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Chromatin immunoprecipitation and high throughput sequencing of SVCV-infected zebrafish reveals novel epigenetic histone methylation patterns involved in antiviral immune response

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ABSTRACT

Chromatin immunoprecipitation (ChIP) and high throughput sequencing (ChIP-seq) have been used to assess histone methylation (epigenetic modification) dynamics within the internal organs of zebrafish after spring viremia of carp virus (SVCV) infection. Our results show H3K4me3 up-methylation in gene promoters associated with innate immune response during the first 5 days after SVCV infection. Gene Ontology (GO) enrichment analysis confirmed up-methylation in 218 genes in the "immune system process" category. In particular, the promoters of interferon (ifn), interferon stimulated genes (isg), Toll-like receptors (tlr) and c-reactive protein (crp) multi gene sets were marked with the permissive H3K4 methylation. Higher histone 3 methylation was associated with higher transcription levels of the corresponding genes. Therefore, the evidence presented here suggests that transcriptional regulation at the promoter level of key immune genes of the interferon signaling pathway and c-reactive proteins genes can be modulated by epigenetic modification of histones. This study emphasizes the importance of epigenetic control in the response of zebrafish to SVCV infection.

1. Introduction

In mammalian models viral infection is known to activate gene expression over a period of time. Virus-induced transcriptional activation can be achieved by histone modification in the promoter regions, an epigenetic mechanism probably related to trained immunity, a type of non-specific immunological memory that provides protection against reinfection [1]. Many methylation events have been positively correlated with gene activation. In particular, tri-methylation of histone H3 lysine 4 (H3K4me3) is one of the histone modifications widely associated with genes undergoing active transcription [2,3]. It is currently unknown whether such mechanism may exist in zebrafish, although viral infection does induce large-scale changes in host transcription in zebrafish [4].

The development of biotechnological methods such as ChIP and next generation sequencing provides us with the tools needed to better understand some of the epigenetic mechanisms of zebrafish response to viral disease at the genome level. In the ChIP assay, the DNA coupled to the modified histone is precipitated with the corresponding antibody to H3K4me3. This DNA is then purified and sequenced, which provides a quantitative measure of regions in the genome that were enriched in a particular methylation pattern after infection. Such genome-wide studies on fish epigenetics have contributed to a better understanding of the response to viral pathogenesis and the development of new prophylactic and therapeutic strategies to control viral diseases in aquaculture [5,6].

To investigate the molecular mechanisms underlying cyprinid fish response to viral infection we have chosen zebrafish (*Danio rerio*) because it has become an established model for teleost fish immunology studies where complete genome sequence and gene annotation are required. Furthermore, microarray analysis of immune-related responses to experimental infections of zebrafish with spring viremia of carp virus

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(SVCV) and viral hemorrhagic septicemia virus (VHSV) have been reported [7–12]. SVCV causes an acute disease in cyprinid fish species with outbreaks typically in spring both in farmed and wild fish with a serious impact on the aquaculture industry of common carp and koi [13].

Here we present data that reveal the modulation of the epigenetic methylation pattern of chromatin during SVCV infection of zebrafish. Samples from the four experimental groups (non-infected control, 1, 2 and 5 days post SVCV infection) were analyzed. The analysis of the data showed that SVCV infection was associated with changes in the histone 3 methylation in a large number of genes with a typical role in immune signaling pathways and host pathogen response. Our findings suggest that epigenetic reprogramming may be an underlying mechanism in fish to achieve an enhanced protective response to subsequent exposure to pathogens.

2. Materials and methods

2.1. Cell culture and virus

The zebrafish embryonic fibroblast ZF4 cells were purchased from the American Type Culture Collection (ATCC number CRL-2050). ZF4 cell lines were maintained at 28 °C in a 5% CO_2 atmosphere in RPMI Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum (Sigma, St. Louis, USA), 1 mM piruvate, 2 mM glutamine, 50 μg/ml gentamicin and 2.5 μg/ml fungizone.

The spring viremia of carp virus SVCV isolate 56/70 was grown in the ZF4 cell line at 22 °C by using the same cell culture media mentioned above except for 2% fetal calf serum. Supernatants from SVCV infected ZF4 cell monolayers were harvested at 7 days p. i. and clarified by centrifugation at 4000 r.p.m. for 30 min and kept in aliquots at −70 °C. SVCV titers were measured by a methylcellulose plaque assay [8,14]. Briefly, serial dilutions of SVCV were used to infect ZF4 cell monolayers in 24-well plates for 1.5 h. Then, the media were removed and each well was covered with a solution of 2% methylcellulose (Sigma) in RPMI 2% fetal calf serum. Plates were incubated at 22 °C for 5 days. Finally, the methylcellulose overlay was removed and the wells stained with crystal violet to visualize plaques due to virus-induced lysis.

2.2. Infection of zebrafish with SVCV

Animal trials procedures were approved by the local government ethics committee on animal experimentation (Dirección General de Agricultura, Ganadería y Pesca, Generalitat Valenciana) and registered under permit number 2016/VSC/PEA/00182. Zebrafish (40 fish, 0.35–0.4 g average body weight) were exposed by bath immersion to a dose of SVCV (2×10^4 pfu/ml) for 90 min at 21 °C. At 1, 2 and 5 days post infection the fish were sacrificed by overexposure to the anesthesia (MS-222, Sigma) and whole head kidney, liver, spleen and heart of 3 fish per treatment were extracted and pooled. Two pools were generated for each experimental condition (6 fish total). The procedure is outlined in Supplementary Fig. 1. Mortality was monitored for 15 days.

2.3. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Imprint® ChIP Kit (Sigma) following the instructions described by the manufacturer. Samples were placed in tubes containing 1% paraformaldehyde (PFA) and fixed for 20 min. After this time, glycine (1.25 M) was added for 5 min to stop the reaction followed by centrifugation and wash with phosphate buffered saline to remove PFA. The washed samples were sonicated to shear the chromatin in small fragments, centrifuged and aliquots analyzed by agarose gel electrophoresis to verify DNA cleavage into 200–500 bp fragments. ChIP was performed using anti-trimethyl histone H3 (Sigma). Once the DNA was immunoprecipitated, the binding reaction was reversed and the DNA was purified using the columns provided by the Imprint® ChIP kit.

2.4. Sequencing of immunoprecipitated chromatin (ChIP seq)

Chromatin precipitated DNA was sent to Bioarray Co. (Elche, Spain) for high-throughput sequencing. SVCV-infected and control uninfected samples (2 pools of 3 fish each) were sequenced. The four experimental groups were: uninfected control; SVCV-1dpi (days post-infection); SVCV-2dpi and SVCV-5dpi. The genetic libraries were built using the NEBNext Ultra II DNA kit for genetic libraries for Illumina (Lucigen, England). The libraries were then sequenced using an Illumina NextSeq500 in 2×75 mode. Sequence reads were subjected to a quality control by the program FastQC and a standard filtering by the program Trimmomatic. Total number and average length of sequence reads are shown in Fig. S2. An alignment of the readings to the *Danio rerio* GRCz10.85 genome version was then performed using the Bowtie2 program. Data analysis of the ChIP seq experiment was performed using the MACS program. This program employs a computational algorithm that identifies transcription factor/chromatin/histone binding genome regions (promoter sequences) from the ChIP seq data. Sequencing data was provided in an excell spreadsheet containing the list of the corresponding genes, gene identification in the UNIPROT database, number of reads, and the relative fold increase versus uninfected control.

2.5. Analysis of ChIP sequence data to identify differentially methylated gene promoters

Initially we performed a crude cleansing analysis of the data, eliminating those readings that were not assigned to any specific gene. Once this process was completed for each of the samples, we proceeded to global gene analysis and verification of the gene sets that were enriched in expression. The analysis was performed by two methods: a) global analysis of all the genes obtained and using the application of GESTALT (http://www.webgestalt.org/option.php). b) Gene Set Enrichment Analysis (GSEA) application (http://software.broadinstitute.org/gsea/index.jsp).

To determine the gene sets enriched in the list of genes obtained by sequencing, we used the GESTALT gene cluster analysis tool. This list of genes was generated for each of the experimental groups (non-infected, SVCV-1dpi, SVCV-2dpi, SVCV-5dpi), including only the annotated genes.

To perform the Gene Set Enrichment Analysis, the 12500 genes were analyzed using the gene sets (GS) described in Estepa and Coll (2015) (Table S1). The list of unique genes and values was ranked by the *t*-test statistic metric (similar results were obtained using the Signal-to-Noise ratio statistic) and the ranked list was used to calculate Enrichment Scores (ES) by comparing the following zebrafish phenotypes: SVCV-1d versus Control, SVCV-2d versus control and SVCV-5d versus control. The GSEA calculated individual gene enrichment scores (ES), overall ES for each GS and finally normalized ES (NES) to correct for the number of genes present in each GS. As suggested by GSEA, the most stringent cut-off value of $p < 0.05$ value was used for NES significance. The FDR method was chosen because only FDR corrected for both gene size and multiple hypothesis (null distribution from 1000 random gene combinations per GS). Because the zebrafish GS were derived from human/zebrafish orthologous pathways and that might be inaccurate, the Leading Edge Gene Analysis (LEGA) was used to search for empirically clustered genes (GS/LEGA matrixes) indicative of novel fish GSs.

2.6. RNA isolation and gene expression analysis by RT-qPCR

For total RNA extraction the E. Z.N.A. HP Tissue RNA kit (Omega Bio-tek) was used following the manufacturer's instructions. DNAse

treatment was done in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.), following the manufacturer's instructions. RNA samples were stored at −80 °C until use. One microgram of RNA, as estimated by a Nanodrop 1000 spectrophotometer (Thermo-fisher Scientific, Inc), was added to the cDNA synthesis reaction with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Corporation, UK). Quantitative PCR (qPCR) was performed using the ABI PRISM 7300 System (Applied Biosystems) Primers used for quantitative real-time PCR analysis are listed in Table S3. The reaction was performed in a total volume of 20 µL, comprising 2 µL cDNA reaction mixture, 900 nM each primer, 10 µL of SYBR Green Master Mix (Applied Biosystems) and 6 µL of water. The cycling conditions were 50 °C for 2 min, and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Expression of gene transcript levels was calculated by the $2^{-\Delta Ct}$ method [15] where ΔCt is determined by subtracting the Ct value from the eukaryotic translation elongation 1 alpha (ef1a) gene, used as endogenous control, to the target Ct value. Every sample was run in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism v5.0 software. To compare the datasets of the different treatments with their respective uninfected controls, Tukey tests were used. When applicable, significant differences were represented as asterisks (*, **) indicating $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Infection of zebrafish with SVCV

To further characterize zebrafish as an experimental model for SVCV infection a bath immersion challenge was performed as described earlier [8,14]. Under our experimental trial conditions symptoms of SVCV disease started at 3 days post-infection (dpi), with external hemorrhages in the mouth, gills, lateral skin and fins found on infected fish (Fig. 1A). Survival in the SVCV-infected group went down to 10% after 14 days whereas in the control group only one dead fish was recorded whose dead was not infection-related (Fig. 1B). Replication of SVCV was monitored by N gene RNA quantitation by real-time RT-PCR (RT-qPCR). SVCV-N RNA increases with time after infection (Fig. 1C), indicating a viral productive infection in the diseased fish.

3.2. Genes with differential methylation in histone 3 after SVCV infection

At each time point after SVCV infection two samples made of a pool of the internal organs from 3 fish each were analyzed. Briefly, chromatin was extracted, fragmented and immunoprecipitated with the anti-H3K4me3 antibody. Immunoprecipitated DNA was sequenced and genes were annotated with UNIPROT using the *Danio rerio* genome as a reference. The number of reads of each annotated sequence was converted to fold-enrichment over the non-infected control group. Genes with a fold-enrichment value ≥ 1.5 over the non-infected control group were considered differentially methylated genes (DMGs). There were 11907 unigenes with a fold-enrichment value ≥ 1.5 in the SVCV-infected groups compared to the control groups ($p < 0.05$). A Venn diagram was drawn to show the DMGs in organs of SVCV-infected fish at different time points (Fig. 2A). The number of genes with the H3 trimethylation mark remained constant within the experiment time frame (5 days).

Functional classification and Gene Ontology (GO) analysis of enriched genes were performed. Genes were grouped into three categories: biological processes, cellular component and molecular function. We focused our attention to the first category (biological processes), which includes the genes involved in the response to the stimulus (Fig. 2B). A subset of 218 genes with fold-enrichment values ≥ 1.5 in the three SVCV groups (1, 2 and 5 dpi) was identified within the “immune system process” keyword in UNIPROT Biological process category (Fig. S4).

Gene set enrichment analysis (GSEA) showed a general response to external stimuli and stress already at 1 dpi (Table 1). At later times (2 and 5 days after SVCV infection) the activated gene sets have a more concrete profile associated with adaptive and innate immune responses to a viral infection. Thus, there was a rapid promoter activation in genes of the Toll-like receptors (*tlr*), interferon (*ifn*), transforming growth factor beta (*tgfb*) and tumor necrosis factor alpha (*tnfα*) signaling pathways, amongst others. A particularly interesting group of genes, c-

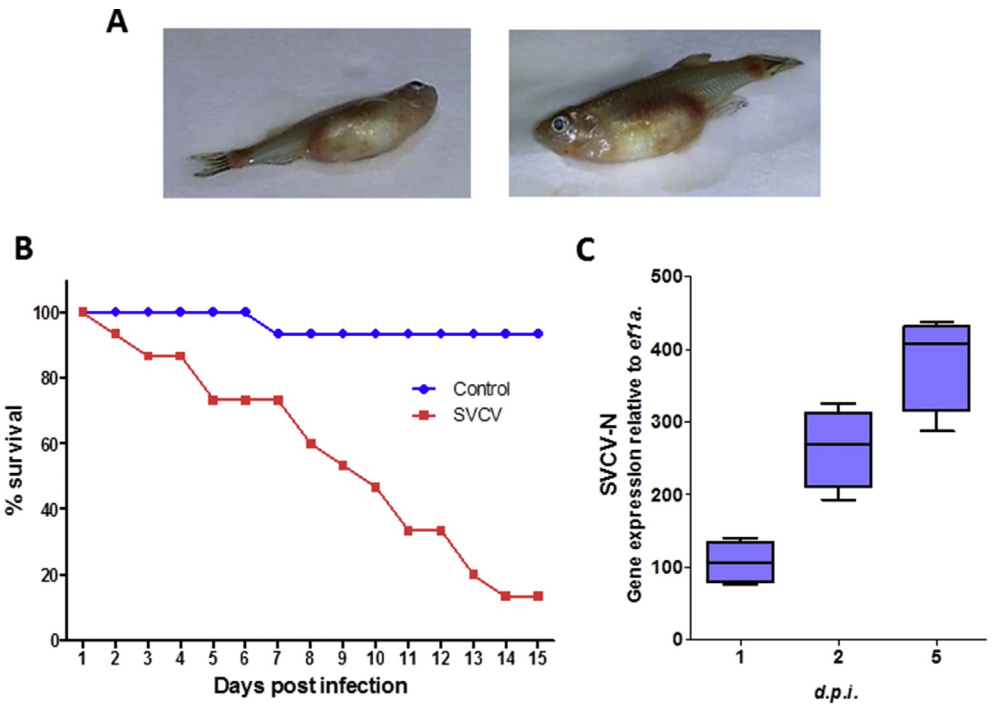

Fig. 1. SVCV challenge of zebrafish. A) Hemorrhagic symptoms induced by SVCV infection. The images show representative fish taken at day 8 post challenge. B) Cumulative percent survival of zebrafish infected with SVCV by bath immersion. C) Viral load in SVCV-infected fish examined by RT-qPCR using specific primers for the N gene. Mean ± SD (n = 5 fish).

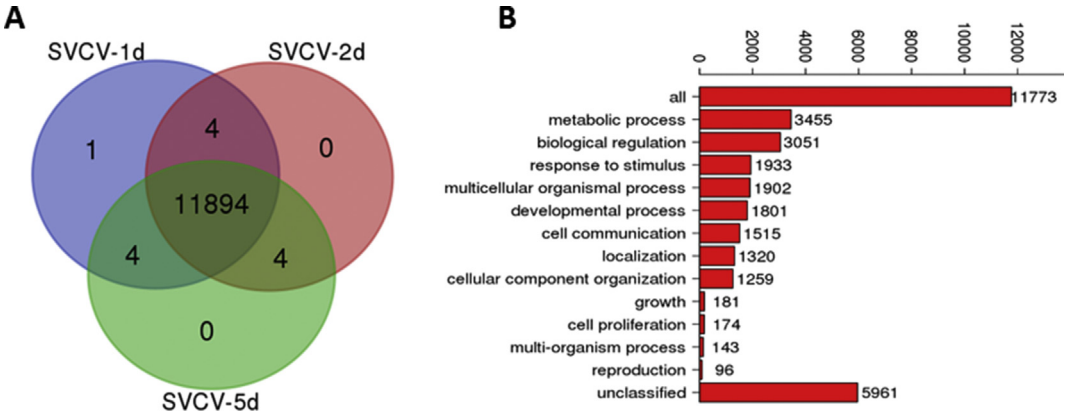

Fig. 2. Genes targeted by the H3K4me3 histone modification in SVCV-infected zebrafish. Number of genes with fold-increments ≥ 1.5 compared to non-infected fish. A) Venn Diagram of genes displaying differential H3K4me3 signal at different time points after infection. B) GESTALT analysis showing the "biological processes" functional categories with activated genes. N = number of genes.

Table 1
GSEA analysis. List of enriched gene sets in the SVCV-infected groups.

SVCV	Gene set	SIZE	NOM p-val	FDR q-val
1 dpi				
	TLR	*9*	*0.06629834*	*1.0*
	CYTOSOLIC DNA-SENSING PATHWAY	*14*	*0.15240642*	*1.0*
	ALPHA6-BETA4 INTEGRIN SIGNALING	*12*	*0.13477089*	*1.0*
	HOM	*15*	*0.17032968*	*1.0*
	APOPTOSIS	*16*	*0.19473684*	*1.0*
	IFN	*17*	*0.19261214*	*1.0*
	TGF-BETA SIGNALING PATHWAY	*14*	*0.21899736*	*1.0*
	CHK	*6*	*0.22424242*	*1.0*
	IGS	*7*	*0.27019498*	*1.0*
	COM	*8*	*0.34472933*	*1.0*
	MEASLES	*34*	*0.3358396*	*1.0*
	RIG-I-LIKE RECEPTOR SIGNALING PATHWAY	*22*	*0.36503857*	*1.0*
	APOPTOSIS MODULATION BY HSP70	*17*	*0.4458763*	*1.0*
	PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM	*30*	*0.4275*	*1.0*
	TOLL-LIKE RECEPTOR SIGNALING PATHWAY	*34*	*0.4508816*	*1.0*
	NATURAL KILLER CELL MEDIATED CYTOTOXICITY	*21*	*0.4716495*	*1.0*
	TGFB RECEPTOR SIGNALING WIKIPATHWAY	*39*	*0.5252525*	*1.0*
	PROTEASOME DEGRADATION	*30*	*0.56171286*	*1.0*
2 dpi				
	CRP	***7***	***0.014778325***	***0.07955655***
	TLR	*9*	*0.1131579*	*1.0*
	IGS	*7*	*0.23684211*	*1.0*
	TGF-BETA SIGNALING PATHWAY	*14*	*0.23626374*	*1.0*
	MAPK SIGNALING PATHWAY WIKIPATHWAY	*10*	*0.31989247*	*1.0*
	APOPTOSIS	*16*	*0.32448378*	*1.0*
	EGFR1 SIGNALING PATHWAY	*43*	*0.27972028*	*1.0*
	ALPHA6-BETA4 INTEGRIN SIGNALING	*12*	*0.33854166*	*0.942*
	TOLL-LIKE RECEPTOR SIGNALING PATHWAY	*34*	*0.41025642*	*1.0*
	HOM	*15*	*0.4469914*	*1.0*
	PROTEASOME DEGRADATION	*30*	*0.47727272*	*1.0*
	TOLL-LIKE RECEPTOR SIGNALING WIKIPATHWAY	*33*	*0.62903225*	*1.0*
	IFN	*17*	*0.61904764*	*1.0*
	JAK-STAT SIGNALING PATHWAY	*11*	*0.61403507*	*1.0*
5 dpi				
	CRP	***7***	***0.0***	***0.0023***
	NITR	*6*	*0.9756098*	*0.9797*

reactive proteins (*crp*) showed the H3K4me3 mark. CRPs are serum proteins synthesized in the liver and released to blood during inflammation or infection with pathogens. At 5 dpi the *crp* and *nitr* (novel immune-type receptor) gene families were the only gene sets significantly enriched in the H3K4 methylation (Table 1).

KEGG pathway analysis of DMGs identified the Toll-like receptor signaling pathway enriched in all SVCV-infected groups (Table S2).

3.3. Activation of immune-relevant genes

We took a closer look to the H3K4 methylation status of those genes with an especially relevant role in immune response to viral infection. The fold-increase values of the H3K4me3 mark over non-infected controls are shown in Fig. 3.

All the Toll-like receptors (*tlr*) gene promoters were activated already at 1 dpi and maintained more or less constant levels of methylation up to 5 dpi, with the exception of *tlr1* that continued increasing at 2 and at 5 dpi. The activation profile of pro-inflammatory cytokines gene promoters (*tnfa, il1b, il6*) was also enhanced in the SVCV-infected fish samples.

SVCV infection led to a change in the methylation of interferon genes promoters: there was a rapid activation of *ifnphi3* at 1 dpi followed by a decline. Coincidentally, the stimulator of interferon genes (*sting*) showed the same pattern as *ifnphi3*. the Interferon regulatory factors (irf) *irf3* and *irf7* were up-methylated from day 1 post-infection. With respect to interferon-stimulated gene promoters (ISGs) the myxovirus resistance (*mx*) gene promoters were up-methylated from 1 dpi onwards. Other ISGs genes such as the gene encoding the reovirus-induced protein *Gig2* was also up-methylated at contant levels from 1 to 5 dpi as well as the proteins containing tetratricopeptide repeats (IFITs) *ifit8* and *ifit14* were found up-methylated. The *rsad2* (also called *viperin*) gene promoter showed H3K4 methylation enrichment increasing with time.

Interestingly, of all examined genes the most highly up-methylated genes were those encoding for c-reactive proteins (*crps*), showing a maximal 9.9-fold H3K4me3 enrichment at 5 dpi (Fig. 3F), which is in good agreement with the GSEA data.

Other selected genes associated with host responses to viral pathogens such as antimicrobial peptides beta-defensin and hepcidin, were also examined, both of them showing some degree of up-methylation.

3.4. RT-qPCR analysis of immune-relevant genes expression

Trimethylation of the lysin 4 in histone H3 should result in active regulation in the promoter region of a gene with a corresponding increase in its transcription levels. To further substantiate the ChIPseq

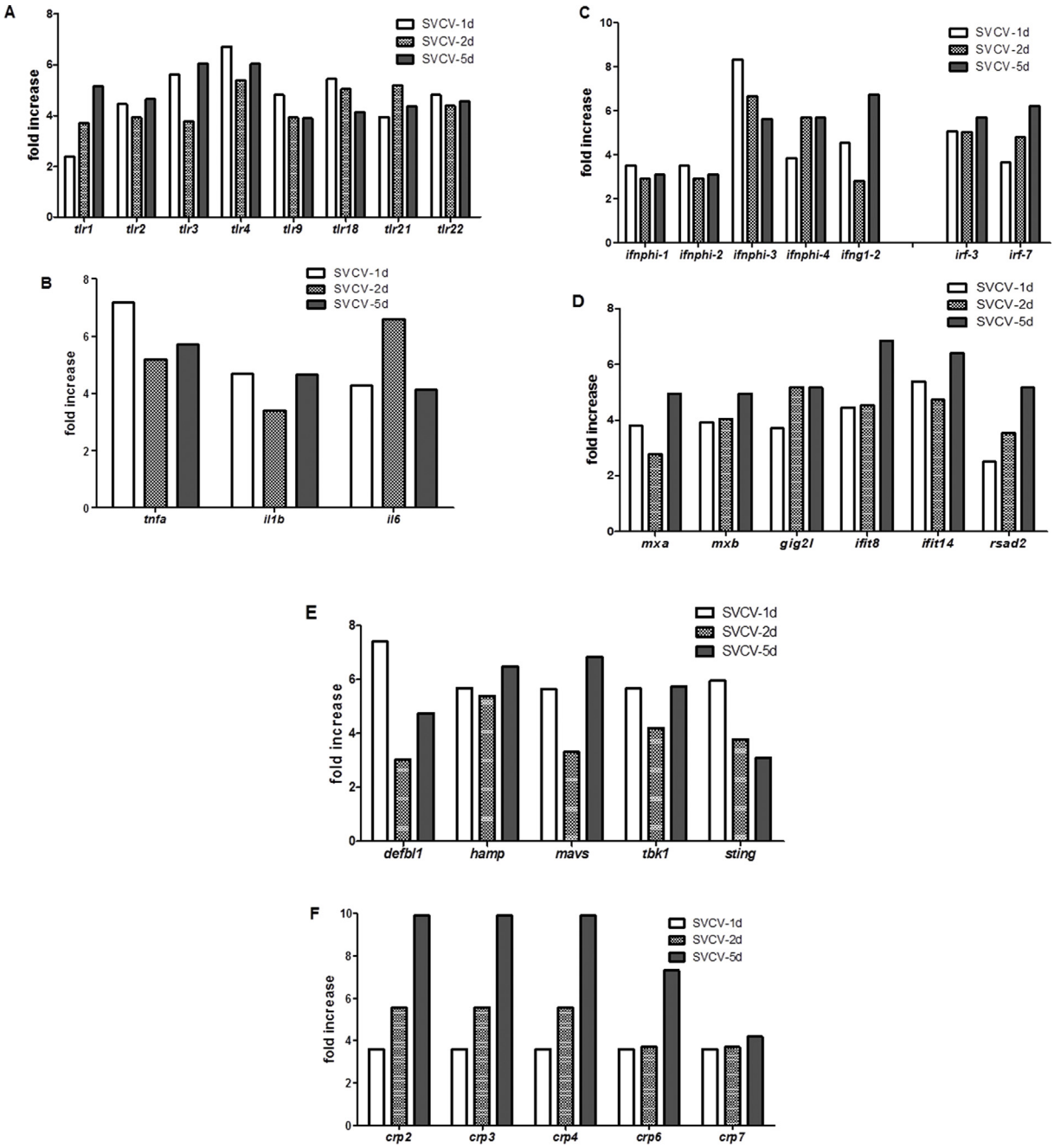

Fig. 3. Changes in H3K4 methylation of immune-related genes. Relative increase profiles over the control group in response to SVCV infection. A) TLRs; B) Inflammatory cytokines; C) Interferons and interferon regulatory factors; D) Interferon-stimulated genes (ISGs) E) Antimicrobial/antiviral genes; F) C-reactive proteins.

observations, RNA from the SVCV-infected zebrafish internal organs was isolated and gene expression analyzed by real-time RT-PCR (Fig. 4).

Despite the intrinsic high variability of in vivo experiments, a time-dependent pattern of gene expression could be drawn for every gene. Thus, we found that all the selected up-methylated genes showed transcriptional activation. The expression values over non-infected controls of the selected genes were in agreement with the ChIPseq analysis. For instance, those genes previously associated with response to viral infection in fish such as *tlr3* or *tmem173* (sting) were up-regulated at all times post-infection. Other genes involved in innate immune activation pathways like *mavs* and in inflammatory responses (*il1b*, *il6*) were also up-regulated from 1 to 5 dpi. Similarly, genes directly related to the interferon response (*irf3*, *ifnphi1*, *gig2*, *rsad2*) were also transcriptionally activated, particularly *irf7* and *mxab* as well as the antimicrobial peptide beta-defensin (*bdef2*) gene were also up-regulated from 2 to 5 dpi. The expression of *ifn* and *mx* genes displayed ever increasing values matching the SVCV N gene RNA measurements, likely reflecting the progression of the disease. All the *crp* genes exhibited increasing transcription from day 1–5 post-infection: *crp2* and *crp6* showing a strong increase at the latest time (5 dpi) while *crp1*, *crp3*, *crp4* and *crp7* showed sustained activation.

Taken together, RT-qPCR data confirmed that H3K4me3 histone methylation patterns were associated with the up-regulation of innate immune signaling pathways genes caused by SVCV infections.

4. Discussion

ChIP sequencing is an effective method to identify transcriptionally

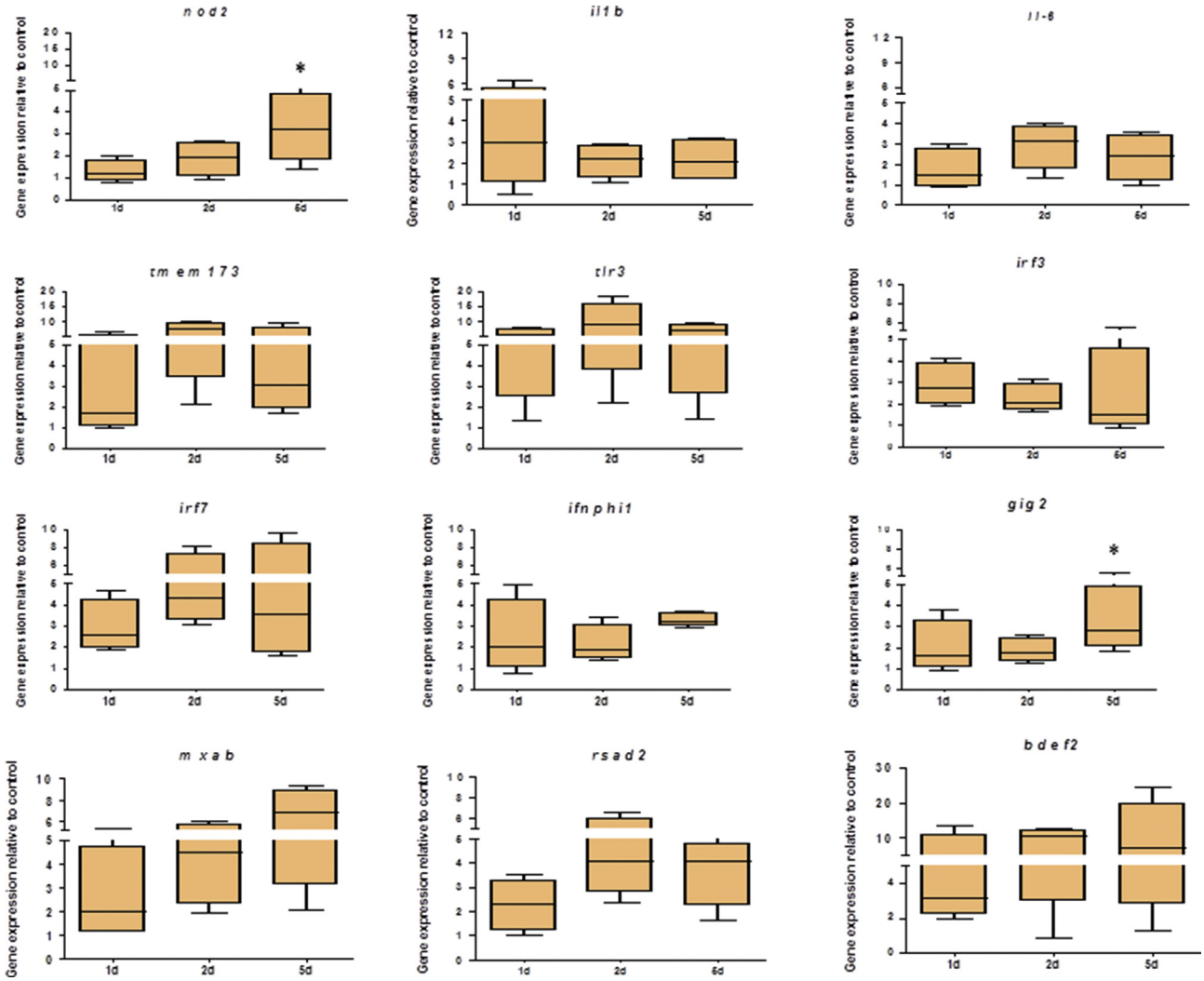

Fig. 4. Changes in the expression profiles of several genes in zebrafish infected with SVCV. Transcript levels of selected genes were evaluated by qRT-PCR and expressed as number of fold increases over the non-infected control group. Mean ± SD data from 3 fish are shown (*p < 0.05; **p < 0.01). A) Selected immune-related genes. B) C-reactive proteins multigene family.

activated genes and therefore obtain information on the organism response to a given stimulus, such as pathogen infection. To our knowledge this is the first study on the changes in histone methylation in the promoters of genes induced in virus-infected zebrafish. This work was aimed to get a better understanding of the host factors involved in response to SVCV infection, and perhaps identifying those epigenetic marks that would lead to an enhanced immune response upon a secondary infection.

Bath immersion was our choice on the way of SVCV infection because it resembles the natural infection route. Zebrafish infected with SVCV by bath immersion [8,14] show characteristic symptoms of the disease [11] and experience mortality. In other works the challenge was done by intraperitoneal injection of the virus [16]. This may lead to some inconsistencies compared to bath immersion data regarding the exact pattern and time-course of gene expression after viral infection [8].

In the present study, ≈12000 genes showed differential methylation of histone H3 in those samples taken from zebrafish infected with SVCV. Data analysis identified GO terms related to host defense mechanisms (“immune system process”) and KEGG pathways (Toll-like receptor signaling) involved in innate immune response. GSEA analysis revealed that only two gene sets (*crp*, *nitr*) were enriched in differentially methylated genes at 5 dpi. This may reflect the host gene expression going back to basal levels after an early response to the virus challenge.

Increased levels of H3K4me3 in the promoter region should correlate with enhanced transcription and stimulation of the marked genes. Other system-wide evaluations of virus-host interactions (such as microarray data) have shown a large impact of viral infection on the fish transcriptome [4–6,8,17]. Viral infection of fish stimulates the production of type I IFN, one key factor in mounting an antiviral response to protect the host from disease. Rhabdoviruses are powerful inducers of interferon gene expression in cyprinid fish, both in vitro and in vivo [18–21]. Our data show the transcriptional activation of *ifnphi3* which is in accordance with other authors' findings [4,9,22]. Upstream of the interferon genes in the interferon response pathway, the interferon regulatory factors (*irf*) constitute a family of transcription factors that regulate the transcription of interferon genes. We found a close relationship between activation of promoters and gene expression for *irf3* and *irf7* in SVCV-infected fish that correlates with the up-regulation of *ifnphi3* [23,24]. IRF3 and IRF7 are known to be involved in response against rhabdovirus infection in fish [25] with *irf3* reaching a peak of expression earlier than *irf7* [26] similar to what we found in here. IRF3 has also been associated with the enhancement of the antiviral gene *gig2* expression in fish [27]. Gig2 is an important antiviral protein in fish, providing resistance to SVCV infection when overexpressed in

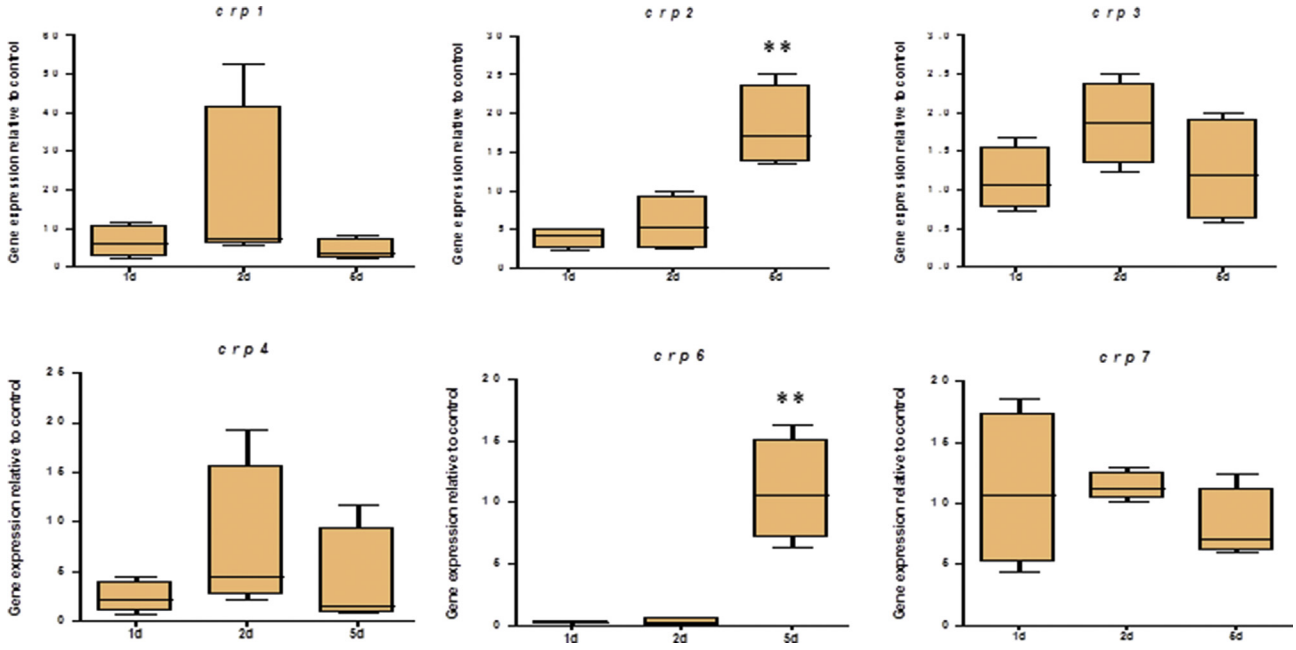

Fig. 4. (*continued*)

zebrafish cells [27].

In addition to *gig2*, other genes that have been proven to play an important role in antiviral response in fish such us *rsad2/viperin* [16,28] or *sting* [29] were rapidly up-methylated after SVCV infection. The interferon inducible proteins IFITs have been proposed to have an antiviral role in fish [30]. Several *ifit* genes were found transcriptionally active in the SVCV-infected group, with *ifit8* and *ifit14* showing the highest fold increase over the non-infected controls.

One relevant interferon-stimulated gene, *mx*, showed an increasing transcriptional activation over time in SVCV-zebrafish. Up-regulation of mx expression after rhabdovirus infection of cyprinid fish has been previously found in other experimental trials [8,21].

Similar to mammalian Toll-like receptors (TLRs) piscine TLRs play a key role in the initial recognition of the virus alerting the fish about danger and triggering immune responses [31]. As stated before, promoters of genes in the Toll-like receptor signaling pathway were activated upon SVCV infection. In particular, *tlr2* and *tlr3* have been found to be rapidly induced in kidney of SVCV-infected common carp although their levels decreased after 5 days [26].

Toll-like receptors involved pathways activate transcription of proinflammatory cytokines: *il1b, il6* and *tnfa* gene promoters were up-methylated in the SVCV-infected samples compared to non-infected samples at all time points. The *tnfa* gene is usually expressed at an early stage after virus infection of fish [32], while *il6* has been associated with immune responses of fish to virus challenge [33]. Here, a rapid inflammatory response was triggered by SVCV in zebrafish internal organs.

One multigene family not usually associated with host antiviral defense has been identified in this work: c-reactive proteins (*crp*) confirming a previous report [4] describing a possible relationship between the CRPs and virus infection. CRPs were discovered to increase their circulating levels in blood in response to bacterial infection [34]. Previous studies by our group have hinted that CRPs may have an antiviral role in fish [4,35,36]. In connection with the up-regulation of *il6* found in here, this interleukin has been associated with stimulation of *crp* expression in zebrafish before [35].

In summary, we have explored the promoter-activating epigenetic modification H3K4me3 in response to SVCV infection in zebrafish. The histone methylation occurs rapidly, in the first 24 h after infection. This is in agreement with other reports of epigenetic changes being established as fast as 5 h post-stimulus [37]. The maintenance of a hypermethylated (up-regulated) chromatin profile along time could help to establish a memory of the innate immune system, similar to what has been termed “trained immunity” [38]. This state of readiness of the innate immune system would render the host more resistant to a subsequent infection [39,40]. Another of the landmarks of mammalian trained immunity is the enhanced expression of proinflammatory cytokines similar to what has been described in here. This study provides novel evidence for the role of epigenetics in the immune response of teleost fish. In the future, the modulation of other transcription associated marks (either methylation or acetylation of histones) may be investigated to get a comprehensive view of the epigenetic reprogramming phenomenon. This knowledge will likely contribute to the understanding of the molecular basis of disease control in aquaculture.

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Conflicts of interest

The authors declare that there are no potential competing interests that may influence this publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.08.056.

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