV nsP2-specific MAb and β-actin-specific antibodies, followed by treatment with infrared dye-labeled secondary antibodies. For quantitative analysis, membranes were scanned on the LI-COR imager.

Firefly luciferase activity was measured with Luciferase assay system (Promega) according to manufacturer’s instructions. Briefly, at the indicated time points cells were washed with PBS and lysed with Reporter lysis buffer (Promega) and overnight freezing at -80°C. The luminescence was measured for 10μl of 100-fold diluted lysates using a FB12 Luminometer (Berthold). All of the measurements were performed in triplicates, mean and SD were calculated.

RESULTS

A VEEV variant with mutated capsid genes can be cleared from NIH 3T3 cells, but not from IFN-α/βR−/− MEFs. In our previous studies, we developed VEEV TC-83 variants containing a set of redundant, attenuating point mutations in the capsid gene (5). One of these variants, VEEV/GFP/C1, was used in this new study. It contained mutations in the capsid protein-specific nuclear localization signal (NLS) and in the peptide connecting the supraphysiological nuclear export signal (supraNES) and the NLS (Fig.
These mutations made the capsid protein incapable of interfering with nucleocytoplastic trafficking and inhibiting cellular transcription (3, 5). The control virus, VEEV/GFP, was identical to VEEV/GFP/C1 in terms of genome organization, but encoded the wt VEEV TC-83 capsid protein (Fig. 1A). Both recombinant viruses contained a GFP-coding sequence under the control of an additional subgenomic promoter.

In both NIH 3T3 cells, which have no defects in type I IFN production and signaling, and IFN-α/βR−/− MEFs, VEEV/GFP infection caused cytopathic effect and total cell death within 24-48 h post infection (Fig. 1B). In contrast, the VEEV/GFP/C1 mutant replicated without detectable CPE in both NIH 3T3 cells and IFN-α/βR−/− MEFs (Fig. 1B), as well as in STAT1−/− MEFs, Vero and BHK-21 cells (data not shown and see (5)). In NIH 3T3 cells, this mutant replicated to essentially the same titers as VEEV/GFP (Fig. 1B); however, the infected cells rapidly released high levels of IFN-β (Fig. 1C). Within 5-6 days post infection, NIH 3T3 cells were able to downregulate virus replication to an undetectable level. Virus replication was then reactivated after day 9 (Fig. 1B). The latter stage of the infection was characterized by the presence of a low concentration of IFN-β in the media and inefficient virus release by a small percentage of the cells (5). IFN-α/βR−/− MEFs were unable to clear the noncytopathic mutant and in multiple, reproducible experiments, continued to produce infectious virus for at least 21 days, until termination of the experiments (Fig. 1B) (5).

Thus, the VEEV variant with mutated capsid protein, VEEV/GFP/C1, induced an antiviral state in cells with intact type I IFN production and signaling, which led to the downregulation of viral replication to undetectable levels. However, the same virus was
able to persistently replicate in the cells defective in type I IFN signaling, suggesting that autocrine IFN signaling and activation of ISGs must play critical roles in noncytopathic VEEV/GFP/C1 clearance. Experiments similar to those presented above have been performed not only in NIH 3T3 cells, but also in primary and immortalized MEFs. The latter cells also demonstrated VEEV/GFP/C1 clearance. However, it was almost impossible to synchronously infect these wt MEFs at reasonable MOIs (10-20 PFU/cell). This effect was observed with VEEV/GFP/C1 and particularly Sindbis virus (SINV) (see (5) and data not shown). This strongly complicated interpretation of the data from the long-term infection-based experiments and those based on microarray analysis. Therefore, the experiments presented in the following sections, were performed using NIH 3T3 cells as a representative wt mouse cell line. They unambiguously demonstrated the ability of these cells to clear replication of noncytopathic alphaviruses.

**Microarray analysis defined a set of genes differentially expressed during virus clearance, but not in persistently infected cells.** The experiments described above suggested that in the absence of autocrine IFN signaling the PRR-mediated antiviral response in IFN-α/βR−/− MEFs is insufficient for clearance of VEEV/GFP/C1 infection. This indicated that the infected NIH 3T3 cells activate an important component(s) of the cellular response that is induced by released type I IFN, and these cellular factor(s) play a critical role in the downregulation of virus replication. Thus, the rationale of the following analysis, presented in Supplementary Fig. 1, was to identify the cellular gene(s) that were i) activated in the NIH 3T3 cells by VEEV/GFP/C1 replication and secreted
type I IFN, ii) activated by IFN-β treatment itself, but iii) not activated in the IFN-α/βR⁻/⁻ MEFs by persistently replicating virus; and to further assess their antiviral function. To achieve this, we infected NIH 3T3 cells and IFN-α/βR⁻/⁻ MEFs with VEEV/GFP/C1, isolated total cellular RNAs at different times post infection and analyzed changes in the mRNA profiles using a microarray-based approach. The same analysis was applied to NIH 3T3 cells that were treated with 1000 IU/ml of IFN-β for 24 h (the first time point of RNA isolation). The IFN-β-treated cells were then incubated in IFN-free media, and RNA was isolated at 24 h and 48 h after IFN-β withdrawal (time points 2 and 3, respectively in Fig. 2). Mock-infected NIH 3T3 cells and IFN-α/βR⁻/⁻ MEFs were used to isolate control RNAs. To ensure statistical significance, all of the microarray experiments were performed in duplicates or triplicates. Several time points were analyzed on both Affymetrix and Illumina gene chips, which generated very similar results (data not shown).

In the NIH 3T3 cells infected with VEEV/GFP/C1, gene expression profiles were assessed and analyzed starting from 8 h post infection, until day 16. However, in order to simplify the data presentation, we only present samples harvested until day 7 (Fig. 2), when the virus was no longer detectable in the media (Fig. 1B). The ANOVA statistical test revealed that the VEEV/GFP/C1 infection in the NIH 3T3 cells significantly changed expression of 1575 genes. The first panel of Fig. 2A displays the expression profiles of these 1575 genes. The second and third panels represent the expression profiles of the same genes in IFN-β-treated NIH 3T3 cells and in VEEV/GFP/C1-infected IFN-α/βR⁻/⁻ MEFs. In all three cases, the highest activation of gene expression occurred by 24 h post
infection, and at this time, in the infected NIH 3T3 cells, 388 different mRNAs were present at 2-207-fold higher levels than in the mock-infected cells.

Further analysis narrowed down the spectrum of genes of interest by identifying the subset of those activated more than 2-fold in both infected NIH 3T3 cells and cells treated with IFN-β (Fig. 2B and Supplementary Tables 1 and 2). This subset contained only 140 genes. Their expression reached the highest level by 24 h post infection and then gradually decreased. The rates of decrease were faster in cells activated by IFN-β treatment only. In 24 h, after removing IFN-β from the media, the NIH 3T3 cells demonstrated a rapid return of gene expression back to normal, pre-treatment levels (Figs. 2A and B). By this time, cells previously treated with type I IFN become susceptible to virus infection (5).

Next, we excluded from further analysis the genes that were activated by virus replication in IFN-α/βR−/− MEFs (Fig. 2C). We concluded that these genes were activated by virus infection only, and expressed proteins that were incapable of stopping virus replication (at least at their observed levels of expression). After applying the above analytical criteria, 98 IFN-inducible genes, activated by VEEV/GFP/C1 replication and produced IFN-α/β in the NIH 3T3 cells, but not in the IFN-α/βR−/− MEFs, were left (Fig. 2C).

To further narrow down the analysis, we divided the remaining 98 genes into two groups (Fig. 3A). The first group included genes that are closely located in mouse genome and expressed as families, such as the OAS genes. We theorized that it was likely that these genes have related functions, and if expressed separately, should have no significant effects on viral replication. The second group contained genes that are
separated in the genome and activated independently, such as IRF7, STAT1, LGP2, PKR, RIG-I, TLR3, etc (7, 30, 39, 46, 55). The latter group contained only 47 genes. We selected from them a small subset of genes demonstrating the most efficient induction and long-term activation in the VEEV/GFP/C1-infected NIH 3T3 cells and also excluded the genes encoding proteins with previously characterized antiviral functions, such as PKR and RIG-I. We also excluded proteins involved in type I IFN signaling pathways, and those mediating the downregulation of the IFN response, such as Usp18. The latter gene was used in some of the experiments presented below, but only as a control having no antiviral function.

After applying these final exclusion filters, the final group of selected genes was represented by PARP12, Ifi27, Trim30 and Bst2. Their expression profiles in i) VEEV/GFP/C1-infected NIH 3T3 cells, ii) IFN-β-treated NIH 3T3 cells and iii) VEEV/C1/GFP-infected IFN-α/βR−/− MEFs are presented in Fig. 3B.

**Murine PARP12 demonstrates antiviral activity.** The first step of further analysis was to confirm gene activation by RT-qPCR. In these experiments, we used the RNA samples isolated from the NIH 3T3 cells at 24 h post infection with VEEV/GFP/C1, and previously used for the microarray analysis presented in Fig. 2. RT-qPCR was performed using gene-specific primers (see Materials and Methods for details). Data was normalized to the concentrations of β-actin mRNA. The results presented in Fig. 4, confirmed activation of the genes, which had previously been defined using gene-chip analysis (Fig. 3B). However, for each of the 4 genes tested, the induction levels determined by RT-qPCR were higher than those found in the microarray experiments. Usp18 and ISG20
mRNA templates, used as additional controls, were also found at higher concentrations than those expected from the microarray data. Thus, the expression of PARP12, Ifi27, Trim30 and Bst2 is activated in NIH 3T3 cells during VEEV/GFP/C1 replication. Alphaviruses represent a convenient system for simultaneous expression of the genes of interest and testing their effects on virus replication. The genes can be introduced under the control of an additional subgenomic promoter into the viral genome, and the effect of their products on replication then evaluated by comparing the titers of released viruses (30, 55). While it is true that cloned heterologous genes are not expressed at very early times post infection and their effect on early events of virus replication cannot be tested, this system mimics the events occurring during ISG induction and allows the experiments to be performed in different cell types. To prevent argument over the validity of the experimental system, a number of different approaches were undertaken to confirm the antiviral effect (see following sections for details). Thus, we synthesized murine Ifi27, Trim30, Bst2 and PARP12 genes by RT-PCR and cloned them into the VEEV/GFP/C1 genome under control of additional subgenomic promoter, replacing the GFP-coding sequence (Fig. 5A). Sequencing of PARP12-specific cDNA revealed that its mRNA is present in the cells in two splice forms (see Fig. 5B), and, thus, two corresponding proteins are likely to accumulate. We termed them long and short isoforms, PARP12L and PARP12S, respectively (Figs. 5A and B). The completely spliced PARP12L RNA encoded a 711-aa-long protein, and PARP12S-specific RNA encoded a 485-aa-long polypeptide that contained the same five Zn fingers as PARP12L, but lacked the PARP domain. In order to detect the antiviral functions of these two forms, both PARP12L and PARP12S genes were separately cloned into the viral genome.
Because the insertion size might affect RNA replication and/or packaging, we used cDNA fragments of the same length. Thus, in the case of PARP12S, the cloned gene contained a large, 3’ terminal nucleotide sequence, which was not translated due to presence of a short intron-mediated frame shift. The recombinant viral genomes were synthesized \textit{in vitro} and transfected into BHK-21 cells. All of the designed double subgenomic viruses were viable, and most of them produced homogenous plaques with size approximating that of VEEV/GFP/C1. The only two viruses that produced noticeably smaller plaques were those expressing PARP12L and Bst2 proteins (data not shown). The smaller plaque-forming phenotype was unlikely to be attributed to the insertion sizes, as the PARP12S-specific sequence was of the same length as that of PARP12L, but did not affect plaque size and virus titers. The Bst2 gene was only 519-nt-long and, thus, even smaller than the control GFP-coding sequence.

From all of the tested constructs, only PARP12L- and Bst2-encoding viruses reproducibly demonstrated lower rates of replication in NIH 3T3 cells (Fig. 5C). In this and numerous other tests, viruses expressing PARP12S, Trim30 and Ifi27 exhibited growth rates almost identical to those of VEEV/GFP/C1 (Fig. 5C and data not shown). The experiments with double-subgenomic viruses could not rule out the possibility that in order to demonstrate an antiviral effect, Trim30, Ifi27 and PARP12S need to be overexpressed before infection or at very early times postinfection. However, in other experiments, based on transient expression of these proteins from plasmid DNA, they also failed to demonstrate replication inhibitory functions (data not shown). Thus, taken together, the data suggested that expression of Trim30, Ifi27 and PARP12S to higher levels alone has no detectable antiviral function.
During the course of the above experiments, we noticed that, while the MOI used for controls had no effect on final virus titers, the inhibitory effect of PARP12L and Bst2 was dependent on the MOI and the cell type used, suggesting that the antiviral effect may depend on the time of expression during VEEV replication. To clearly demonstrate this and express the proteins of interest at earlier times post infection, we infected NIH 3T3 and BHK-21 cells at different MOIs with VEEV/PARP12L/C1 and VEEV/Bst2/C1 viruses and assessed the rates of virus release. In these experiments, virus encoding the entire PARP12L gene in reverse orientation under the duplicated subgenomic promoter, VEEV/PARPrev/C1, was used as a control. The later time points of virus replication were excluded from the analysis in these and most of other experiments, because PARP12L expression could potentially reach unphysiological levels. We were unable to confirm this conjecture as the commercially available PARP12-specific Abs we tested were unable to recognize either PARP12L or PARP12S on Western blots. Thus, we could not analyze the levels of endogeneous and exogeneous PARP expression. Therefore, we focused our analysis of virus replication on the early times post infection, 12 h PI.

The results presented in Fig. 6 clearly demonstrate more efficient and earlier antiviral activities of both PARP12L and Bst2 proteins at higher MOIs, which appeared to promote earlier expression of PARP12L and Bst2 from VEEV genomes. The antiviral effect was also more prominent in BHK-21 cells, which, based on our previous experience, support more efficient viral RNA replication and higher levels of heterologous protein expression from the alphavirus genome-based constructs than do NIH 3T3 cells (data not shown). This suggested that inhibition of virus replication
depends on the concentration and/or rates of accumulation of the studied proteins in the cells. Bst2 protein is known to affect release of some enveloped viruses from the cell surface (40, 41). Therefore, in these experiments, its expression was mostly used as a positive control to additionally demonstrate that the applied virus-based expression system is able to detect the inhibitory activity of the studied gene products. Moreover, the experiments described above demonstrated that PARP12L possesses more efficient inhibitory function(s) than Bst2. Therefore, the experiments described in the following sections were focused exclusively on further analysis of the PARP12L-specific antiviral effect.

To rule out the possibility that the virus replication inhibitory functions of PARP12L are specific to replication of VEEV with mutated capsid protein, we cloned the corresponding gene and PARP12L in reverse orientation into VEEV TC-83 containing the wt capsid protein, VEEV/PARP12L and VEEV/PARPrev, respectively (Fig. 7A). As we expected, expression of PARP12L affected VEEV replication in both BHK-21 and NIH 3T3 cells (Fig. 7B). This was a strong indication that the inhibitory activity of PARP12L is not limited to VEEV variants encoding mutated capsid protein.

Expression of PARP12L in-trans downregulates virus replication. To further assess the antiviral activity of PARP12L, we attempted to produce recombinant lentiviruses encoding the PARP12L and PARP12S genes. However, the PARP12L-encoding constructs, but not the constructs encoding PARP12S or other genes, were packaged to
very low titers (data not shown), suggesting that the antiviral effect of PARP12L might be broader than expected, and affect replication of other viruses.

We also transiently expressed PARP12L, PARP12S, Bst2 and Trim30 and GFP genes in NIH 3T3 cells from RNA polymerase II promoters, and tested their effects on virus replication. Plasmid DNAs encoding the genes of interest under control of the CMV promoter were electroporated into NIH 3T3 cells. At 24 h post transfection, cells were infected with the VEEV/GFP/C1 mutant, and replication rates of this virus were assessed. The results were encouraging. PARP12L expression led to a 4-6-fold decrease in virus titers (data not shown). However, plasmid transfection of NIH 3T3 cells is not very efficient, and the CMV promoter normally generates a very high level of heterologous gene expression, which could be higher than the standard physiological level.

In order to exclude potential effects of high PARP12L expression on cell metabolism, indirectly affecting virus replication, we applied another experimental system, which allowed the study of the antiviral effect of PARP12L at early times after the initiation of its expression. PARP12L and PARPrev sequences were cloned into VEEV replicons to generate the constructs VEErep/PARP12L and VEErep/PARPrev, respectively (Fig. 8A). The in vitro-synthesized RNAs were co-transfected into BHK-21 cells together with helper RNAs (see Materials and Methods for details), and replicon-containing viral particles were harvested at 24 h post transfection. Titers of packaged VEErep/PARP12L replicons were at least 10-fold lower than those of VEEV replicons encoding other heterologous genes. However, they were sufficiently high to infect all of the cells in the monolayers in the following experiments.
BHK-21 cells were infected with packaged replicons at an MOI of 20 inf.u/cell. One hour later, before PARP12L expression reached a high level, they were infected with VEEV/GFP, SINV/GFP (double subgenomic Sindbis virus expressing GFP) and CHIKV 181/25 (attenuated chikungunya virus strain 181/25) at an MOI of 1 PFU/cell. In the preliminary experiments, 1 hour between replicon and virus infections was found to be insufficient time for the development of the superinfection exclusion phenomenon, which occurs in vertebrate cells during double infection with alphaviruses. In multiple, reproducible experiments, PARP12L expression had a strong negative effect on the rates of VEEV/GFP release (Fig. 8B presents the results of one of the reproducible experiments). Similarly, it inhibited replication of SINV/GFP and CHIKV 181/25. At 6 h post infection, the CHIKV titers were more than three orders of magnitude lower than those found in the samples harvested from VEErep/PARPrev replicon-containing cells, superinfected with the indicated CHIKV. A similar negative effect of PARP12L expression on virus replication was also detected in NIH 3T3 cells (data not shown). Importantly, the antiviral effect of PARP12L expression was found not to be limited to alphavirus infections. In another set of experiments, the VEErep/PARP12L-containing cells were superinfected with i) vesicular stomatitis virus (VSV), a negative-strand RNA genome virus; ii) encephalomyocarditis virus (EMCV), a positive-strand RNA genome virus; and Rift Valley fever virus (RVFV) MP12, an ambisense RNA genome virus. All of the indicated viruses replicated slower in the presence of VEErep/PARP12L replicon than in cells containing the control VEErep/PARPrev (Fig. 8C). This was particularly evident at early times post infection where a few orders of
magnitude difference was observed. Thus, PARP12L protein expression appears to have a broad antiviral effect.

In additional experiments, we investigated whether a lower level of expression than that achieved in the experiments with VEErep/PARP12L would be sufficient to inhibit virus replication. To achieve this, the PARP12L sequence was cloned in direct and reverse orientations under control of the subgenomic promoter into the VEEV replicon VEEVrepL, containing a point mutation in the nsP2 coding sequence (Q739L). This mutation was previously shown to have a strong negative effect on replicon RNA replication and decreased expression of the replicon-encoded, heterologous genes 10- to 20-fold (36). Upon transfection into the cells, replication of VEErepL/PARP12L did not noticeably alter either cell growth or morphology, but replication of the tested viruses was strongly affected (Supplementary Fig. 2), indicating that even lower levels of PARP12L expression are sufficient for developing the antiviral effect.

PARP12L expression affects intracellular replication rather than alphavirus release. The PARP12L inhibitory function could potentially result from its ability to interfere with either intracellular virus replication or release. In our earlier experiments (Fig. 8), VEEV/GFP and SINV/GFP demonstrated very poor GFP expression in cells expressing PARP12L, but not in the VEErep/PARPrev-containing cells. This was a strong indication that PARP12L expression may have a profound effect specifically on viral RNA replication, rather than other processes in the viral life cycle. To confirm this possibility, we infected BHK-21 and NIH 3T3 cells with packaged VEErep/PARP12L or VEErep/PARPrev replicons, and at 1 h post infection, infected them with packaged VEE...
replicons encoding luciferase and GFP genes under the subgenomic promoters (VEErep/Luc/GFP) (Fig. 9A). At all times post superinfection, luciferase activity in PARP12L-expressing cells was lower than that detected in the VEErep/PARPrev replicon-containing cells. This was an indication that PARP12L expression strongly affected expression of the replicon-encoded proteins, most likely due to its effect on viral RNA replication/transcription (Fig. 9B). We also indirectly measured the effect of PARP12L expression on RNA replication by assessing the accumulation of the nsP2 protein in the cells infected with packaged VEErep/PARP12L and VEErep/PARPrev. Starting from 5 h post infection, the replicon-encoded nsP2 was always found in VEErep/PARP12L-infected cells at a lower concentration, indicating a negative effect of PARP12L expression on replicon replication itself (Fig. 9C). These experiments successfully demonstrated PARP12L’s effect on alphavirus replication. However, they were unable to ascertain whether the difference was mediated by direct effects of PARP12L on function of virus-specific replication complexes, or an indirect negative effect on replication through inhibition of translation of virus-specific RNA, or both. These possibilities are now under detailed investigation.

Other PARPs demonstrate a negative effect on VEEV replication. PARP12L is a member of a reasonably large family of proteins containing the PARP domain (42), in the subfamily of CCCH-type Zn finger-containing PARPs (CCCH-PARPs). Other members of this subfamily include PARP7 and PARP13. The short splice form of PARP13 was previously termed ZAP (42). In the above-described microarray experiments, the PARP7 and PARP13 genes became activated in the NIH 3T3 cells upon their treatment with IFN-
β or during infection with VEEV/GFP/C1 (Fig. 10B). Further analysis of their antiviral functions was not given the highest priority, because of detectable activation of the same genes in the IFN-α/βR−/− MEFs upon their infection with the same VEEV mutant. However, this does not necessarily mean that one or more other PARP proteins have no anti-alphavirus functions. Therefore, to test the possible antiviral activity of other PARPs, we replaced the GFP gene in the VEEV/GFP/C1 genome with PARP7- and PARP13-coding sequences (Fig. 10A). In addition to CCCH PARPs, we also cloned a PARP10-coding sequence into the viral genome under control of an additional subgenomic promoter (Figs. 10A and B). PARP10 also contains a putative, albeit different, RNA-binding sequence (42) and showed an increase in transcriptional activity following treatment of NIH 3T3 cells with IFN-β or infection with VEEV/GFP/C1 (Fig. 10B). We then tested the effect of the expressed proteins on the rates of virus replication (Fig. 10C). VEEV/PARPrev/C1, containing the PARP12 gene in the reverse orientation, was used as a control. Expression of PARP7 inhibited VEEV replication with an efficiency similar to that described above for PARP12L (Fig. 6B), while PARP13 expression had no effect on VEEV replication. PARP10 demonstrated intermediate inhibitory activity. Thus, the ability to interfere with alphavirus replication appears not to be limited to PARP12L, and other members of the PARP superfamily may also be involved in development of the antiviral response.

**DISCUSSION**

The identification of new cellular genes, whose products demonstrate anti-alphavirus activities, is critical for further understanding of alphavirus pathogenesis and virus-host
interactions. Traditionally, replication of alphaviruses is considered to be highly
cytopathic for cells of vertebrate origin (45). This cytopathic phenotype is determined by
a combination of different factors, such as high-level expression of viral structural
proteins (10), rapidly induced transcriptional shutoff (10), and the ability to inhibit
translation and transcription in infected cells (20). Some of the cell lines used in
laboratory practice also develop apoptotic changes (31). However, current data suggest
that cytopathic effect is not necessarily an inevitable result of infection, and at least in the
murine brain, almost complete clearance of some alphaviruses can occur without wide-
spread neuronal death and pathological changes (6). Moreover, in spite of the virus’
ability to efficiently inhibit transcription and translation in vitro, infected animals are
capable of responding with type I and type II IFN production, which for some alphavirus
infections, can reach very high levels (13, 28, 48, 49). This suggests that, during in vivo
replication or in less permissive cells in vitro, alphavirus replication might not completely
inhibit transcription and translation, nor profoundly affect activation of the antiviral
response. As a result, the release of IFN and other cytokines and chemokines can be
detected.

Our previous studies (5, 18, 19), as well as those of other research groups (1, 8, 9), demonstrated that point mutations in the alphavirus genes encoding proteins with
transcription inhibitory functions make mutants dramatically less pathogenic and less
cytopathic. Both Old World alphaviruses containing point mutations in nsP2, and New
World alphaviruses with mutations in the capsid-specific amino terminal peptide, or
chimeric viruses encoding the Old World alphavirus structural protein genes and the New
World alphavirus-derived nsPs, can persistently replicate in cells defective in IFN-α/β
production or signaling without developing a profound CPE (5, 13, 27). However, in type I IFN-competent cells, replication of such mutated or chimeric viruses is downregulated to undetectable levels within a few days post infection (5). Moreover, addition of IFN-α/β-specific Abs to the media of infected type I IFN signaling-competent cells also makes replication persistent and similar to that observed in IFN-α/βR\(^{-/-}\), or STAT1\(^{-/-}\) MEFs (5, 13). This is a strong indication that the cellular antiviral response developed in the absence of autocrine IFN signaling is insufficient for virus clearance, even though these cells contain endogenous PRRs. In the experiments presented in this study, IFN-α/βR\(^{-/-}\) MEFs demonstrated activation of numerous cellular genes, including those encoding proteins with known antiviral activities, in response to mutant virus replication (Fig. 2). However, this response was insufficient for virus clearance (Fig. 1). In NIH 3T3 cells, which have no defects in type I IFN production and signaling, VEEV variants with mutated capsid protein induced type I IFN at readily detectable levels by 4-6 h post infection, and it reached a maximum level by 24 h post infection (Fig. 1 and (5)). The autocrine IFN signaling leads to a gradual decrease in VEEV/GFP/C1 replication, and within 5-6 days post infection, replication becomes undetectable, and cells remain viable (Fig. 1). This is a strong indication that the IFN treatment induces an additional, and most likely a multicomponent response that plays a critical role in the inhibition of virus replication. Therefore, in this study we made an attempt to define the previously underappreciated type I IFN-inducible genes that are involved in VEEV clearance from IFN-competent cells.

The microarray-based profiling and subsequent bioinformatic analysis pointed out the set of genes expressed in the IFN-competent, but not in the IFN-α/βR\(^{-/-}\) cells, during
VEEV/GFP/C1 mutant replication. These genes were also activated in the uninfected IFN-β-treated cells that demonstrated complete protection against subsequent viral infection (Figs. 2 and 3). Many of the selected 47 genes were previously shown to express proteins with virus inhibitory functions, and this was a good indication that the bioinformatic analysis was correct. After further analysis of the identified group of genes, Bst2 and PARP12 attracted more attention. Bst2, a tetherin, was previously found to have an anti-HIV-1 function. Bst2 interferes with release of HIV-1, as well as other enveloped viruses, from the cell surface (40, 41). Thus, its anti-VEEV activity detected in the experimental tests, was expected and mostly served as a control aimed at showing that the double subgenomic viruses and VEEV replicons represent adequate experimental systems for demonstrating antiviral functions of the genes of interest. In contrast, the antiviral function of PARP12 has not been previously described. From the PARP superfamily, only PARP13 (and its short splice form) were previously characterized as having an intrinsic immunological function, and were found in organisms of broad taxonomic range (26, 34). The inhibitory functions of PARP13 were demonstrated on murine leukemia virus and two alphaviruses, Sindbis and Semliki Forest virus (16, 34). Interference of PARP13 with virus replication appears to be based on its ability to bind and degrade virus-specific RNAs (56). PARP12 protein demonstrates a number of similarities with PARP13 in terms of domain structure (Fig. 10A), such as a PARP domain at the carboxy terminus, and five Zn finger domains. Additionally, PARP13 and PARP12 are expressed in two splice variants, with the short variants lacking the carboxy terminal PARP-encoding fragment. However, unlike PARP13, PARP12 has not been previously shown to possess virus inhibitory functions. In our experiments, the long
isoform of PARP12, PARP12L, was capable of downregulating replication of a broad range of evolutionarily distinct alphaviruses and other RNA viruses that do not belong to the alphavirus genus at all (Fig. 8). The mechanism of this broad inhibitory effect needs to be further investigated. The previously published data suggested that PARP13 mediates RNA degradation (56) and, thus, indirectly inhibits virus replication. We can speculate that based on its similarities to PARP13, PARP12 may have a similar function. However, our preliminary data indicate that it inhibits translation by a different mechanism (data not shown), which is now under detailed investigation (manuscript in preparation). Another possibility comes from the recent finding that a conserved domain of the alphavirus nonstructural protein nsP3 is structurally similar to a PARP domain (35). The exact function of nsP3 in virus replication is not completely understood, but regardless of nsP3’s activity, overexpression of PARP12L might interfere with the function of this alphavirus nonstructural protein. However, this hypothesis needs additional experimental support and does not explain the negative effect of PARP12L expression on other RNA viruses. Experiments with other PARPs have indicated that in the PARP superfamily, PARP12 and PARP13 are not the only proteins exhibiting anti-alphavirus activities. PARP7 and PARP10 also interfere with VEEV replication. Thus, the importance of the PARP superfamily in the development of an antiviral response is likely to be greater than we currently know. In additional experiment, we decreased PARP12 expression almost 10-fold using RNAi, but, as expected, failed to affect virus clearance significantly. The ability of other PARPs to inhibit VEEV replication and activation of a very wide variety of other ISGs during VEEV/GFP/C1 replication, explain
the lack of an observable effect of PARP12L knock down on the rates of virus clearance from NIH 3T3 cells (data not shown).

The results of this study suggest that PARPs are members of a growing group of IFN-inducible genes whose products exhibit antiviral effects (7, 30, 33, 39, 43, 46, 47, 55). So far, all of these proteins, expressed individually, demonstrate low but detectable antiviral activities. Thus, despite these antiviral effects, overexpression of these individual proteins is insufficient for complete protection of cells against viral infections. Additionally, knockout of the individual genes usually does not have a deleterious effect on the development of the innate immune response to viral infections (38). Thus, it appears that there is a highly redundant system involving numerous gene families, as well as multiple genes within these families, in the development of the antiviral response. This redundancy and lack of a dominant antiviral gene product, makes evolution of resistant virus variants an almost impossible task. This also complicates analysis of antiviral proteins, because dissecting the small effects of numerous individual gene products make accurate attribution of their particular antiviral functions very difficult. As a result, we are probably still in the early stages of understanding how hundreds of innate immune response genes interact in a coordinated fashion to regulate and thwart viral infections.

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29. **Lemm, J. A., R. K. Durbin, V. Stollar, and C. M. Rice.** 1990. Mutations which alter the level or structure of nsP4 can affect the efficiency of Sindbis virus replication in a host-dependent manner. J. Virol. 64:3001-3011.


**FIGURE LEGENDS**

Fig. 1. The VEEV variant with mutated capsid gene persistently replicates in IFN-α/βR−/− MEFs, but is cleared from IFN-competent cells. (A) The schematic representation of the VEEV genomes encoding wt and mutated capsid proteins. The mutated amino acids are presented in red. The NLS and the carboxy terminus of supraNES are indicated by boxes.
(3). Underlined aa indicate connecting peptide. nsP1-4 indicate nonstructural genes. C, E2 and E1 indicate structural genes. SG indicates subgenomic RNA promoter. (B) NIH 3T3 cells and IFN-α/βR−/− MEFs were infected with the indicated viruses at an MOI of 20 PFU/cell. Media were replaced at the indicated time points and titers of the released viruses were measured by plaque assay on BHK-21 cells. (C) Concentrations of IFN-β were measured in the samples (see panel B) harvested from the infected NIH 3T3 cells.

Fig. 2. Microarray data analysis. A, B and C present the sequential analysis used to reduce the number of genes to analyze further in this study (see text for details). The first panel shows the gene expression profiles in the VEEV/GFP/C1-infected NIH 3T3 cells. The expression profiles of the same genes in IFN-β-treated NIH 3T3 cells and in the infected IFN-α/βR−/− MEFs are presented in the second and the third panels, respectively. Each line reflects expression levels of a single gene at different time points. The red lines represent the genes whose expression was activated the most, and the blue color indicates the genes whose expression was downregulated the most in the VEEV/GFP/C1-infected compared to mock-infected NIH 3T3 cells. Other colors indicate intermediate levels of activation and downregulation of gene expression. Arrow indicates the end of IFN-β treatment.

Fig. 3. Four cellular genes were selected for further analysis. (A) The scheme used to define the final set of genes with putative antiviral functions. (B) The expression profiles of cellular genes that were selected for testing of their antiviral activities.
Fig. 4. qPCR analysis confirms the microarray data. The selected genes demonstrate higher levels of activation in RT-qPCR tests than those detected in the microarray experiments. The experiments were performed in triplicates (see Materials and Methods for details).

Fig. 5. PARP12L and Bst2 expression has a negative effect on VEEV mutant replication. (A) The schematic representation of recombinant viral genomes. HG indicates a position of cloned heterologous genes. (B) The schematic representation of long and short isoforms of PARP12 protein. (C) Replication of recombinant VEEV variants expressing the indicated heterologous genes in NIH 3T3 cells. Cells were infected at an MOI of 10 PFU/cell, media were replaced at the indicated times, and virus titers were determined by plaque assay on BHK-21 cells.

Fig. 6. The antiviral effects of PARP12L and Bst2, expressed from the viral genome, is dependent on the applied MOI and cell type. (A) The schematic representation of recombinant VEEV genomes encoding mutated capsid protein, Bst2 and the PARP12L genes in direct or reverse orientations. (B) NIH 3T3 and BHK-21 cells were infected with recombinant viruses encoding PARP12L, Bst2, or PARP12L in reverse orientation at the indicated MOIs. Media were harvested at the indicated times post infection, and virus titers were determined by plaque assay on BHK-21 cells.
Fig. 7. Expression of PARP12L affects replication of VEEV encoding wt capsid protein.
(A) The schematic representation of recombinant VEEV genomes encoding wt capsid protein and the PARP12L genes in direct or reverse orientations. (B) Analysis of replication of the recombinant viruses in BHK-21 and NIH 3T3 cells. Media were harvested at different times post infection, and virus titers were determined by plaque assay on BHK-21 cells.

Fig. 8. Expression of PARP12L affects replication of different alpha- and other RNA viruses. (A) The schematic representation of VEEV replicon expressing the PARP12L gene, control VEEV replicon, and the genomes of VEEV, SINV and CHIKV used for superinfection. PAC indicates a puromycin acetyl transferase gene. (B and C) BHK-21 cells were infected with packaged VEEV replicons, encoding PARP12L either in direct or reverse orientation, at an MOI of 20 inf.u/cells. One hour later, they were infected with the indicated viruses at an MOI of 1 PFU/cell, and media were replaced at the indicated times. Titers were determined by plaque assay on BHK-21 cells for all viruses.

Fig. 9. Expression of PARP12L strongly affects expression of genes encoded by the recombinant VEEV genome. (A) The schematic representation of VEEV replicons encoding PARP12L in either direct or reverse orientation or fire fly luciferase (Luc). (B) NIH 3T3 and BHK-21 cells were infected with packaged VEEV replicons expressing PARPrev and PARP12L an MOI of 20 inf.u/cell. After incubation for 1 h at 37°C, they were superinfected with packaged VEErep/Luc/GFP replicon at an MOI of 1 inf.u/cell. Luciferase activity was measured at indicated time points. (C) BHK-21 and NIH 3T3
cells were infected with packaged VEErep/PARP12L or VEErep/PARPrev replicons at an MOI of 20. Intracellular accumulation of VEEV nsP2 was measured at the indicated time points by Western blot as described in the Materials and Methods. The data were normalized to the level of β-tubulin.

Fig. 10. Expression of some other PARP proteins also downregulates VEEV replication. (A) The schematic representation of the VEEV genome encoding different PARP proteins, and the schematic representation of different PARPs, used in this study. Zn fingers and domains are indicated. (B) Activation of different PARP genes in the NIH 3T3 cells infected with VEEV/GFP/C1 or treated by IFN-β for 24 h. Activation of these genes was measured in the microarray experiments presented in Figs. 2 and 3. (C) Replication of recombinant PARP-expressing viruses and VEEV/PARPrev in BHK-21 and NIH 3T3 cells after infection at an MOI of 10 PFU/cell. Media were replaced at the indicated time points, and virus titers were determined by plaque assay on BHK-21 cells.
Step 1.

1575 genes demonstrated different expression profiles in virus-infected NIH 3T3 cells compared to uninfected cells.

Step 2.

140 genes were induced more then 2-fold in both infected and IFN-β-treated NIH 3T3 cells.

Step 3.

Expression of 98 IFN-β-dependent genes was not upregulated in persistently infected IFN-α/βR-/- MEFs, but was upregulated in infected NIH 3T3 cells.
A) 98 genes identified at the last step of analysis were divided into two groups

51 genes located in groups in the genome
47 genes with discrete, independent locations

<table>
<thead>
<tr>
<th>Gene families</th>
<th>Number of genes</th>
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<td>Nuclear body complex</td>
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<td>Torsin family</td>
<td>3</td>
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<td>Ilf 200 family</td>
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<td>7</td>
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</table>

IRF1, IRF7, IRF9, STAT1, LGP2, PKR, RIG-1, TLR3, and others.

B)
A) VEEV genome

Zn finger domain PARP domain PARP7
Zn finger domain PARP domain PARP12L
Zn finger domain PARP domain PARP13
Zn finger domain PARP domain PARP10

B) NIH 3T3 VEEV/GFP/C1 NIH 3T3 IFN-β-treated

Fold difference in RNA expression (log2)

Days post infection

Days post treatment

PARP12  PARP10  PARP7  PARP13

C) BHK-21 MOI 10

Virus release (PFU/h)

Hours post infection

NIH 3T3 MOI 10

Virus release (PFU/h)

Hours post infection