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RNA-Seq analysis reveals that spring viraemia of carp virus induces a broad spectrum of Pim kinases in zebrafish kidney that promote viral entry

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Abstract

PIM kinases are a family of serine/threonine protein kinases that potentiate the progression of the cell cycle and inhibit apoptosis. Because of this, they are considered to be proto-oncogenes, and they represent an interesting target for the development of anticancer drugs. In mammals, three PIM kinases exist (PIM-1, PIM-2 and PIM-3), and different inhibitors have been developed to block their activity. In addition to their involvement in cancer, some publications have reported that the PIM kinases have pro-viral activity, and different mechanisms where PIM kinases favour viral infections have been proposed. Zebrafish possess more than 300 Pim kinase members in their genome, and by using RNA-Seq analysis, we found a high number of Pim kinase genes that were significantly induced after infection with spring viraemia of carp virus (SVCV). Moreover, analysis of the miRNAs modulated by this infection revealed that some of them could be involved in the post-transcriptional regulation of Pim kinase abundance. To elucidate the potential role of the 16 overexpressed Pim kinases in the infectivity of SVCV, we used three different pan-PIM kinase inhibitors (SGI-1776, INCB053914 and AZD1208), and different experiments were conducted both *in vitro* and *in vivo*. We observed that the PIM kinase inhibitors had a protective effect against SVCV, indicating that, similar to what is observed in mammals, PIM kinases are beneficial for the virus in zebrafish. Moreover, zebrafish Pim kinases seem to facilitate viral entry into the host cells because when ZF4 cells were pre-incubated with the virus and then were treated with the inhibitors, the protective effect of the inhibitors was abrogated. Although more investigation is necessary, these results show that pan-PIM kinase inhibitors could serve as a useful treatment for preventing the spread of viral diseases.

Keywords: zebrafish, SVCV, Pim kinases, pan-PIM kinase inhibitors, viral entry, antiviral

Introduction

The proviral insertion site in Moloney murine leukaemia virus (PIM) kinases are a family of serine/threonine protein kinases involved in the regulation of different cellular processes [1]. In mammals, three PIM kinase members exist (PIM-1, PIM-2 and PIM-3). They are considered proto-oncogenes due to their ability to promote cell survival and proliferation by inhibiting apoptosis and positively regulating cell cycle progression, among other functions [1]. Because of these functions, PIM kinases are involved in tumourigenesis and represent an attractive target for pharmacological anticancer therapy [2]. PIM protein expression is induced via the JAK/STAT signalling pathway, which is activated by several cytokines after cytokine-receptor interaction [3]. Therefore, these kinases are induced after a variety of immune stimuli, indicating their role in the immune response [4].

PIM kinases lack a regulatory domain [5]; therefore, it is thought that these proteins are constitutively active when expressed in cells and, consequently, that their activity is directly correlated with their transcription level [3]. Due to their involvement in cancer progression, many efforts have been made to develop efficient PIM kinase inhibitors. Most of them block the activity of all three PIM isoforms, so they serve as pan-PIM kinase inhibitors [1]. Moreover, although only a few inhibitors have been tested in human clinical trials, the inhibition of all the PIM isoforms in murine models revealed very modest side effects, maintaining animal viability and fertility [6].

PIM kinases have also been linked to the progression of certain viral pathogens. A few publications reported that PIM kinase inhibition ameliorated the resolution of a variety of viral diseases, although this effect was tested exclusively *in vitro*, and the mechanisms by which PIM kinases promote viral replication seem to be different [7-11].

Whereas mammals possess three highly evolutionarily conserved PIM kinase isoforms, more than 300 Pim kinases have been identified in zebrafish by computational analysis [12]. The expansion of gene families is frequent in teleost species [13, 14], which sometimes makes the establishment of functional equivalences between mammalian and fish proteins difficult. Nevertheless, despite this great diversity of Pim kinases in zebrafish, a high conservation of functionally important residues has been observed between human and zebrafish PIM kinases [12].

As a model species, the zebrafish is a very useful organism to study a multitude of biological processes, including infectious diseases [15]. Among viruses, one of the most commonly used to challenge zebrafish is the spring viraemia of carp virus (SVCV) [16]. SVCV is an enveloped, bullet-shaped, negative-sense, single-stranded RNA virus belonging to the Rhabdoviridae family [17]. This family also includes other viruses causing relevant economic losses in the fish aquaculture industry [18-20]. Moreover, humans and mammals are also affected by rhabdoviruses, such as the rabies virus and the vesicular stomatitis virus [17]. Therefore, advances in the knowledge of the antiviral immune response in zebrafish could help to gain a better understanding of the defence mechanisms against rhabdoviruses or viruses in general in other species.

High-throughput sequencing technologies have emerged as a powerful tool to thoroughly analyse the transcriptome response to a specific stimulus or condition. In this work, we conducted RNA-Seq analysis of kidney samples from SVSV-infected and uninfected zebrafish to evaluate the gene modulations (mRNA changes) induced after infection. The differential expression analysis between infected and control individuals showed that in addition to a multitude of immune-related genes, a broad spectrum of Pim kinases is induced after SVSV challenge. Additionally, the microRNA (miRNA) profile was also obtained. We found that at least five different miRNAs affected by the infection have Pim kinase mRNA as a potential target, revealing that the level of these kinases could be regulated by miRNAs after viral challenge. To better understand the involvement of Pim kinases in SVCV progression, we tested three different pan-Pim kinase inhibitors both *in vivo* and *in vitro.* Our results clearly showed that blocking Pim kinase activity reduces SVCV entry into the cells and consequently ameliorates the survival of infected zebrafish larvae.

Material and methods

Animals, virus and cell lines

Six-month-old wild-type zebrafish were obtained from the facilities at the Instituto de Investigaciones Marinas (Vigo, Spain), where zebrafish are maintained following established protocols [21-22]. Zebrafish were euthanized using a tricaine methanesulfonate (MS-222) overdose (500 mg/l). Fish care and challenge experiments

were conducted according to the guidelines of the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG. Wild-type zebrafish larvae were also obtained in the same facilities.

SVCV isolate 56/70 was propagated in epithelioma papulosum cyprini (EPC) carp cells (ATCC CRL-2872) that were maintained in MEM (Gibco) supplemented with 2 % FBS (Gibco) and 1 % penicillin/streptomycin solution (Gibco), and the cells were titrated in 96-well plates. The $TCID_{50}$/ml was calculated according to the Reed and Muench method [23].

For *in vitro* assays, the zebrafish fibroblastic cell line ZF4 (ATCC CRL-2050) was maintained in DMEM (Gibco) supplemented with 10 or 2 % FBS (Gibco) and 1 % penicillin/streptomycin solution (Gibco), and the cells were kept at 27°C.

Experimental design and samples for sequencing

Twelve adult zebrafish were injected intraperitoneally (i.p.) with 20 µl of SVCV (3×10^2 $TCID_{50}$/ml), and as a control group, the same number of fish were inoculated with an equivalent volume of MEM + 2 % FBS + penicillin/streptomycin. That viral concentration was previously tested and resulted in a survival rate of 20 % [24]. Kidney samples were collected at 24 h post-infection (hpi), and the same quantity of tissue from 4 animals was pooled, obtaining 3 biological replicates (4 fish/replicate) per condition. Samples were stored at -80°C until RNA extraction.

High-throughput transcriptome sequencing (mRNA and miRNA)

Total RNA from the different samples was extracted using a Maxwell 16 LEV simplyRNA Tissue kit (Promega) with an automated Maxwell 16 Instrument in accordance with the instructions provided by the manufacturer. The quantity of RNA was measured in a NanoDrop ND-1000 (NanoDrop Technologies, Inc.), and RNA integrity was analysed in an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. All the samples passed the quality control tests and were used for Illumina library preparation.

For mRNA sequencing, double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA), and sequencing was performed using Illumina HiSeq 4000 technology. For miRNA-Seq, a

TruSeq small RNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used, and sequencing was conducted with HiSeq 2500 technology. Both types of sequencing were conducted by Macrogen Inc. (Seoul, Republic of Korea).

The read sequences obtained with both methodologies were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA532380.

Trimming, mapping, RNA-Seq and differential expression analysis of mRNAs

CLC Genomics Workbench, v. 11.0.2 (CLC Bio, Aarhus, Denmark) was used to filter and trim reads, map the high-quality reads against the last version of the zebrafish genome (GRCz11) and perform the RNA-Seq statistical analyses. Raw reads were trimmed to remove adaptor sequences and low-quality reads (quality score limit 0.05 on the Phred scale). RNA-Seq analyses were performed using the zebrafish genome with the following parameters: length fraction = 0.8, similarity fraction = 0.8, mismatch cost = 2, insertion cost = 3 and deletion cost = 3. The expression values were set as transcripts per million (TPM). Finally, a differential expression analysis test was used to compare gene expression levels and to identify differentially expressed genes (DEGs). Transcripts with absolute fold change (FC) values > 2 and p-values < 0.05 were retained for further analyses. A heat map showing hierarchical clustering of gene expression (TPM values) was constructed using the complete linkage method with Euclidean distance.

Gene Ontology (GO) enrichment, KEGG pathways and domain analyses

For the up- and downregulated DEGs between SVCV-infected and uninfected zebrafish, we conducted GO enrichment analysis of biological processes, KEGG pathway analysis and domain enrichment using DAVID software [25, 26]. The significance level was set at 0.05 ($p < 0.05$) in all cases. For domain enrichment, the Protein Information Resource (PIR) database [27] was used. The representation of the different categories was based on the fold-enrichment value.

Sequence alignment and identity/similarity matrix

Protein sequences for the Pim kinase genes modulated after infection were obtained from the zebrafish genome via Ensembl

(http://www.ensembl.org/Danio_rerio/Info/Index) [28]. The region corresponding to the Pim kinase domain was selected, and alignment was conducted using the ClustalW server [29]. Sequence similarity and identity scores were calculated with the software MatGAT [30] using the BLOSUM62 matrix.

Analysis of the zebrafish miRNome and target prediction

miRNAs are small non-coding RNAs that are evolutionarily conserved, and they regulate gene expression at the post-transcriptional level by interacting with the 3'UTR of mRNAs and recruiting molecular machinery that degrades the target mRNAs [31, 32]. Therefore, miRNAs could serve as key mechanisms of post-transcriptional gene silencing.

The CLC Genomics Workbench, v. 11.0.2 (CLC Bio, Aarhus, Denmark) was also used for small RNA analysis. The raw reads were also filtered (quality score limit 0.05 on the Phred scale) and trimmed to delete adaptor sequences. High-quality reads with lengths ranging from 15 to 30 nucleotides were retained as small RNAs. RNA-Seq analyses were conducted using the zebrafish mature miRNAs database downloaded from the miRBase 22.1 (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=dre) [33] as reference sequences. For the analysis, the following settings were used: mismatches = 2, length fraction = 0.6, similarity fraction = 0.5. The expression values were set as transcripts per million (TPM). Finally, a differential expression analysis test was used to compare gene expression levels. Those miRNAs with an FC > 2 were selected for further analyses (statistical restriction was not applied due to the presence of only one biological replicate per condition).

Based on the probability of interaction between the different mature miRNAs and the 3'UTR of the zebrafish genes, the prediction of the potential gene targets for the differentially expressed miRNAs was conducted using TargetScanFish v6.2 [34] and mirMAP [35]. For the TargetScanFish, those potential targets with a total context+ score < -0.3 were considered, and for the mirMAP, those with a mirMAP score > 90 were considered.

Quantitative PCR (qPCR) validation of RNA-Seq and miRNA data

For DEG validation, cDNA synthesis of the samples was performed with an NZY First-Strand cDNA Synthesis kit (NZYTech) using 0.5 µg of total RNA. A total of

4 genes were used to validate the RNA-Seq results. Specific qPCR primers were designed using Primer 3 software [36], and their amplification efficiency was calculated with the threshold cycle (CT) slope method [37]. Primer sequences are listed in Supplementary Table S1. Individual qPCR reactions were carried out in a 25 µl reaction volume that contained 12.5 µl of SYBR GREEN PCR Master Mix (Applied Biosystems), 10.5 µl of ultrapure water, 0.5 µl of each specific primer (10 µM) and 1 µl of two-fold diluted cDNA template; reactions were performed in MicroAmp optical 96-well reaction plates (Applied Biosystems). Reactions were conducted using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems). qPCR conditions consisted of an initial denaturation step (95°C, 10 min), which was followed by 40 cycles of a denaturation step (95°C, 15 s) and one hybridization-elongation step (60°C, 1 min). The relative expression levels of the different genes were normalized following the Pfaffl method [37]; *18s ribosomal RNA* (*18s*) was used as a reference gene. Fold-change units were calculated by dividing the normalized expression values in SVCV-infected zebrafish by the normalized expression values of the controls.

For miRNA validation, RNA samples (0.25 µg) were reverse transcribed with a miScript II RT kit (Qiagen). Primers for 4 miRNAs were purchased based on the exact sequence of the zebrafish mature miRNA deposited in the mirBASE [33]. We selected the U6 snRNA (5'-ATGACACGCAAATCCGTGAAG-3') as a reference sequence for normalization. qPCR reactions were conducted with a miScript SYBR Green PCR Kit (Qiagen) following the manufacturer's recommendations. Reactions were conducted using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems). qPCR conditions consisted of an initial denaturation step (95°C, 15 min), which was followed by 40 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s) and extension (70°C, 34 s). Fold-change units were calculated by dividing the normalized expression values in SVCV-infected zebrafish by the normalized expression values of the controls.

Pimr106 expression after SVCV or Poly I:C challenge

Adult (9 month) zebrafish were i.p. injected with 10 µl of an SVCV suspension (3×10^6 $TCID_{50}$/ml), and the corresponding controls were injected with the same volume of culture medium (MEM + 2 % FBS + penicillin/streptomycin). The same experiment was conducted using polyinosinic:polycytidylic acid (Poly I:C) (1 mg/ml in

PBS; Sigma–P1530), and the corresponding controls were injected with PBS. Both the SVCV and Poly I:C concentrations were previously tested for the induction of a significant immune response [38,39]. To analyse the induction of the *pim proto-oncogene, serine/threonine kinase, related 106* (*pimr106*) gene by qPCR, kidney samples were taken from anaesthetized fish at 3, 6 and 24 h post-stimulation, and 4 biological replicates (4 fish/replicate) per time point were obtained. Additionally, the expression of two pivotal genes involved in the type I interferon response was analysed in the Poly I:C-stimulated fish and the corresponding controls to confirm the activation of the typical antiviral response: *interferon phi 1* (*ifnphi1*) and *interferon-stimulated gene 15* (*isg15*). The primers used for gene amplification are listed in Supplementary Table S1.

Pan-PIM kinase inhibitors

PIM kinase inhibitors used in this work were SGI-1776 (Calbiochem; Ref. 526528), INCB053914 (Selleckchem; Ref. S8800) and AZD1208 (Sigma-Aldrich; Ref. SML2595). The compounds were resuspended in DMSO.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay in ZF4 cells

Based on the literature [40-42], we selected the following concentrations of PIM kinase inhibitors to conduct the functional assays: 5 µM SGI-1776, 2 µM INCB053914 and 10 µM AZD1208. ZF4 cells were seeded in 96-well plates and treated for 24 h with these concentrations of inhibitors; as a control condition, cells were treated with the vehicle alone (0.002 % DMSO). A total of 12 wells per treatment were included. To test cell viability, an MTT assay was conducted with a Vybrant MTT Cell Proliferation Assay Kit (Life Technologies). Briefly, the cell medium was replaced with 100 µl of fresh medium, and then 10 µl of MTT stock solution (12 mM) was added to each well and incubated for 4 h. After this period, a 25 µl volume was removed from each well, and 50 µL of DMSO was added. After 10 min, the absorbance was measured at 540 nm with a spectrophotometer microplate reader (iEMS reader MF; Labsystems).

Effect of PIM kinase inhibitors on the expression of cell cycle-related genes

ZF4 cells were seeded in 24-well plates, and on the next day, they were treated for 24 h with the pan-PIM kinase inhibitors SGI-1776 (5 µM), INCB053914 (2 µM), AZD1208 (10 µM) or vehicle alone (0.002 % DMSO). Total RNA was isolated (3

biological replicates/treatment), and qPCRs were conducted to detect the expression of the cell cycle-related genes *cellular tumour antigen p53 (tp53)*, *cyclin-dependent kinase inhibitor 1a (p21)* and *e3 ubiquitin-protein ligase mdm2 (mdm2)*. Moreover, we also analysed whether the inhibition of Pim kinase activity is compensated for by higher gene expression of these proteins. For this, we selected *pimr106* as a prototypical gene. The primer pairs used are listed in Supplementary Table S1.

Treatment of ZF4 cells with pan-PIM kinase inhibitors and infection with SVCV

ZF4 cells were seeded in 96-well plates, and on the next day, the media was removed and was replaced by new media (DMEM + 2 % FBS + P/S) containing 5 µM SGI-1776, 2 µM INCB053914, 10 µM AZD1208 or 0.002 % vehicle (DMSO). These concentrations were previously reported to inhibit the activity of the PIM kinases in a variety of cell lines [40-42]. After incubation for 24 h at 27°C, new treatments were added that contained seven 10-fold serial dilutions of SVCV (highest concentration: 3×10^7 $TCID_{50}$/ml) for viral titration, which was performed in triplicate according to the Reed and Muench method [23]. Non-infected controls were also included. This experiment was conducted five times. In parallel, 24-well plates were also seeded with ZF4 cells and treated with the different pan-PIM kinase inhibitors or the vehicle alone. After 24 h, new treatments were also added together with the SVCV (3×10^5 $TCID_{50}$/ml). At 24 h post-infection (hpi), the media was removed, the cells were washed with PBS, total RNA was isolated (4 biological replicates/treatment) and qPCR was conducted to detect the SVCV N gene (the primers used are listed in Supplementary Table S1).

To elucidate if the pan-PIM kinase inhibitors could affect viral entry, ZF4 seeded onto 96-well plates were infected with seven 10-fold serial dilutions of SVCV (highest concentration: 3×10^9 $TCID_{50}$/ml) in triplicate; after 5 h, the media was removed, the wells were washed twice with PBS and then cells were treated with the pan-PIM inhibitors or the vehicle alone at the same concentrations mentioned above. The viral titer for the different conditions was estimated based on the visualization of cytopathic effect (CPE) according to the Reed and Muench method [23]. This experiment was replicated three times. As in the previous experiment, 24-well plates were also seeded with ZF4, and in this case they were infected with the virus (3×10^7 $TCID_{50}$/ml) for 5 h, washed twice with PBS and then treated with the different pan-PIM

kinases. At 24 h post-infection (hpi), the media was removed, the cells were washed with PBS, total RNA was isolated (4 biological replicates/treatment) and qPCR was conducted to detect the SVCV N gene.

In vivo treatment of zebrafish larvae with pan-PIM kinase inhibitors

Zebrafish larvae (2 days post-fertilization–dpf) were placed in 6-well plates (10 larvae per well) in a volume of 6 ml. Larvae from 6 wells were pre-treated with SGI-1776 (5 µM), INCB053914 (2 µM), AZD1208 (10 µM) or vehicle alone (0.002 % DMSO). After 24 h (3 dpf larvae), half of the larvae from each treatment were infected via the duct of Cuvier with 2 nl of an SVCV suspension (5×10^4 $TCID_{50}$/ml; 10 % phenol red), and the other half were inoculated with the same volume of PBS with 10 % phenol red, as previously described [39]. For microinjections, we used glass capillaries coupled to a micromanipulator (MN-151, Narishige, Japan) and a FemtoJet 4x microinjector (Eppendorf, Germany). Mortality was assessed through 6 dpi using three biological replicates comprised of 10 larvae each. This experiment was replicated three times. In parallel, samples were also taken after 24 h (3 biological replicates, 4-5 larvae replicate) to analyse the viral replication in infected larvae by qPCR.

Statistical analyses

For qPCR experiments, the results are represented graphically as the mean ± standard error of the biological replicates. Significant differences were determined with the computer software package IBM SPSS Statistics v25 using Student's t-tests. Kaplan-Meier survival curves were analysed with a log-Rank (Mantel-Cox) test. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Results

Sequencing and mapping information of the coding RNA

A summary of the reads per sample, trimming results, and mapping information is included in Table 1. A total of 543.596.316 million reads were obtained from the different samples of zebrafish, with an average of 90 million per sample, and over 99 %

of raw reads passed the quality control. From these high-quality reads, 97.28 % successfully mapped to the zebrafish genome, with an average value of 97.27 % per sample. Therefore, only 2.72 % of the reads remained unmapped, with an average value of 2.73 % per sample.

Differentially modulated genes, GO enrichment, KEGG pathways and domain enrichment analysis

When we analysed the expression of the different zebrafish genes in those individuals infected with SVCV compared to the uninfected fish, a total of 714 DEGs were observed (Supplementary Table S2; Figure 1). A heat map representing the TPM values of the DEGs across the different samples showed well-differentiated clusters of genes (Figure 1A) one of the clusters corresponded to those genes overexpressed in the control samples and another to those overexpressed in infected fish. The three biological replicates of each condition clustered together (Figure 1A), indicating a good consistency of the results.

Whereas 343 DEGs were significantly upregulated after viral infection, a total of 371 were inhibited following the viral challenge (Supplementary Table S2; Figure 1B). These RNA-Seq results were validated by qPCR of 4 genes. The qPCR results for the tested genes exhibited the same modulation pattern that was observed in the RNA-Seq data (Supplementary Table S3A).

For the genes that were downregulated after SVCV infection, GO biological process enrichment showed a variety of terms, but a large number were related to the synthesis of corticosteroids, muscle contraction/formation, cytoskeleton organization and calcium transport (Figure 2A). This was also reflected in the KEGG pathways analysis (Figure 2B). Domain enrichment analysis showed the “Kelch-like protein, gigaxonin type” and “ATP-gated ion channel P2X4 receptor” as the domain families enriched for the downregulated genes (Figure 2C).

As expected, GO enrichment analysis of the upregulated genes revealed a high representation of biological processes related to immunity, especially to the antiviral response (Figure 3A). These significantly enriched immune terms were “response to the virus”, “cell chemotaxis”, “neutrophil chemotaxis”, “defence response to virus”, “negative regulation of apoptotic process”, “inflammatory response”, “immune

response" and "innate immune response". This elevated representation in immune terms was also reflected in the KEGG pathways, where all the pathways significantly enriched for the genes induced after SVCV were related to the antiviral response: "Cytosolic DNA-sensing pathway", "NOD-like receptor signalling pathway", "Toll-like receptor signalling pathway", "Cytokine-cytokine receptor interaction", "Jak-STAT signalling pathway" and "Herpes simplex infection" (Figure 3B). Interestingly, when the domain enrichment analysis was conducted, only one domain family was represented for the overexpressed genes, and it corresponded to "Proto-oncogene serine/threonine-protein kinase Pim-1" (Figure 3C).

A variety of Pim kinases are induced after SVCV infection

Because the "Proto-oncogene serine/threonine-protein kinase Pim-1" domain was the only domain overrepresented among the genes induced after viral challenge, we wanted to analyse in a more detailed way this family of proteins identified in our RNA-Seq results. We found a total of 16 Pim kinases upregulated in zebrafish kidneys 24 h after SVCV challenge (Table 2). The fold-change values ranged from 3.94 to 89.15. The TPM values of the different replicates are represented in Figure 4.

To further understand the potential implication of the 16 Pim kinases modulated in response to the virus, we first analysed whether they effectively correspond to the PIM kinase family. We searched the Pim kinase domain and the characteristic adenosine triphosphate (ATP)-binding site in all of these sequences. We conducted an alignment of the Pim kinase domain of these 16 Pim kinases, although the domain was incomplete for some partial sequences due to genome sequencing ambiguities (Figure 5A). In general, the Pim kinase domain was relatively well conserved across the different zebrafish Pim kinases induced upon SVCV challenge. Indeed, the similarity percentage was always above 40 % for the different comparisons (Figure 5B). Therefore, we confirmed that these proteins corresponded to the Pim kinase family.

Pimr106 is induced early after SVSV infection, and its increase is not mediated by viral nucleic acids

We selected one of the most overexpressed Pim kinases, *pimr106* (*pimr106*, FC = 60), to analyse its expression pattern after viral challenge. We analysed its transcription in adult zebrafish infected with SVCV or stimulated with Poly I:C for 3, 6

and 24 h. Poly I:C, as a synthetic analogue of viral dsRNA, was also inoculated to determine if viral nucleic acids affected the expression of Pim kinases. The gene *pimr106* was already overexpressed in SVCV-infected fish at 3 hpi, and its expression remained higher than the uninfected control until 24 hpi (Figure 6). Poly I:C did not induce significant differences in the expression of *pimr106* at the tested sampling points (Figure 6), although this compound significantly increased the expression of the antiviral genes *ifnphi1* and *isg15* (Supplementary Figure S1). Based on this, viral nucleic acids do not seem to induce Pim kinases during viral infection.

miRNAs as potential modulators of Pim kinase expression

In addition to mRNA, we wanted to test the miRNA profile after infection. Due to the presence of only one biological replicate, these results should be carefully considered. Nevertheless, we validated 4 different miRNAs in three independent biological replicates, and a very comparable expression pattern was observed between the RNA-Seq and qPCR results (Supplementary Table S3B). For the control zebrafish, more than 27 million raw reads were obtained, and 59.18 % passed the filter parameters (Table 3A). For the infected fish, we obtained more than 25 million raw reads, and 82.46 % passed the filters (Table 3A). Because we annotated our results using the mature miRNA database of zebrafish, only 597 and 546 reads from the control and infected samples, respectively, were successfully annotated to one of the 355 mature miRNAs (Table 3A).

We found 47 mature miRNAs modulated (FC >2) in the kidney after SVCV infection (Figure 7; Supplementary Table S4); 24 of them were particularly overexpressed, whereas 23 were downregulated. By analysing the potential 3' untranslated region (3'UTR) targets of these miRNAs, we found that 5 of the modulated miRNAs could interact with Pim kinases (Table 3B). Although only one of the predicted targets corresponded to a significantly modulated Pim kinase, this opens the door to further studies of whether zebrafish Pim kinase expression is regulated by miRNAs.

Assay to determine ZF4 cell viability after treatment with pan-PIM kinase inhibitors

To confirm the non-cytotoxic effect of the concentrations of pan-PIM kinase inhibitors used in this work, we conducted an MTT assay in ZF4 cells treated for 24 h

with the three inhibitors. The tested concentrations seemed not to be cytotoxic to ZF4 cells, and even a slight increase in the formazan precipitation was observed for the cells treated with the inhibitors compared to the control cells (Supplementary Figure S2). Therefore, the inhibitors were not cytotoxic to ZF4 cells at the tested concentrations.

PIM kinase inhibitors do not alter the expression of pimr106, but they do affect the expression of genes related to the cell cycle

We analysed whether the use of pan-PIM kinase inhibitors could affect the expression of zebrafish Pim kinases. When we analysed the expression of *pimr106* in ZF4 cells treated with the inhibitors for 24 h, we did not observe significant differences in the expression of this gene (Supplementary Figure S3). This could indicate that the use of the inhibitors is not compensated for by increased transcription of the Pim kinase genes.

Due to the involvement of PIM kinases in the progression of the cell cycle, we wanted to confirm the alteration of this process by pan-PIM kinase inhibitors in zebrafish cells. To do this, we analysed the expression of three genes directly involved in the cell cycle; two of them act as tumour suppressors (*tp53* and *p21*), and another one has oncogenic activity (*mdm2*), similar to that of the PIM kinases. Independent of whether their impact on the cell cycle is positive or negative, the three genes were inhibited by the three pan-PIM kinase inhibitors (Supplementary Figure S4).

Pim kinase inhibition reduces the SVCV titer in ZF4 cells

We wanted to study the potential effect of the repertoire of zebrafish Pim kinases in SVCV infection. For this, we conducted two different assays in ZF4 cells. In one of them, we pre-treated the cells with three pan-PIM kinase inhibitors (SGI-1776, INCB053914 or AZD1208). Then, after 24 h, we infected the cells with seven 10-fold dilutions of SVCV in the presence of the inhibitors. We observed a significant reduction in the viral titer in the presence of the three inhibitors, especially SGI-1776 (Figure 8A). For this compound, the reduction was 3-log compared to the untreated cells, whereas for INCB053914 and AZD1208, the viral titer was reduced by 2-log or more than 2-log, respectively (Figure 8A). When the expression of the N gene from SVCV was analysed by qPCR at 24 h post-infection, a significant reduction in the viral nucleoprotein gene levels was also observed following treatment with the three drugs (Figure 8B).

Interestingly, when the cells were infected with serial dilutions of SVCV for 5 h, washed and then treated with the inhibitors, these differences in the viral titer almost disappeared; differences were less than 1-log, or there was no difference (Figure 8C). This was also confirmed by qPCR at 24 h post-infection (Figure 8D). Therefore, it seems that Pim kinases mainly mediate SVCV entry.

Pan-PIM kinase inhibitors protect zebrafish larvae from SVCV infection

To better understand the implication of the Pim kinases in the death caused by SVCV, we pre-treated 2 dpf larvae with the PIM kinase inhibitors for 24 h, and they were infected by microinjection into the duct of Cuvier and returned to the water containing the different inhibitors. Kaplan-Meier survival curves showed that the three drugs increased the survival of the larvae (Figure 9A). Whereas the untreated larvae showed a 33.3 % survival, this percentage increased to 70.4 % with SGI-1776, 55.6 % with INCB053914 and 53.6 % with AZD1208. Although the differences in survival were significantly different only between the control and SGI-1776 groups, as assessed by a log-Rank (Mantel-Cox) test, if we analyse the survival at the end of the experiment with a Student's t-test, the three inhibitors significantly protected larvae from SVCV. For the uninfected larvae, a mean survival of 95 % was achieved. qPCR analysis of the SVCV N gene at 24 h post-challenge showed a significant reduction in the viral detection in the groups treated with the three inhibitors (Figure 9B).

Discussion

SVCV is a Rhabdovirus predominantly affecting cyprinid fish, and it is a cause of death and, consequently, economic losses in the aquaculture industry [43]. Moreover, due to the high susceptibility of the model species zebrafish to this virus, the SVCV-zebrafish interaction could be a useful tool for studying antiviral mechanisms or potential treatments for Rhabdovirus infecting mammals.

Some previous publications reported the transcriptome of zebrafish in response to SVCV. This is the case for the microarrays conducted for kidney samples [44-46] or an RNA-Seq analysis of the brain and spleen [47]. However, these transcriptome studies were mainly focused on the typical immune response and on the effect of certain mutations or immunostimulants in the response to SVCV. Some publications also

reported the modulation of non-coding RNAs after SVCV challenge, as seen in long non-coding RNAs (lncRNAs) [24] or the miRNA profile, which was analysed *in vitro* using the carp cell line EPC [48].

In this work, we conducted RNA-Seq analysis of kidney samples from adult zebrafish infected or not infected with SVCV for 24 h. Both the mRNA and miRNA profiles were analysed. A total of 714 DEGs were significantly modulated (343 upregulated and 371 downregulated) following the infection. Whereas the downregulated genes were mainly involved in the synthesis of steroid hormones and muscle and cytoskeleton organization, the genes overexpressed following virus treatment were directly related to the antiviral immune response. Interestingly, GO terms related to the negative regulation of apoptosis were also enriched, and these were mainly conformed by several Pim kinase proteins. As mentioned in the introduction, PIM kinases are inhibitors of apoptosis and are positive regulators of cell cycle progression [1]. Moreover, the Pim kinase domain was the only domain significantly enriched in the upregulated genes. In addition, four miRNAs affected by the SVCV challenge are potential modulators of different members of the PIM kinase family. Although further functional studies would help us to determine if the interaction of these miRNAs and the 3'UTR of certain zebrafish Pim kinases exist, this observation could shed some light on the mechanisms regulating the mRNA levels of PIM kinases.

The involvement of PIM kinases in the context of viral infections has hardly been studied. To the best of our knowledge, only a few publications have investigated the potential role of PIM kinases in the progression of viral diseases. The first publication reporting the pro-viral effect of PIM kinases was published by Rainio et al. [7], and it was based on the role that PIM kinases played in the ability of Epstein-Barr virus to immortalize B-cells and predispose them to malignant growth. PIM-1 and PIM-3 also induce reactivation of a herpes virus, Kaposi's sarcoma herpesvirus (KSHV), from its latency due to the phosphorylation of the KSHV latency-associated nuclear antigen (LANA) on specific serine residues [8]. After that, another study revealed that the inhibition of PIM-1 reduced viral replication in primary bronchial epithelial cells, and this was attributed to enhanced apoptosis upon viral infection, limiting viral replication and spread [9]. In the same year, Park et al. [10] found that the hepatitis C virus (HCV) nonstructural 5A protein interacts with PIM kinases and stabilizes them, and PIM kinases regulate HCV entry via unknown mechanisms without affecting the

other steps of the HCV life cycle. Finally, it has been shown that PIM kinases phosphorylate the human immunodeficiency virus (HIV) protein Vpx, which in turn promotes the ubiquitin-mediated proteolysis of Sterile alpha motif and histidine-aspartate domain-containing protein 1 (SAMHD1), an inhibitor of the transcription of several lentiviruses, including HIV [11]. Therefore, inhibition of the PIM kinases by treatment with the inhibitor AZD1208 allowed increased SAMHD1 activity and, as a consequence, decreased lentivirus replication [11].

Although the potential mode of action for PIM kinases in favouring viral progression varies enormously among the different publications, all of them reported a beneficial effect of blocking the PIM kinases to reduce viral progression. For that reason, pan-PIM kinase inhibitors are promising drugs not only for cancer therapy but also as new treatments against certain viral infections. Many of these inhibitors are PIM kinase ATP-competitive inhibitors. Because the Pim kinases induced in zebrafish conserved the characteristic ATP-binding site at the beginning of the PIM kinase domain, we tested its effectiveness in zebrafish cells. This is the first time that PIM kinase inhibitors have been used in zebrafish. Due to the existence of more than 300 Pim kinases in this species, we first confirmed that SGI-1776, INCB053914 and AZD1208 were not cytotoxic to ZF4 cells at the concentrations used in this work, and we showed that they were able to downregulate the expression of three pivotal genes involved in the cell cycle (*p53*, *p21* and *mdm2*). The MDM2/p53/p21 axis is a core pathway controlling the cell cycle [49], and it is known that mammalian PIM kinases interact with this axis [50-52]. Although PIM kinases do not directly affect the expression of these genes, modulations in their mRNA levels are indicative of alterations in the cell cycle. Therefore, we can assume that pan-PIM kinase inhibitors effectively affect the functionality of zebrafish Pim kinases.

Based on the high number of Pim kinases induced in zebrafish after SVCV infection, we wanted to evaluate whether this family of proteins in this model organism is also involved in SVCV infectivity. We first tested the effect of the pan-PIM kinase inhibitors *in vitro* using a zebrafish fibroblast cell line, ZF4. When the cells were pre-incubated for 24 h with the inhibitors and then infected with SVCV in the presence of the inhibitors, we observed a large reduction in the viral titer and viral replication in these cells. The results showed a pro-viral effect of the PIM kinases and an antiviral activity of the pan-PIM kinase inhibitors. Interestingly, when the cells were pre-

incubated with the virus for 5 h and then treated with the inhibitors, these differences were dramatically reduced and even abrogated, which is consistent with an effect of the PIM kinases in the SVCV entry and is in agreement what was observed with HCV [10].

Due to the potential use of these inhibitors in prophylaxis and/or treatment of viral infections, we also wanted to analyse their effect *in vivo* using zebrafish larvae. Larvae were pre-treated for 24 h with SGI-1776, INCB053914 or AZD1208 diluted in the water, and then they were microinjected with SVCV and returned to the water containing the inhibitors. These treated larvae showed an increase in survival after infection compared to the untreated and infected larvae. Moreover, larvae treated with the drugs showed significantly lower SVCV detection. As observed in the *in vitro* experiments, SGI-1776 was the most protective pan-PIM kinase inhibitor. Therefore, although larvae were microinjected into the duct of Cuvier, these results could indicate that PIM kinase inhibitors penetrate into the larvae and avoid the entry of the SVCV into the host cells for efficient replication. However, due to the effects that PIM kinases can exert on mammalian immune cells, we cannot rule out the activation of additional antiviral mechanisms in the whole organism. Nevertheless, the activity of the PIM kinases on immune cells was mainly investigated in T-lymphocytes [53-55], which are absent in zebrafish larvae [56].

The different bioactivity of the inhibitors observed both *in vitro* and *in vivo* cold be conditioned by the concentrations used, but also by the different inhibition constant (Ki) values. The Ki is the concentration of an inhibitor that gives half maximal rate of inhibition and therefore, it is an indicative of how potent an inhibitor is. Indeed, pan-PIM kinase inhibitors possess different Ki values against the three PIM isoforms described in mammals [57]. In zebrafish, this could be much more complex due to the expansion of this gene family.

In conclusion, in this work, we conducted RNA-Seq analysis of kidneys from adult zebrafish that were i.p. infected or not with SVCV for 24 h. We observed a high induction of typical antiviral immune genes (type I interferon-related genes, chemokines, pro-inflammatory cytokines, etc.) However, one of the observations that drawn our attention was the high representation of Pim kinases overexpressed during SVCV infection. *In vitro* and *in vivo* assays with three different pan-PIM kinase inhibitors allowed us to corroborate previous observations with mammalian viruses [7,

578 9-11], showing that Pim kinase activity is beneficial for SVCV. Moreover, in the
579 particular case of this virus, the zebrafish Pim kinases seem to facilitate the entry of the
580 SVCV into the cells. For that reason, PIM kinase inhibitors deserve certain attention due
581 to their antiviral effect, and their use could help to control the spread of different viral
582 diseases. However, future investigations will be needed to test the potential pernicious
583 collateral effects of these drugs.

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591 **References**

592 [1] M. Narlik-Grassow, C. Blanco-Aparicio, A. Carnero, The PIM family of
593 serine/threonine kinases in cancer, Med. Res. Rev. 34 (2014) 136–159.

594 [2] R. Swords, K. Kelly, J. Carew, S. Nawrocki, D. Mahalingam, J. Sarantopoulos, D.
595 Bearss, F. Giles, The Pim kinases: new targets for drug development, Curr. Drug
596 Targets. 12 (2011) 2059–2066.

597 [3] N.A. Warfel, AS Kraft, PIM kinase (and Akt) biology and signaling in tumors,
598 Pharmacol. Ther. 151 (2015) 41-49.

599 [4] G.G. Jinesh, S. Mokkapati, K. Zhu, E.E. Morales, Pim kinase isoforms: devils
600 defending cancer cells from therapeutic and immune attacks, Apoptosis. 21 (2016)
601 1203–1213.

602 [5] K.C. Qian, J. Studts, L. Wang, K. Barringer, A. Kronkaitis, C. Peng, A. Baptiste, R.
603 LaFrance, S. Mische, B. Farmer, Expression, purification, crystallization and
604 preliminary crystallographic analysis of human Pim-1 kinase, Acta Crystallogr. Sect F:
605 Struct. Biol. Cryst. Commun.61 (2005) 96–99.

606 [6] H. Mikkers, M. Nawijn, J. Allen, C. Brouwers, E. Verhoeven, J. Jonkers, A. Berns,
607 Mice deficient for all PIM kinases display reduced body size and impaired responses to
608 hematopoietic growth factors, Mol. Cell. Biol. 24 (2004) 6104–6115.

609 [7] E.M. Rainio, H. Ahlfors, K.L. Carter, M. Ruuska, S. Matikainen, E. Kieff, P.J.
610 Koskinen, Pim kinases are upregulated during Epstein-Barr virus infection and enhance
611 EBNA2 activity, Virology. 15 (2005) 201–206.

612 [8] F. Cheng, M. Weidner-Glunde, M. Varjosalo, E.M. Rainio, A. Lehtonen, T.F.
613 Schulz, P.J. Koskinen, J. Taipale, P.M. Ojala, KSHV reactivation from latency requires
614 Pim-1 and Pim-3 kinases to inactivate the latency-associated nuclear antigen LANA,
615 PLoS Pathog. 5 (2009) e1000324.

616 [9] M. de Vries, NP Smithers, PH Howarth, MC Nawijn, DE Davies, Inhibition of Pim1
617 kinase reduces viral replication in primary bronchial epithelial cells, Eur. Respir. J. 45
618 (2015) 1745–1748.

619 [10] C. Park, S. Min, E.M. Park, Y.S. Lim, S. Kang, T. Suzuki, E.C. Shin, S.B. Hwang,
620 Pim Kinase Interacts with nonstructural 5A protein and regulates hepatitis c virus entry,
621 J. Virol. 89 (2015) 10073–10086.

622 [11] K. Miyakawa, S. Matsunaga, M. Yokoyama, M. Nomaguchi, Y. Kimura, M. Nishi,
623 H. Kimura, H. Sato, H. Hirano, T. Tamura, H. Akari, T. Miura, A. Adachi, T. Sawasaki,
624 N. Yamamoto, A. Ryo, PIM kinases facilitate lentiviral evasion from SAMHD1
625 restriction via Vpx phosphorylation, Nat. Commun. 10 (2019) 1844.

626 [12] R. Rakshambikai, N. Srinivasan, R.A. Gadkari, Repertoire of protein kinases
627 encoded in the genome of zebrafish shows remarkably large population of PIM kinases,
628 J. Bioinform. Comput. Biol. 12 (2014) 1350014.

629 [13] J. Wittbrodt, A. Meyer, M. Schartl, More genes in fish?, BioEssays 20 (1998) 511–
630 515.

631 [14] I.G.Woods, P.D. Kelly, F. Chu, P. Ngo-Hazelett, Y.L. Yan, H. Huang, J.H.
632 Postlethwait, W.S. Talbot, A comparative map of the zebrafish genome, Genome Res.
633 10 (2000) 1903–1914.

[15] M.C. Gomes, S. Mostowy, The case for modeling human infection in zebrafish, Trends Microbiol. In Press (2019). https://doi.org/10.1016/j.tim.2019.08.005

[16] G.E. Sanders, W.N. Batts, J.R. Winton, Susceptibility of zebrafish (*Danio rerio*) to a model pathogen, spring viremia of carp virus, Comp. Med. 53 (2003) 514–521.

[17] P.J. Walker, A. Benmansour, R. Dietzgen, R.X. Fang, A.O. Jackson, G. Kurath, J.C. Leong, S. Nadin-Davies, R.B. Tesh RB, N. Tordo, Family Rhabdoviridae, in: M.H.V. van Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, R.B. Wickner (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses, Academic Press, San Diego, 2000, pp. 563–583.

[18] M.K. Purcell, K.J. Laing, J.R. Winton, Immunity to fish rhabdoviruses, Viruses 4 (2012) 140–166.

[19] P. Dixon, R. Paley, R. Alegria-Moran, B. Oidtmann, Epidemiological characteristics of infectious hematopoietic necrosis virus (IHNV): a review, Vet. Res. 47 (2016) 63.

[20] P. Pereiro, A. Figueras, B. Novoa, Turbot (*Scophthalmus maximus*) vs. VHSV (Viral Hemorrhagic Septicemia Virus): A Review, Front. Physiol. 7 (2016) 192.

[21] M. Westerfield, The zebrafish book: A guide for the laboratory use of zebrafish, University of Oregon Press, 2000.

[22] C. Nusslein-Volhard, R. Dahm, Zebrafish: a practical approach, Oxford University Press, 2002.

[23] L.J. Reed, H. Muench, A simple method of estimating fifty percent endpoints, Am. J. Epidemiol. 27 (1938) 493–497.

[24] V. Valenzuela-Muñoz, P. Pereiro, M. Álvarez-Rodríguez, C. Gallardo-Escárate, A. Figueras, B. Novoa, Comparative modulation of lncRNAs in wild-type and rag1-heterozygous mutant zebrafish exposed to immune challenge with spring viraemia of carp virus (SVCV), Sci. Rep. 9 (2019) 14174.

[25] D.W. Huang, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources, Nature Protoc. 4 (2019a) 44–57.

[26] D.W. Huang, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009b) 1–13.

[27] C.H. Wu, L.S.L. Yeh, H. Huang, L. Arminski, J. Castro-Alvear, Y. Chen, Z. Hu, P. Kourtesis, R.S. Ledley, B.E. Suzek, C.R. Vinayaka, J. Zhang, W.C. Barker, The Protein Information Resource, Nucleic Acids Res. 31(2003) 345–347.

[28] D.R. Zerbino, P. Achuthan, W. Akanni, M.R. Amode, D. Barrell, J. Bhai, K. Billis, C. Cummins, A. Gall, C. García Girón, L. Gil, L. Gordon, L. Haggerty, E. Haskell, T. Hourlier, O.G. Izuogu, S.H. Janacek, T. Juettemann, J. Kiang To, M.R. Laird, I. Lavidas, Z. Liu, J.E. Loveland, T. Maurel, W. McLaren, B. Moore, J. Mudge, D.N. Murphy, V. Newman, M. Nuhn, D. Ogeh, C. Kee Ong, A. Parker, M. Patricio, H. Singh Riat, H. Schuilenburg, D. Sheppard, H. Sparrow, K. Taylor, A. Thormann, A. Vullo, B. Walts, A. Zadissa, A. Frankish, S.E. Hunt, M. Kostadima, N. Langridge, F.J. Martin, M. Muffato, E. Perry, M. Ruffier, D.M. Staines, S.J. Trevanion, B.L. Aken, F. Cunningham, A.Yates, P. Flicek, Ensembl 2018, Nucleic Acids Res. 46 (2018) D754–D761.

[29] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice, Nucleic Acid Res. 22 (1994) 4673–4680.

[30] J.J. Campanella, L. Bitincka, J. Smalley, MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences, BMC Bioinform. 4 (2003), 29.

[31] M. Chekulaeva, W. Filipowicz, Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells, Curr. Opin. Cell Biol. 21 (2009) 452–460.

[32] M. Hafner, M.Jr. Ascano, T. Tuschl, New insights in the mechanism of microRNA-mediated target repression, Nat. Struct. Mol. Biol. 18 (2011) 1181–1182.

692 [33] S. Griffiths-Jones, R.J. Grocock, S. van Dongen, A. Bateman, A.J. Enright,
693 miRBase: microRNA sequences, targets and gene nomenclature, Nucleic Acids Res. 34
694 (2006) D140–D144.

695 [34] D.M. Garcia, D. Baek, C. Shin, G.W. Bell, A. Grimson, D.P. Bartel, Weak seed-
696 pairing stability and high target-site abundance decrease the proficiency of lsy-6 and
697 other microRNAs, Nat. Struct. Mol. Biol. 18 (2011) 1139–1146.

698 [35] C.E. Vejnar, M. Blum, E.M. Zdobnov, miRmap web: comprehensive microRNA
699 target prediction online, Nucleic Acids Res. 41 (2013) W165–W168.

700 [36] S. Rozen, H.J. Skaletsky, Primer3 on the WWW for general users and for biologist
701 programmers, in: S. Krawetz, S. Misener (Eds.), Bioinformatics Methods and Protocols:
702 Methods in Molecular Biology, Humana Press, Totowa, 2000, pp. 365–386.

703 [37] M.W. Pfaffl, A new mathematical model for relative quantification in real-time
704 RT-PCR, Nucleic Acids Res. 29 (2001) e45.

705 [38] P. Pereiro, M. Varela, P. Diaz-Rosales, A. Romero, S. Dios, A. Figueras, B.
706 Novoa, Zebrafish Nk-lysins: First insights about their cellular and functional
707 diversification, Dev. Comp. Immunol. 51 (2015) 148–159.

708 [39] P. Pereiro, G. Forn-Cuni, S. Dios, J. Coll, A. Figueras, B. Novoa, Interferon-
709 independent antiviral activity of 25-hydroxycholesterol in a teleost fish, Antiviral Res.
710 145 (2017) 146–159.

711 [40] J. Li, M. Favata, J.A. Kelley, E. Caulder, B. Thomas, X. Wen, R.B. Sparks, A.
712 Arvanitis, J.D. Rogers, A.P. Combs, K. Vaddi, K.A. Solomon, P.A. Scherle, R. Newton,
713 J.S. Fridman, INCB16562, a JAK1/2 selective inhibitor, is efficacious against multiple
714 myeloma cells and reverses the protective effects of cytokine and stromal cell support,
715 Neoplasia. 12 (2010) 28–38.

716 [41] Q. Yang, L.S. Chen, S.S. Neelapu, V. Gandhi, Combination of Pim kinase inhibitor
717 SGI-1776 and bendamustine in B-cell lymphoma, Clin. Lymphoma Myeloma Leuk. 13
718 (2013) S355–S362.

[42] S. Kreuz, K.B. Holmes, R.M. Tooze, P.F. Lefevre, Loss of PIM2 enhances the anti-proliferative effect of the pan-PIM kinase inhibitor AZD1208 in non-Hodgkin lymphomas, Mol. Cancer. 14 (2015) 205.

[43] W. Ahne, H.V. Bjorklund, S. Essbauer, N. Fijan, G. Kurath, J.R. Winton, Spring viraemia of carp (SVC), Dis. Aquat. Organ. 52 (2002) 261–272.

[44] P. Encinas, P. Garcia-Valtanen, B. Chinchilla, E. Gomez-Casado, A. Estepa, J. Coll, Identification of multipath genes differentially expressed in pathway-targeted microarrays in zebrafish infected and surviving spring viremia carp virus (SVCV) suggest preventive drug candidates, PLoS One. 8 (2013) e73553.

[45] P. García-Valtanen, A. Martínez-López, A. López-Muñoz, M. Bello-Perez, R.M. Medina-Gali, M.D. Ortega-Villaizán, M. Varela, A. Figueras, V. Mulero, B. Novoa, A. Estepa, J. Coll, Zebra fish lacking adaptive immunity acquire an antiviral alert state characterized by upregulated gene expression of apoptosis, multigene families, and interferon-related genes, Front. Immunol. 8 (2017) 121.

[46] M. Álvarez-Rodríguez, P. Pereiro, F.E. Reyes-López, L. Tort, A. Figueras, B. Novoa, Analysis of the long-lived responses induced by immunostimulants and their effects on a viral infection in zebrafish (*Danio rerio*), Front. Immunol. 9 (2018) 1575.

[47] Y. Wang, H. Zhang, Y. Lu, F. Wang, L. Liu, J. Liu, X. Liu, Comparative transcriptome analysis of zebrafish (*Danio rerio*) brain and spleen infected with spring viremia of carp virus (SVCV), Fish Shellfish Immunol. 69 (2017) 35–45.

[48] S. Wu, L. Liu, A. Zohaib, L. Lin, J. Yuan, M. Wang, X. Liu, MicroRNA profile analysis of Epithelioma papulosum cyprini cell line before and after SVCV infection, Dev. Comp. Immunol. 48 (2015) 124–128.

[49] S. Nag, J. Qin, K.S. Srivenugopal, M. Wang, R. Zhang, The MDM2-p53 pathway revisited, J. Biomed. Res. 27 (2013) 254–271.

[50] C. Hogan, C. Hutchison, L. Marcar, D. Milne, M. Saville, J. Goodlad, N. Kernohan, D. Meek, Elevated levels of oncogenic protein kinase Pim-1 induce the p53 pathway in cultured cells and correlate with increased Mdm2 in mantle cell lymphoma, J. Biol. Chem. 283 (2008) 18012–18023.

748 [51] Z. Wang, N. Bhattacharya, P.F. Mixter, W. Wei, J. Sedivy, N.S. Magnuson,
749 Phosphorylation of the cell cycle inhibitor p21Cip1/WAF1 by Pim-1 kinase, Biochim.
750 Biophys. Acta. 1593 (2002) 45–55.

751 [52] Z. Wang, Y. Zhang, J.J. Gu, C. Davitt, R. Reeves, N.S. Magnuson, Pim-2
752 phosphorylation of p21(Cip1/WAF1) enhances its stability and inhibits cell proliferation
753 in HCT116 cells, Int. J. Biochem. Cell Biol. 42 (2010) 1030–1038.

754 [53] X.P. Chen, J.A. Losman, S. Cowan, E. Donahue, S. Fay, B.Q. Vuong, M.C.
755 Nawijn, D. Capece, V.L. Cohan, P. Rothman, Pim serine/threonine kinases regulate the
756 stability of Socs-1 protein, Proc. Natl. Acad. Sci. USA. 99 (2002) 2175–2180.

757 [54] C.J. Fox, P.S. Hammerman, C.B. Thompson, The Pim kinases control rapamycin-
758 resistant T cell survival and activation, J. Exp. Med. 201(2005) 259–266.

759 [55] G. Deng, Y. Nagai, Y. Xiao, Z. Li, S. Dai, T. Ohtani, A. Banham, B. Li, S.L. Wu,
760 W. Hancock, A. Samanta, H. Zhang, M.I. Greene, Pim-2 kinase influences regulatory T
761 cell function and stability by mediating Foxp3 protein N-terminal phosphorylation, J.
762 Biol. Chem. 290 (2005) 20211–20220.

763 [56] C.E. Willett, A. Cortes, A. Zuasti, A.G. Zapata, Early hematopoiesis and
764 developing lymphoid organs in the zebrafish, Dev. Dyn. 214 (1999) 323–336.

765 [57] M.T. Burger, W. Han, J. Lan, G. Nishiguchi, C. Bellamacina, M. Lindval, G.
766 Atallah, Y. Ding, M. Mathur, C. McBride, E.L. Beans, K. Muller, V. Tamez, Y. Zhang,
767 K. Huh, P. Feucht, T. Zavorotinskaya, Y. Dai, J. Holash, J. Castillo, J. Langowski, Y.
768 Wang, M.Y. Chen, P.D. Garcia, Structure guided optimization, in vitro activity, and in
769 vivo activity of pan-PIM kinase inhibitors, ACS Med. Chem. Lett. 4 (2013) 1193–1197.

770 **Tables**

771 **Table 1. Summary of the mRNA Illumina sequencing, trimming and genome**
772 **mapping.**

mRNA				
Sample	**Raw reads**	**Reads after trim**	**Mapped to genome (%)**	**Unmapped (%)**
WT C1	94,617,822	93,976,916	97.21	2.79
WT C2	91,411,306	90,745,708	97.41	2.59
WT C3	89,359,334	88,664,158	97.39	2.61

WT-SVCV 1	90,571,676	90,011,432	97.17	2.83
WT-SVCV 2	95,483,786	94,975,756	97.60	2.40
WT-SVCV 3	82,152,392	81,664,334	96.84	3.16
Average value	90,599,386	90,006,384	97.27	2.73
Total value	543,596,316	540,038,304	97.28	2.72

773

774 **Table 2. Summary of the Pim kinases significantly induced in zebrafish kidney at**
775 **24 h after SVCV infection.**

ENSEMBL ID	Gene Symbol	Description	FC	p-value
ENSDARG00000055056	si:ch73-129a22.11_3	PREDICTED: Serine/threonine-protein kinase pim-1-like	89.15	0.01192
ENSDARG00000094651	pimr106	Pim proto-oncogene, serine/threonine kinase, related 106	60.36	0.019852
ENSDARG00000104164	CABZ01028711.1	PREDICTED: Serine/threonine-protein kinase pim-3-like	34.95	0.045781
ENSDARG00000100992	pimr101_2	Pim proto-oncogene, serine/threonine kinase, related 101	17.56	0.005791
ENSDARG00000069851	pimr152_8	Pim proto-oncogene, serine/threonine kinase, related 152	15.31	0.012636
ENSDARG00000037246	pimr212	Pim proto-oncogene, serine/threonine kinase, related 212	13.25	0.012636
ENSDARG00000070015	pimr173	Pim proto-oncogene, serine/threonine kinase, related 173	10.23	0.031065
ENSDARG00000052677	si:ch211-138g9.2	PREDICTED: Serine/threonine-protein kinase pim-3-like	10.00	0.002023
ENSDARG00000100216	pimr117	Pim proto-oncogene, serine/threonine kinase, related 117	7.91	0.017364
ENSDARG00000092339	pimr61	Pim proto-oncogene, serine/threonine kinase, related 61	6.97	0.012071
ENSDARG00000095386	pimr179	Pim proto-oncogene, serine/threonine kinase, related 179	6.83	0.009817
ENSDARG00000103910	pimr202_2	Pim proto-oncogene, serine/threonine kinase, related 202	5.94	0.036673
ENSDARG00000057265	pimr52	Pim proto-oncogene, serine/threonine kinase, related 52	5.40	0.022613
ENSDARG00000102396	pimr66	Pim proto-oncogene, serine/threonine kinase, related 66	5.00	0.006272
ENSDARG00000093631	pimr20	Pim proto-oncogene, serine/threonine kinase, related 20	4.72	0.037309
ENSDARG00000098638	pimr65	Pim proto-oncogene, serine/threonine kinase, related 65	3.94	0.011634

776

777 **Table 3. Summary of the small RNA sequencing and miRNA annotation to the**
778 **mature miRNAs present in miRBase (A), and the representation of those miRNAs**
779 **that were modulated by the infection with SVCV and have a Pim kinase as a**
780 **potential target (B).**

781

A

miRNA				
Sample	**Raw reads**	**Reads after trim**	**Total annotated reads**	**Unique annotated reads**
WT-C	27,280,984	16,143,997 (59.18 %)	597	551
WT-SVCV	25,817,631	21,289,295 (82.46 %)	546	498

B

miRNA	Target	TargetScanFish score	mirMAP score

dre-miR-2188	ENSDARG00000059001	-0.50	94.77
dre-miR-199	ENSDARG00000059001	-0.65	-
dre-miR-210-5p	ENSDARG00000055129	-	95.94
dre-miR-124	ENSDARG00000055129	-	90.99
dre-miR-181b	ENSDARG00000070015	-0.54	98.59

782

783 **Figure Legends**

784 **Figure 1. Differentially expressed genes in zebrafish kidneys after infection with**
785 **SVCV (FC >2, p-value < 0.05). A)** Heat map representing the expression level of those
786 genes differentially expressed and hierarchical clustering of the different samples
787 constructed based on TPM values. Two well-differentiated clusters are observed: one
788 for those genes inhibited after viral challenge and another for the genes overexpressed
789 after infection. **B)** Stacked column chart reflecting the distribution (up- or
790 downregulated) and intensity (FC value) of regulated genes.

791 **Figure 2. GO enrichment of biological processes (A), KEGG pathways (B) and**
792 **domain enrichment (C) analyses of the downregulated genes in SVCV-infected**
793 **fish.**

794 **Figure 3. GO enrichment of biological processes (A), KEGG pathways (B) and**
795 **domain enrichment (C) analyses of the upregulated genes in SVCV-infected fish.**

796 **Figure 4. Representation of the TPM values of the zebrafish Pim kinases**
797 **overexpressed in kidney after SVCV infection.** Three biological replicates per
798 condition are shown.

799 **Figure 5. Analysis of the PIM kinase domain of the zebrafish Pim kinases induced**
800 **by SVCV. A)** Alignment of the PIM kinase domain of 16 Pim kinases. With the
801 exception of two Pim kinases where the predicted domain was incomplete in the
802 genome, all the Pim kinases contained a typical ATP binding site (highlighted in pink).
803 **B)** Identity/similarity matrix of the zebrafish Pim kinase domains.

804 **Figure 6. Expression of the zebrafish *pimr106* (ENSDARG00000094651) gene in**
805 **kidney samples at 3, 6 and 24 h post-challenge with SVCV or Poly I:C.** Whereas
806 SVCV increased the expression of this gene from the first few hours post-infection,
807 Poly I:C does not significantly affect the expression of *pimr106*.

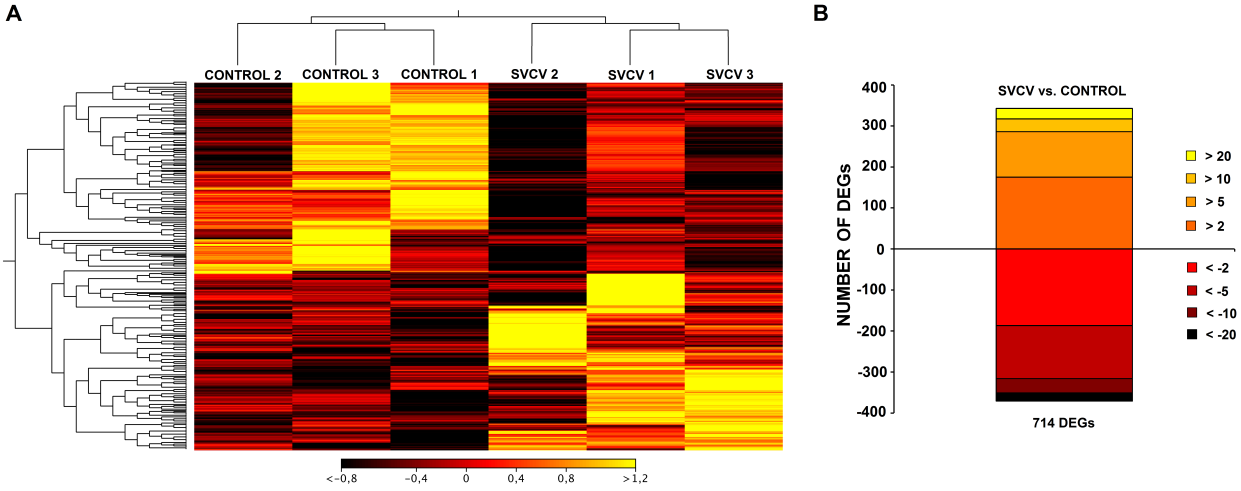
Figure 7. Representation of mature zebrafish miRNAs modulated (FC > 2) by SVCV. In green colour are represented the overexpressed miRNAs and in red colour the down-regulated miRNAs.

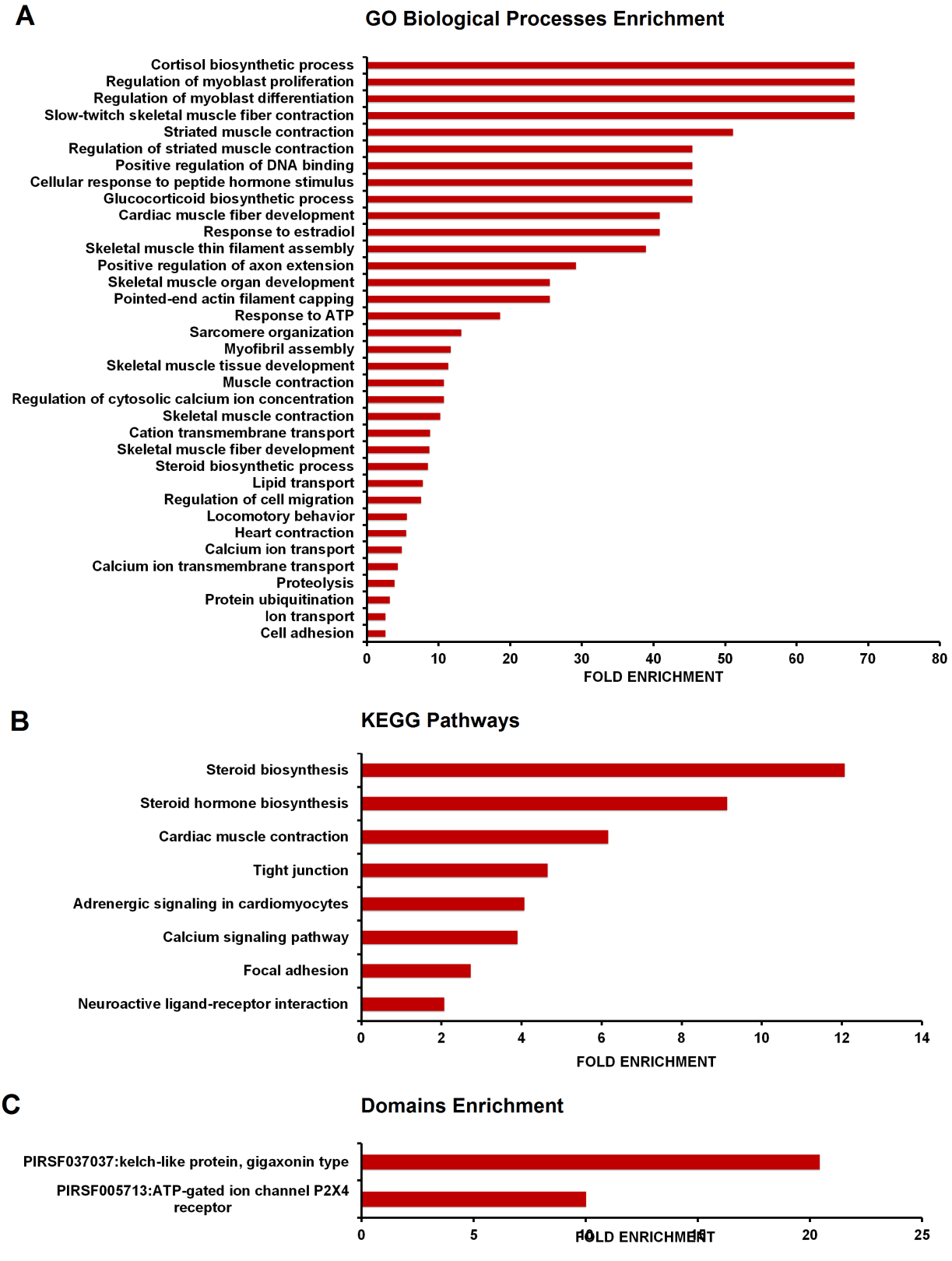
Figure 8. Pan-PIM kinase inhibitors reduce the entry of SVCV into ZF4 cells. A) ZF4 cells were pre-treated with the different PIM kinase inhibitors and were then infected with 1:10 serial dilutions of SVCV in the presence of the inhibitors. Changes in the CPE were checked every day, and the viral titer was calculated following the Reed and Muench method. A 3-log reduction in the viral titer was detected for SGI-1776, 2-log for INCB053914 and more than 2-log for AZD1208. **B)** When ZF4 cells were infected in the presence of the inhibitors, there was a significant reduction in the qPCR detection of the SVCV nucleoprotein (N) gene. **C)** ZF4 cells were pre-incubated for 5 h with 1:10 serial dilutions of SVCV, washed twice with PBS and then treated with the pan-PIM kinase inhibitors. Viral titer was also calculated according to the Reed and Muench method. The antiviral effects mediated by the inhibitors almost disappeared when the cells were first infected and were then treated with the drugs, indicating that Pim kinases are probably involved in the entry of the virus into the cells. A lower titer was obtained for SGI-1776, but the difference compared to the control was less than 1-log. **D)** qPCR detection of the SVCV N gene was performed after ZF4 cells were pre-incubated with the virus for 5 h and then treated with the inhibitors and sampled after 24 h. No significant differences were observed between the control cells and those treated with the different PIM kinase inhibitors.

Figure 9. **Pan-PIM kinase inhibitors protect zebrafish larvae against SVCV infection. A)** Kaplan-Meier survival curves are shown for zebrafish larvae infected with SVCV in the presence of the different inhibitors. Groups of 2 dpf larvae were pre-treated with the inhibitors or the vehicle alone (0.002 % DMSO) diluted in the water. After 24 h, larvae were microinjected via the duct of Cuvier with an SVCV suspension. Control larvae were inoculated with the same volume of viral medium diluted in PBS. Then, larvae were returned to the water containing the PIM kinase inhibitors. Mortality was registered during the next 6 dpi. The three drugs reduced the mortality caused by SVCV, but statistically significant differences were only obtained for SGI-1776. The survival of the uninfected larvae was: 93 % (control), 97 % (SGI-1776), 97 % (AZD1208) and 93 % (INCB053914). **B)** Detection of the SVCV N gene was performed after 24 h in zebrafish larvae treated with the different inhibitors or with the

841 vehicle alone. A lower detection of the viral gene was observed in the zebrafish larvae
842 infected in the presence of the inhibitors.

843

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Regulation of myoblast differentiation
Slow-twitch skeletal muscle fiber contraction
Striated muscle contraction
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Positive regulation of DNA binding
Cellular response to peptide hormone stimulus
Glucocorticoid biosynthetic process
Cardiac muscle fiber development
Response to estradiol
Skeletal muscle thin filament assembly
Positive regulation of axon extension
Skeletal muscle organ development
Pointed-end actin filament capping
Response to ATP
Sarcomere organization
Myofibril assembly
Skeletal muscle tissue development
Muscle contraction
Regulation of cytosolic calcium ion concentration
Skeletal muscle contraction
Cation transmembrane transport
Skeletal muscle fiber development
Steroid biosynthetic process
Lipid transport
Regulation of cell migration
Locomotory behavior
Heart contraction
Calcium ion transport
Calcium ion transmembrane transport
Proteolysis
Protein ubiquitination
Ion transport
Cell adhesion
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70
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Cardiac muscle contraction
Tight junction
Adrenergic signaling in cardiomyocytes
Calcium signaling pathway
Focal adhesion
Neuroactive ligand-receptor interaction
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12
14
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C
Domains Enrichment
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5
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15
20
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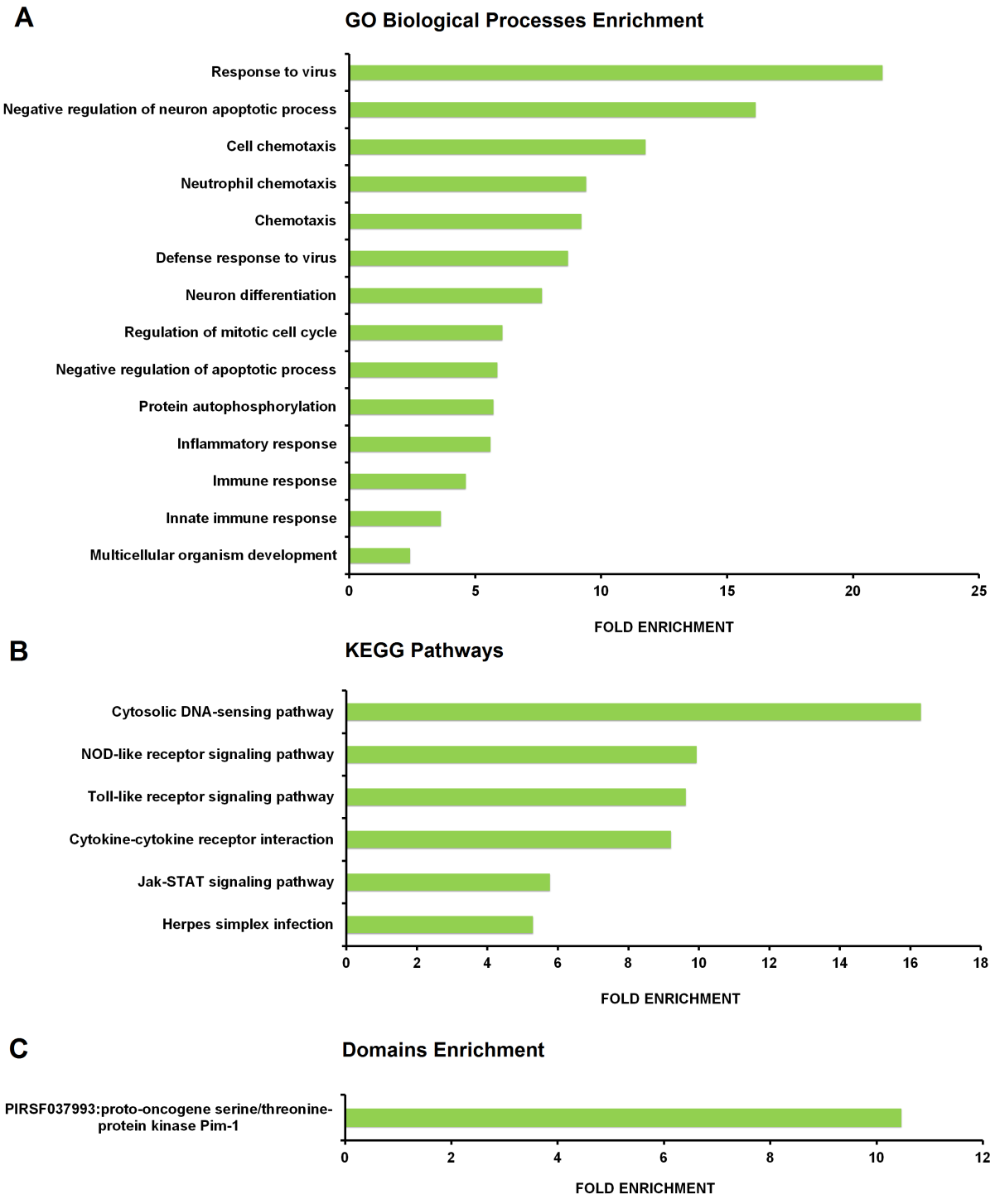
FOLD ENRICHMENT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GO Biological Processes Enrichment
Response to virus
Negative regulation of neuron apoptotic process
Cell chemotaxis
Neutrophil chemotaxis
Chemotaxis
Defense response to virus
Neuron differentiation
Regulation of mitotic cell cycle
Negative regulation of apoptotic process
Protein autophosphorylation
Inflammatory response
Immune response
Innate immune response
Multicellular organism development
0
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10
15
20
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FOLD ENRICHMENT
B
KEGG Pathways
Cytosolic DNA-sensing pathway
NOD-like receptor signaling pathway
Toll-like receptor signaling pathway
Cytokine-cytokine receptor interaction
Jak-STAT signaling pathway
Herpes simplex infection
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4
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FOLD ENRICHMENT
C
Domains Enrichment
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0
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4
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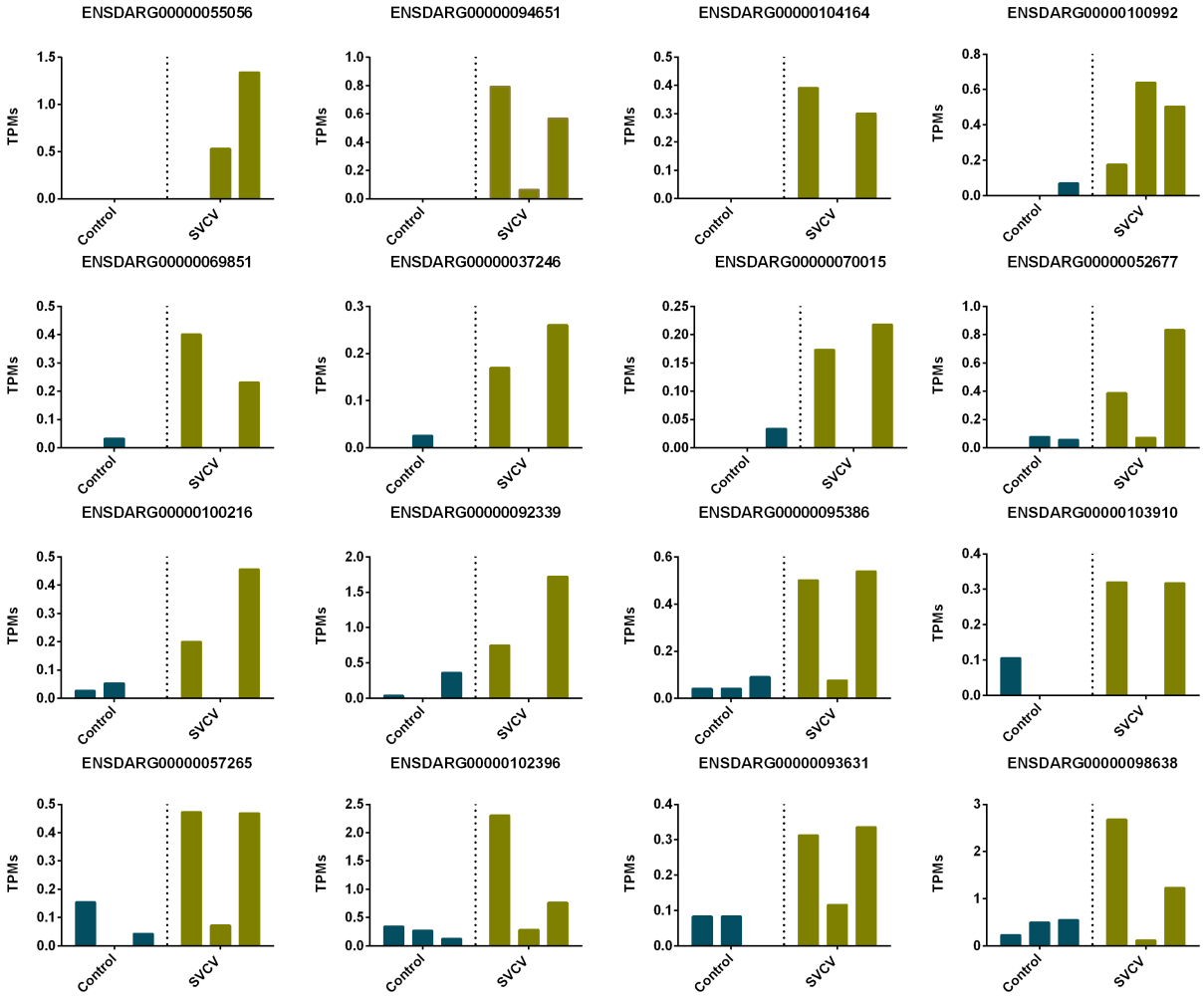
FOLD ENRICHMENT



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B

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3. ENSDARG00000104164	50	44.6		32.8	30.7	28.8	28.8	31.7	28.5	27.9	23.7	48.8	26.2	29.4	28.3	29.9
4. ENSDARG00000100992	72	77.4	52.1		49.8	48.6	44.2	32.4	49.4	39.4	40.5	25.1	36.2	51.8	42.9	51.8
5. ENSDARG00000069851	68.8	54.9	49	69.6		45.1	50.6	30	46.3	39.1	35.2	23	37.9	51.8	39.2	52.5
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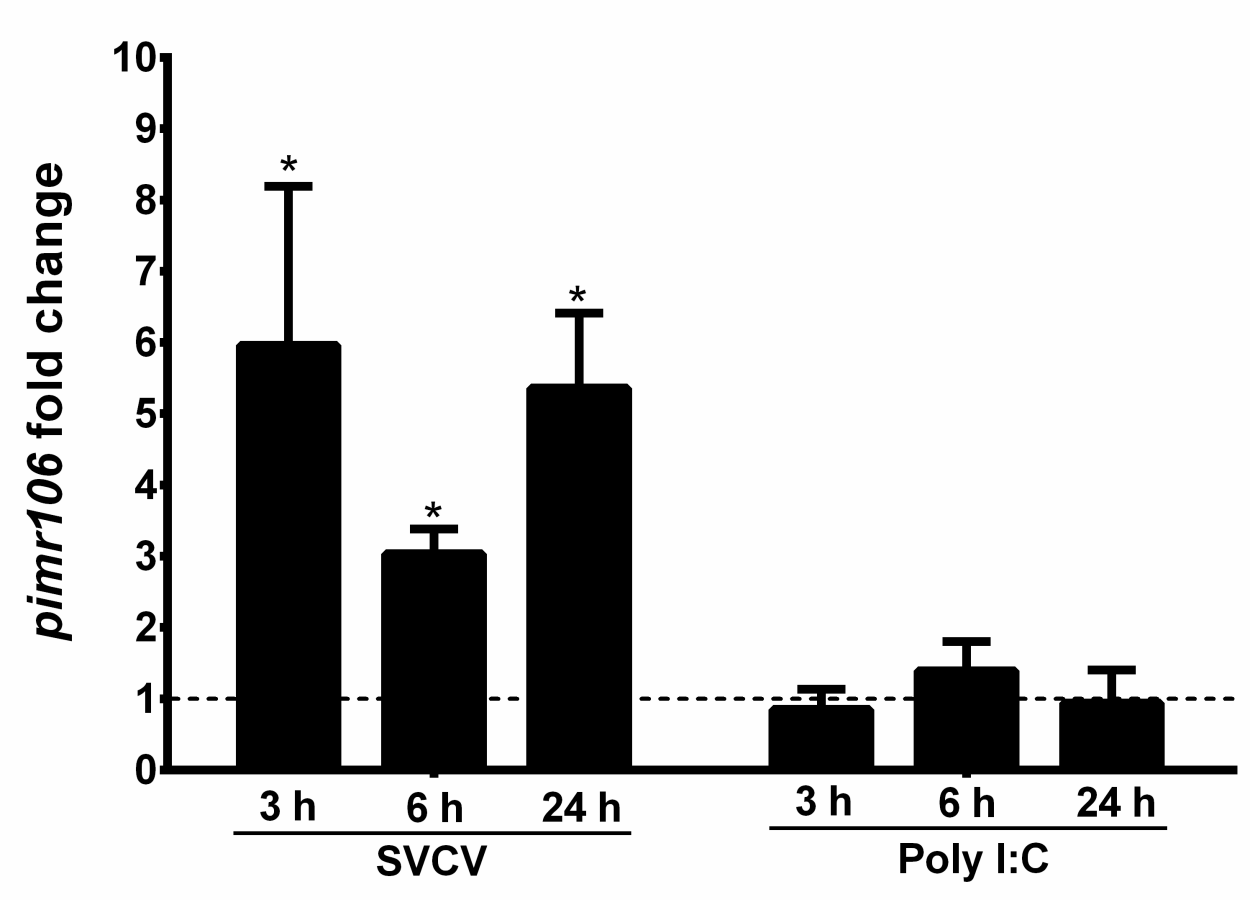
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SVCV
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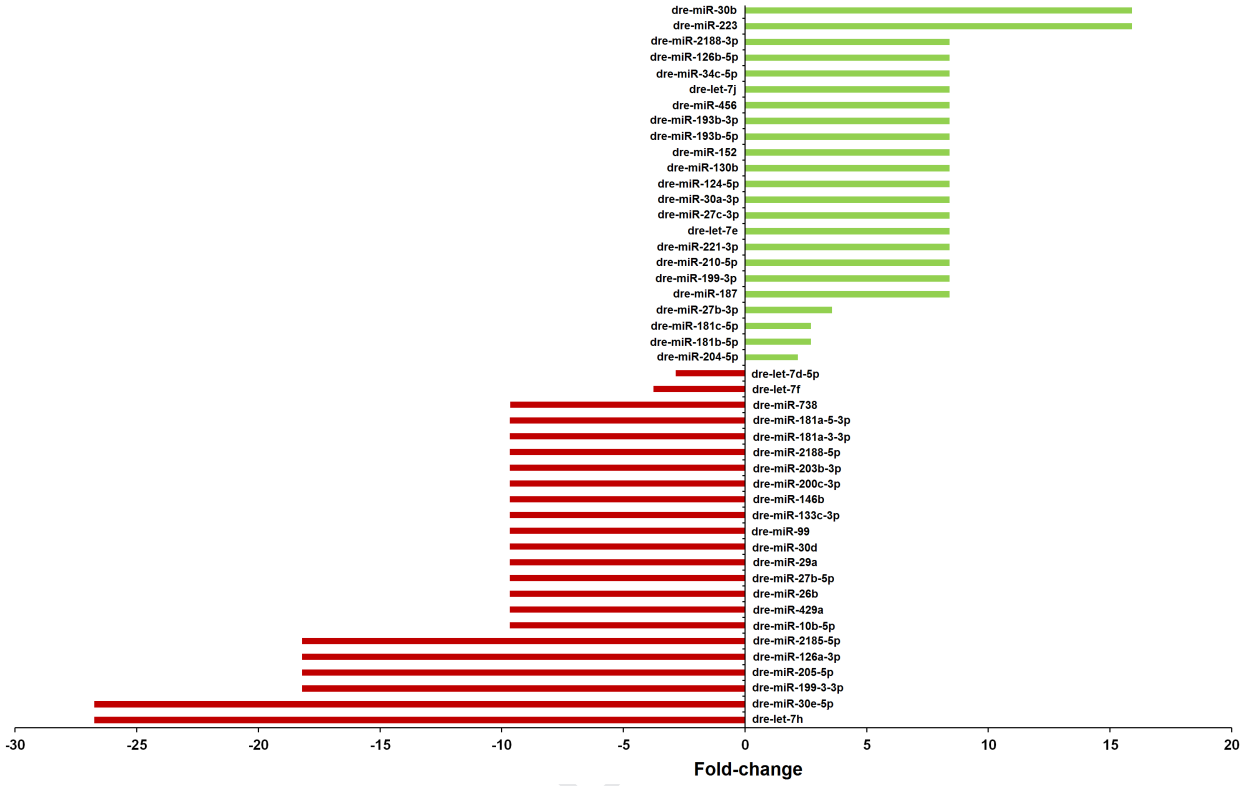

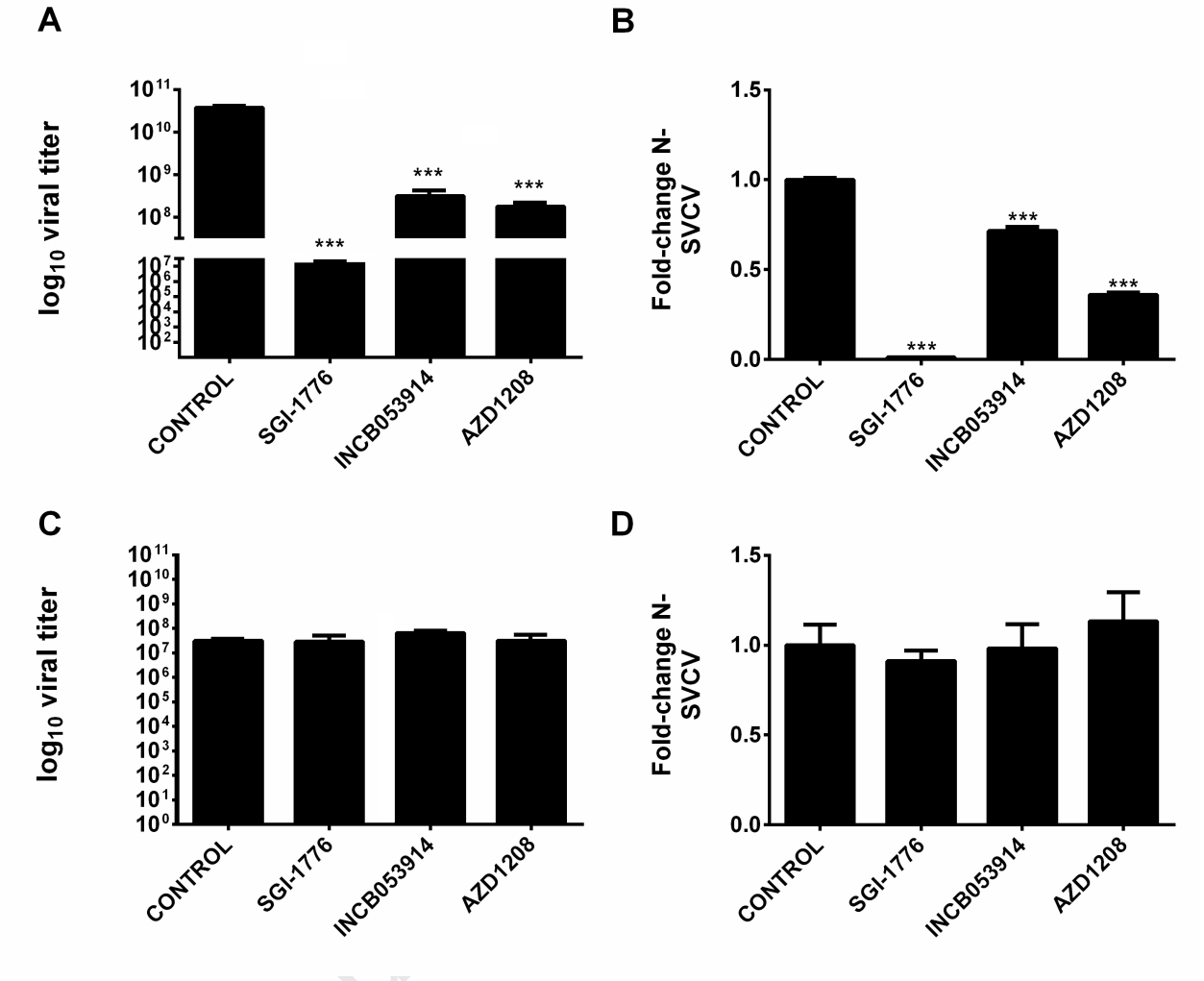
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10^9
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10^7
10^6
10^5
10^4
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10^2
***
***
***
CONTROL
SGI-1776
INCB053914
AZD1208
B
Fold-change N-SVCV
1.5
1.0
0.5
0.0
***
***
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CONTROL
SGI-1776
INCB053914
AZD1208
C
log10 viral titer
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CONTROL
SGI-1776
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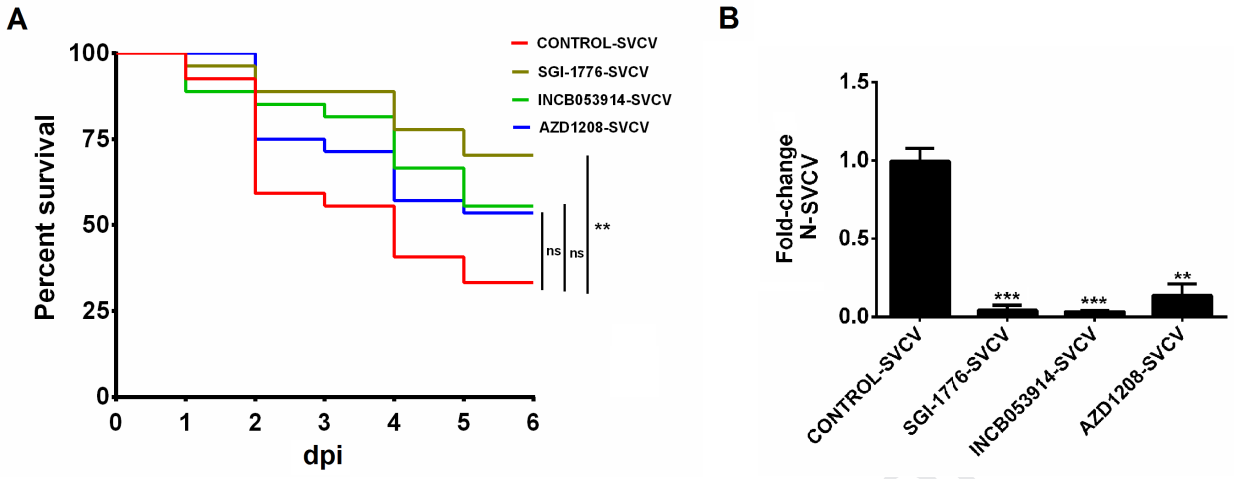

Highlights:

- Numerous genes encoding for Pim kinases were induced in zebrafish after SVCV challenge
- Zebrafish Pim kinases seem to be involved in the SVCV entry step
- Pan-PIM kinase inhibitors reduce SVCV entry into ZF4 cells
- Pan-PIM kinase inhibitors protect zebrafish larvae from SVCV