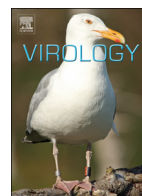




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# Differential transcription of fathead minnow immune-related genes following infection with frog virus 3, an emerging pathogen of ectothermic vertebrates



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## ABSTRACT

Frog virus 3 (FV3) and other ranaviruses are responsible for die-offs involving wild, farmed, and captive amphibians, fish, and reptiles. To ascertain which elements of the immune system respond to infection, we explored transcriptional responses following infection of fathead minnow cells with either wild type (wt) FV3 or a knock out (KO) mutant targeting the 18 kDa immediate early gene (18K). At 8 h post infection we observed marked upregulation of multiple transcripts encoding proteins affecting innate and acquired immunity. Sequences expressed 4-fold or higher in wt-infected cells included transcripts encoding interferon (IFN), IFN regulatory factors (IRFs), IFN stimulated genes (ISGs) such as Mx and MHC class I, and interleukins IL-1 $\beta$ , IL-8, IL-17C and IL-12. Cells infected with the 18K KO mutant ( $\Delta$ 18K) showed qualitative differences and lower levels of induction. Collectively, these results indicate that ranavirus infection induced expression of multiple cellular genes affecting both innate and acquired immunity.

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## Introduction

During the past 30 years, ranaviruses have caused considerable morbidity and mortality among ectothermic vertebrates, i.e., amphibians, fish, and reptiles (Chinchar et al., 2009, 2011). Although infections with the chytrid fungus *Batrachochytrium dendrobatidis* are responsible for the extinction of several amphibian species and have been viewed as the principal pathogen threatening amphibians, ranavirus infections are common and are the leading cause of localized die-offs among amphibians in North America (Gray et al., 2009; Green et al., 2002). Moreover, ranavirus infections affect multiple species throughout the world and it is thought that die-offs may push small populations with limited geographic ranges to extinction.

Frog Virus 3 is the best-characterized member and the type species of the genus *Ranavirus* (family *Iridoviridae*), a group of large, icosahedral, double-stranded DNA viruses (Chinchar, 2002;

Chinchar et al., 2009). Within the family *Iridoviridae*, two genera (*Iridovirus* and *Chloriridovirus*) infect invertebrates, whereas three genera (*Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*) target cold-blooded vertebrates (Jancovich et al., 2012). Among these five genera, ranaviruses and megalocytiviruses are currently viewed as emerging pathogens of fish and amphibians (Chinchar et al., 2009).

Ranaviruses are promiscuous pathogens and infect a wide range of species belonging to one or more taxonomic classes (Jancovich et al., 2012). For example, although originally isolated from leopard frogs, FV3 and FV3-like viruses have been detected in other frog species, as well as from salamanders, fish, and turtles (Chinchar and Waltzek, 2014). Previous studies (Gantress et al., 2003; Tweedell and Granoff, 1968) demonstrated that although immunocompetent adults confine ranavirus infection to the kidney and successfully recover, tadpoles fail to clear infection, develop systemic disease, and succumb to infection. The sensitivity of tadpoles to infection likely reflects the fact that tadpoles lack full expression of MHC class I molecules and are thought to be deficient in the development of T cell responses (Robert and Ohta, 2009). Moreover, metamorphosis imposes considerable metabolic

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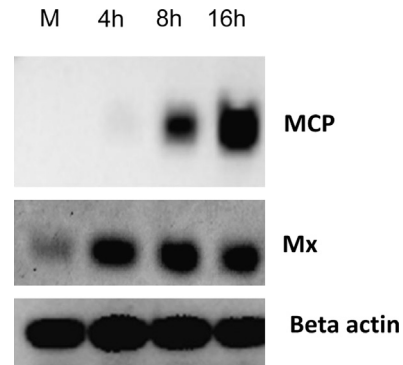
costs on amphibians and heightens their susceptibility to severe ranavirus infections (Rollins-Smith, 1998). In contrast, adult frogs develop protective innate and acquired responses, the latter involving the induction of anti-viral antibodies and cytotoxic T cells (Maniero et al., 2006; Morales and Robert, 2007; Robert et al., 2005). While cellular genes play critical roles in protection and recovery from ranavirus infection, viral genes are thought to play important roles as possible immune antagonists. For example poxviruses encode a dozen or more genes whose function is to circumvent various aspects of the host immune response or enhance replication (Finlay and McFadden, 2006; Johnston et al., 2005; Johnston and McFadden, 2003; Seet et al., 2003; Wang et al., 2009). Ranaviruses likely encode functionally similar genes and, consistent with that view, a FV3 knock out mutant lacking the 18 kDa immediate early gene ( $\Delta$ 18K-FV3) displayed reduced lethality following infection of *Xenopus* tadpoles (Chen et al., 2011). Likewise, an *Ambystoma tigrinum* virus (ATV) mutant lacking the viral homolog of eukaryotic initiation factor 2 ( $vIF-2\alpha$ ), a putative virulence gene, was unable to prevent the phosphorylation and subsequent inactivation of eIF-2 $\alpha$  and displayed slightly less virulence in vivo (Jancovich and Jacobs, 2011).

In this study we explored whether FV3 infection induced expression of immune-related genes in fathead minnow (*Pimephales promelas*, FHM) cells, a permissive epithelial line commonly used to study the replication of FV3 and other ranaviruses. FHM cells were infected with either *wt* FV3 or  $\Delta$ 18K-FV3 and assayed to determine whether immune-related genes were differentially expressed. Expression was monitored using a 60 K-feature FHM microarray and results of several key genes were validated by quantitative real time RT-PCR (qPCR). As discussed below, we observed induction of numerous immune-related genes in cells infected with *wt*- or  $\Delta$ 18K-FV3 virus suggesting that host immune responses likely play critical roles in combating ranavirus infection.

## Results

### Induction of host immune-related transcripts

Ranavirus infections result in the progressive inhibition of cellular RNA and protein synthesis (Raghow and Granoff, 1979; Tannenbaum et al., 1978, 1979). As a consequence, virus-induced host gene expression is likely a transient event that takes place within a window defined by virus-induced onset and virus-mediated inhibition of host protein and RNA synthesis. To determine the time at which cellular immune-related transcripts were present, we infected FHM cells at a multiplicity of infection sufficient to infect all cells (5 PFU/cell) and monitored expression of the viral major capsid protein (MCP) gene and cellular transcripts encoding Mx and  $\beta$  actin by RT-PCR. Expression of the MCP, a late viral gene product, serves as a marker of infection, Mx, a cellular anti-viral protein whose expression is induced by interferon (IFN), is a marker for virus-induced immune-related transcripts, and  $\beta$  actin is a constitutively expressed housekeeping gene that serves as an indicator for RNA integrity. As shown in Fig. 1, MCP transcripts were not detected in uninfected cells, marginally expressed at 4 h p.i., abundant at 8 h, and maximally present at 16 h. Reflective of a low level of constitutive synthesis, Mx transcripts were present at reduced levels in mock-infected cells, but were upregulated by 4 h p.i. and remained at high levels thereafter. As expected,  $\beta$ -actin levels were abundant and constant throughout infection. Based on these results, cellular gene expression was examined at 8 h p.i. as this time point provides an excellent opportunity for detecting cellular genes that were differentially regulated by virus infection.

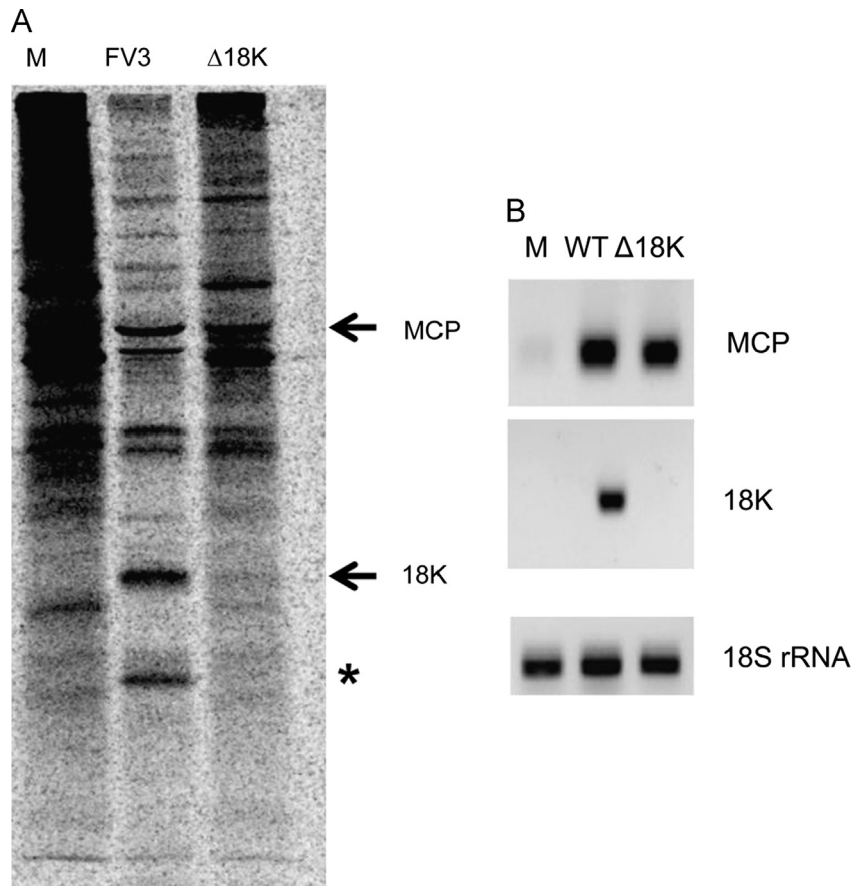


**Fig. 1.** Expression of a representative host immune-related gene, Mx, following FV3 infection. FHM cells were infected with *wt* FV3 at a MOI=5 PFU/cell, and at 4, 8, and 16 h after infection total RNA was isolated and subjected to RT-PCR analysis using primers specific for transcripts encoding viral MCP, FHM Mx, and FHM  $\beta$  actin. Total RNA from uninfected cells (M) served as a measure of constitutive gene expression.

### Ranavirus-induced transcriptional changes

To monitor global cellular gene expression, total RNA was isolated from replicate cultures of mock-, *wt*-, or  $\Delta$ 18K- infected FHM cells at 8 h p.i., and subject to microarray analysis. Prior to array hybridization, we confirmed a productive virus infection by monitoring viral protein and RNA synthesis in replicate cultures. Fig. 2A displays protein synthesis profiles in mock-, *wt*-, and  $\Delta$ 18K-infected cells at 8 h p.i.. Infection by *wt* virus led to a marked reduction of cellular protein synthesis and to the appearance of numerous virus-specific proteins, including the 48 kDa MCP and 18K immediate early protein. In contrast, infection with the  $\Delta$ 18K mutant did not suppress host protein synthesis as strongly as *wt* virus. Nonetheless, indicative of a productive infection, multiple viral proteins, including the MCP, were clearly detected in  $\Delta$ 18K-infected cells. As expected, the 18K protein was not detected due to the deletion of its cognate gene, and a prominent band of lower molecular weight, indicated by the asterisk, was also not detected. The presence of this latter band in *wt*-infected cells suggests that this unknown product might represent either a degradation product of the 18K protein or a polypeptide generated by internal initiation within the 18K coding region. Using a RT-PCR assay to monitor viral transcription (Fig. 2B) we confirmed the results of the protein screen and showed that *wt* virus induced expression of both MCP and 18K transcripts, whereas only MCP messages were present following infection with the KO virus.

Having confirmed a productive infection, total RNA from replicate cultures of mock-, *wt*-, and  $\Delta$ 18K-infected cells was subjected to microarray analysis using a 60K feature oligonucleotide array and validated by qPCR. Microarray analyses identified a total of 10,051 differentially expressed genes (DEGs) (fold change > 2; *p* value < 0.01) in *wt*- infected cells of which 6681 were upregulated and 3370 down regulated (Supplementary Table S1). Within  $\Delta$ 18K-infected cells 5386 DEGs were detected with 3572 showing upregulation and 1814 downregulation (Supplementary Table S2). Note that because some genes are present at multiple sites on the array, the actual number of differentially expressed genes is likely lower than the values shown here. Surprisingly, despite the marked downturn in cellular protein synthesis depicted in Fig. 2A, approximately twice as many gene transcripts were upregulated than downregulated. This finding suggests that following viral infection cellular transcription is transiently upregulated prior to the onset of virus-induced transcriptional and translation shutdown. Moreover, although genes identified in *wt* virus-infected cells were generally also upregulated in  $\Delta$ 18K-infected cells, the response in the latter was not as



**Fig. 2.** Confirmation of a productive FV3-infection by SDS-PAGE and RT-PCR analysis. (A) SDS-polyacrylamide gel electrophoresis of lysates from mock-, wt FV3-, and  $\Delta$ 18K-infected FHM cells. FHM cells were mock-infected or infected at a MOI=5 PFU/cell. Cells were radiolabeled with [ $^{35}$ S]methionine/cysteine from 6 to 8 h after infection, and radiolabeled proteins were separated by electrophoresis and visualized by phosphorimaging. Positions of the viral MCP and 18K proteins are indicated. A lower mol wt protein seen only in wt-infected cells is indicated by an asterisk. (B) Total RNA from mock-, wt-, and  $\Delta$ 18K-infected FHM cells was isolated at 8 h after infection and subjected to RT-PCR using primers targeting the viral MCP and 18K transcripts, and host 18S rRNA. Amplicons were separated by agarose gel electrophoresis and visualized using ethidium bromide.

strong as following infection with wt virus (Table 2). With few exceptions, putative immune related genes were generally up-regulated after viral infection. However, some immune-related genes (NKEF, NK cell enhancing factor; ATG7, autophagy related gene 7; EIF2AK1, an eIF-2 $\alpha$  kinase; C9, complement component 9, TLR5A, toll-like receptor 5A, and TRIM25, tripartite motif containing protein 25) were modestly downregulated 2- to 4-fold after infection. While a number of genes involved in DNA (DNA polymerase A, POLA2; DNA polymerase B, POLB; DNA polymerase E, POLE) and RNA synthesis (the transcription factor SP1) were down-regulated after infection, other genes linked to RNA (e.g., the transcription factors JUND and JUNB) and protein synthesis (e.g., EIF2S2, the  $\beta$  subunit of eukaryotic initiation factor 2) were upregulated (Tables 2 and 3). It should be noted that the FHM cultures used in these experiments were confluent and therefore not actively engaged in the synthesis of cellular macromolecules. We speculate that genes that underwent at least transient induction were likely those that represent an immune response to infection or play critical roles in the synthesis of viral gene products and/or viral replication and assembly. A selection of differentially expressed immune-related and metabolic genes is listed in Tables 2 and 3 and their putative roles in viral replication are described more fully in the Discussion.

Cluster analyses of five replicate cultures from mock and wt virus-infected cells and four samples from cells infected with the  $\Delta$ 18K mutant are shown in Fig. 3. Gene expression patterns displayed good agreement among mock- wt-, and  $\Delta$ 18K-infected

replicates. Expression profiles in virus-infected samples were clearly distinct from those in mock-infected cultures, and although similar, profiles in wt and  $\Delta$ 18K cultures were distinct.

To validate the microarray findings, we analyzed four differentially expressed genes by qPCR. Validation studies (Fig. 4) confirmed the microarray findings and showed that IL-8, INF, IRF3 and HSPB8 were differentially expressed in virus-infected cultures. The levels of expression of IL-8, IRF3, and HSPB8 were comparable in both microarray and qPCR studies, whereas IFN levels in wt-infected cells were markedly higher when monitored by qPCR (70-fold upregulated) compared to microarray results (11-fold upregulated). The congruence of microarray and qPCR results indicates that microarray analysis provides an accurate measure of cellular message levels after infection.

#### Functional analysis

FHM cells infected with wt FV3 displayed differential regulation of multiple pathways especially those involving molecular mechanisms of cancer, TGF- $\beta$  signaling, acute phase response signaling, and IL-8 signaling among others (Supplementary Table S3). Likewise, cells infected with  $\Delta$ 18K-FV3 differentially expressed genes involved in PI3K/AKT signaling, acute phase response signaling, molecular mechanisms of cancer, and cell cycle regulation (Supplementary Table S4). A total of 170 pathways displaying differential expression were common to both exposures, with 67 unique to wt exposure, and 15 unique to  $\Delta$ 18K exposure. Pathways



**Fig. 3.** Heat map analysis of mock-, wt-, and  $\Delta$ 18K KO-infected FHM cells. Levels of gene expression are indicated by color: blue lowest, yellow intermediate, red highest. Brackets across the top of the map indicate relatedness (clustering) among samples.

unique to wt FV3 infected cells involved signaling controlled by thrombin, p38 MAPK, IL-2, or death receptors. Unique pathways displaying differential regulation after  $\Delta$ 18K-FV3 infection were those for glycogen degradation and PXR/RXR activation. Collectively, pathway analysis suggests that FV3 infection induces, at least transiently, the expression of multiple genes that regulate various aspects of cellular and viral metabolism.

## Discussion

### Differential gene expression in wild-type and $\Delta$ 18K infected cells

FV3 infection resulted in the marked upregulation of multiple cellular transcripts including those that encode immune-related proteins such as IFN and the products of various IFN-stimulated genes (ISGs). These results are consistent with those seen following infection with influenza virus, smallpox virus, and a number of ranaviruses including FV3 where infection of *Xenopus laevis* was shown to trigger the expression of TNF $\alpha$ , IL-1 $\beta$ , and IFN $\gamma$  (Chen et al., 2011; Holopainen et al., 2012; Huang et al., 2011; Kash et al., 2004; Randall and Goodbourn, 2008; Rubins et al., 2004; Wu et al., 2012; Xu et al., 2010a). The marked upregulation of immune-related transcripts in FV3-infected FHM cells is in marked contrast to the marked progressive inhibition of cellular protein synthesis that accompanies infection. The presence of immune-related transcripts at a time of diminished protein synthesis likely reflects

their earlier induction and continued stability. We speculate that these transcripts are translated prior to the profound inhibition of host protein synthesis seen at later times after infection and serve to either reduce viral replication in infected cells or, if released from cells as is the case for IFN, trigger induction of an anti-viral state in surrounding uninfected cells.

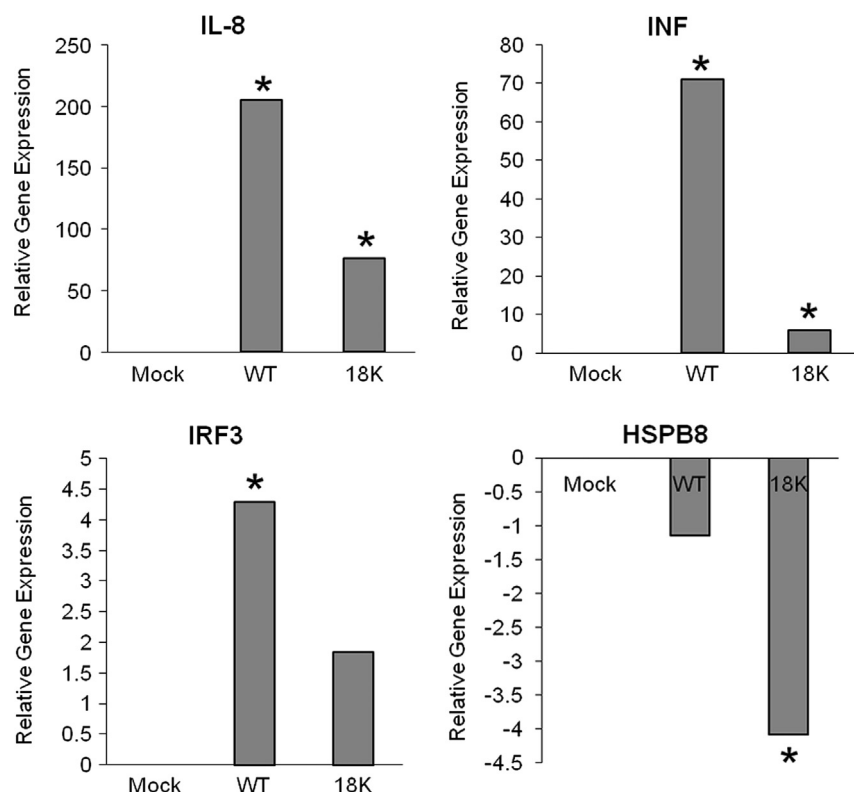
Despite the apparently large number of upregulated putative immune-related transcripts, FV3 infected FHM cells ultimately undergo cytopathic effect and die within 24 h. It is not known whether cultures infected at a lower MOI, where the vast majority of cells are initially uninfected, would respond to the IFN induced in, and released by, infected cells and display reduced levels of viral replication due to the induction of anti-viral ISGs such as PKR (protein kinase R), OAS (2'5' oligoadenylate synthase), RNase L (RNase L), ISG15, and other antiviral molecules (Randall and Goodbourn, 2008; Sadler and Williams, 2008). We speculate that despite the rapid replication of FV3 in vitro, the small size of FV3 plaques in FHM monolayers after 5–7 days of culture may reflect the inhibitory effects of IFN on surrounding uninfected cells. Moreover, while such protective effects might not be seen after high multiplicity infection in vitro, they may play important protective roles in vivo where even a modest inhibition of virus replication may allow host innate and adaptive immune systems sufficient time to successfully contain and resolve an ongoing infection.

We observed that the level of induction of immune-related genes in  $\Delta$ 18K KO-infected FHM cells was lower than that seen after wt infection (Table 2) and that, with the exception of the 18K protein, there was little to no difference in the expression of virus-specific polypeptides in wt and  $\Delta$ 18K KO-infected cultures (Fig. 2A). Consistent with these observations, we previously showed that knock down of 18K expression using anti-sense morpholino oligonucleotides had little to no effect on viral gene expression and virus production (Sample et al., 2007). Based on those results, we concluded that 18K was not essential for replication in FHM cells and hypothesized that 18K only played an essential replicative role in certain cell types or that 18K modulated anti-viral immunity and virulence in vivo. The observation that lower levels of immune-related transcripts were induced following infection with  $\Delta$ 18K may explain its reduced virulence in vivo. Perhaps the viral 18K protein, by a mechanism yet to be elucidated, triggers a robust induction of cellular immune-related transcripts (i.e., the amphibian version of a “cytokine storm”) that is responsible for the marked morbidity and mortality seen in vivo after wt infection. Conversely, mutants lacking the 18K gene trigger a modest induction of immune-related genes which protects the animal and contributes to viral clearance.

### Immune-related genes upregulated after infection

Transcripts induced by FV3 infection include a number associated with innate immune responses to viral and bacterial infections. In mammalian cells, engagement of TLR3 by viral double-stranded RNA (dsRNA) triggers signaling through IFN regulatory factor 3 (IRF3) and results in the induction of IFN $\beta$ , which acts in an autocrine fashion to induce IFN $\alpha$  (Murphy et al., 2008). IFN $\alpha$  subsequently binds the IFN $\alpha$ / $\beta$  receptor and triggers signaling through the JAK/STAT pathway leading to the synthesis of 200 or more IFN-stimulated genes (ISGs) that inhibit viral replication (Sadler and Williams, 2008). In this study, we saw evidence for induction of IFN $\alpha$  (11-fold upregulated) in wt-infected cells and several ISGs including MXD (9-fold up) and MHC class I (5-fold up), but did not detect upregulation of a piscine homolog of PKR, a cellular product that in most mammalian systems lead to phosphorylation of eukaryotic initiation factor 2 $\alpha$  and the global





**Fig. 4.** Validation of microarray results by qPCR analysis. Four cellular transcripts (IL-8, INF, HSPB8, and IRF3) showing differential expression between mock- and virus-infected samples were monitored. Levels of expression were determined using the  $\Delta\Delta C_t$  method. Asterisks indicate fold changes that were statistically significant at  $p < 0.05$ .

inhibition of protein synthesis (Sadler and Williams, 2008). MX proteins have been shown to inhibit the replication of influenza and other viruses and may play a similar role here (Fernandez-Trujillo et al., 2013; Sadler and Williams, 2008). MX proteins are wide spread among both mammals and lower vertebrates and a putative MX gene has been identified in FHM cells (GenBank AY751300.1). We also noted that transcripts encoding putative homologs of IL-1 $\beta$  (9-fold), IL-8 (145-fold), and IL12 (12-fold) were upregulated by FV3 infection. All three are important elements of the innate immune response. IL-1 $\beta$  is a pro-inflammatory cytokine which in mammals is involved in the induction of fever, the production of IL-6, and activation of the vascular endothelium which increases access of effector cells to sites of infection (Murphy et al., 2008). IL-8 is a chemokine involved in the recruitment of neutrophils to sites of infection, whereas IL-12 is a cytokine, synthesized by macrophages, that activates NK and T cells to produce IFN- $\gamma$  and which induces the differentiation of CD4 T cells into T<sub>H</sub>1 cells (Murphy et al., 2008; Trinchieri, 1995; Xu et al., 2010b). Collectively, IL-1 $\beta$ , IL-8, and IL-12 may play critical roles in controlling FV3 infection during the early stages of infection through activation of a pro-inflammatory response.

Two gene products that showed the greatest upregulation are the aforementioned IL-8 (145-fold up) and a member of the TNF Receptor superfamily (TNFRSF) annotated by BLAST analysis as TNFRSF14 or TNFRSF1A (151-fold up). TNFRSF1A activates NF-KB, mediates apoptosis, and functions as a regulator of inflammation. Likewise, TNFRSF14 functions in a variety of ways (Steinberg et al., 2011). On one hand, it regulates T-cell immune responses by activating both inflammatory and inhibitory signaling pathways through its interaction with diverse ligands (Steinberg et al., 2011). In addition, TNFRSF14 binds, along with nectin-1, herpesvirus glycoprotein D (gD) and mediates viral entry (Kopp et al., 2013). It is not clear whether the marked upregulation of TNFRSF14 expression in FHM cells is an immune response to virus infection,

or an attempt by the virus to expand the number of viral receptors on target cells. Interestingly, transcripts encoding caveolin (CAV1) were also upregulated (14-fold) after infection. Caveolin-mediated entry has been suggested to take place with some iridoviruses and may reflect the fact that promiscuous pathogens such as FV3 have evolved multiple ways to enter cells (Guo et al., 2011).

In addition to MX, IFN, and the triad of IL-1 $\beta$ , IL8, and IL-12, FV3 infection also induced high levels of gamma interferon inducible lysosomal thiol reductase (GILT) and IL-17C. GILT (30-fold up) is thought to play an important role in antigen presentation by reducing disulfide bonds, thus facilitating protein unfolding and the subsequent generation of antigenic peptides (Arunachalam et al., 2000; Hastings and Cresswell, 2011). IL-17C (20-fold up) is a member of the IL-17 family of cytokines, which in mammals is induced by bacterial challenge and inflammatory stimuli and acts in an autocrine fashion to trigger expression of proinflammatory cytokines, chemokines, and antimicrobial peptides (Gaffen, 2011; Pappu et al., 2011; Ramirez-Carrozzi et al., 2011). Moreover, the increased expression of IL-8 and IL-1 $\beta$  seen in FV3-infected FHM cells may be triggered by IL-17C expression (Beklen et al., 2007). We also observed modest to marked up-regulation of several matrix metalloproteinases (MMPs) such as MMP14 (9-fold up), MMP9 (2-fold up) and MMP3 (2-fold up) in wt-infected cells. MMPs are important mediators of connective tissue destruction and are involved in collagen degradation (Beklen et al., 2007).

Modest down-regulation of host transcripts was also noted (Table 3). It was not surprising that host DNA polymerases were downregulated because the cells used in this study were confluent and likely not engaged in extensive DNA synthesis, and because FV3 encodes its own DNA polymerase. Modest reductions in the levels of transcripts associated with autophagy (ATG7), complement (C9), NK cell enhancing factor (NKEF), and TRIM25 were also detected. The down-regulation of TRIM25 transcripts may represent an attempt by the virus to block an IFN response since

TRIM25, an E3/ISG15 ligase, ubiquitinates the N-terminal CARD domain of RIG-I (DDX58) and triggers IFN induction (Gack et al., 2007). The importance of this mechanism is underscored by the observation that influenza A virus targets TRIM25 in an attempt to evade recognition by RIG-I (Gack et al., 2009). In FV3, down regulation of TRIM25 may allow the virus to escape detection. Likewise, down-regulation of the autophagy-related protein ATG7 may be critical for successful replication because cytoplasmic viral assembly sites may be viewed as danger signals and trigger autophagosome formation. In contrast to the down regulation noted above, we detected marked upregulation of several transcription factors (JUNB and JUND) as well as one of the subunits of eIF-2. Upregulation may reflect the involvement of host RNA polymerase II and associated transcription factors in early viral transcription, and the subsequent need to ramp up viral protein synthesis in contact-inhibited cells as they transition from host to viral protein synthesis.

#### Modulation of signaling pathways following FV3 infection

To identify host elements that modulate viral infection, we examined specific pathways within *wt*- and the  $\Delta$ 18K-infected cells that play key roles in cellular and viral metabolism. Since *wt*- and  $\Delta$ 18K-FV3 differed in virulence following infection of *Xenopus laevis* tadpoles (Chen et al., 2011), we hypothesized that differences in their ability to modulate the host immune response could explain this dichotomy. Several pathways common to both infections were related to immune responsiveness, cancer, or apoptotic death. Some of these pathways involved signaling by acute phase proteins, mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK/MAPK), PI3K/AKT, TGF- $\beta$ , and IL-8. Although elements of these pathways were differentially expressed following both *wt*- and  $\Delta$ 18K-infection, *wt*-infected cells displayed a larger number of differentially expressed genes (compare Supplementary Tables S1 and S2). A number of viruses have been found to affect key signaling pathways such as those involving MAPK/ERK and PI3K/AKT. The MAPK/ERK pathway is involved in cell growth and proliferation and has been suggested to modulate replication of several viruses including human immunodeficiency virus (HIV), bovine herpesvirus, and hepatitis B virus (Jacque et al., 1998; Zheng et al., 2003; Zhu et al., 2011). In addition, dysregulation of this pathway has been linked to viral pathogenicity (Zhao et al., 2005). Interestingly, integrins, used as receptors by several viruses (Schmidt et al., 2013) and involved in MAPK/ERK signaling (Yee and Hamerman, 2013), were down-regulated in both exposures. Likewise, the PI3K/AKT pathway is required for the efficient replication of several viruses including influenza virus (Shin et al., 2007a, b) and human cytomegalovirus (Johnson et al., 2001). As indicate therein, inhibition of the MAPK/ERK and PI3K/AKT pathways led to reductions in virus yield, suggesting that elements of these pathways might be necessary for virus multiplication.

An example of a pathway that was differentially regulated following *wt*- and  $\Delta$ 18K-infection was that involving TGF- $\beta$  signaling (Fig. 5). Notably activins/inhibins, members of the TGF- $\beta$  superfamily, were upregulated after *wt* infection, but unaffected following infection with  $\Delta$ 18K-FV3. Activins are cytokines with critical roles in infection and inflammation, and their induction following *wt* infection suggests a virus-induced immune response (de Kretser et al., 2012; Ebert et al., 2010; Robson et al., 2009). Another part of this signaling pathway, the Raf/MEK/ERK cascade, has an important role in cell growth, differentiation and survival. Pleschka et al. (Pleschka et al., 2001) showed that infection of cells with influenza A virus led to activation of the Raf/MEK/ERK cascade, and inhibition of Raf signaling resulted in inhibition of virus replication. While the cascade is mostly upregulated in

*wt*-infected cells, Raf is downregulated after  $\Delta$ 18K infection, which could explain its decreased virulence in vivo (Chen et al., 2011).

Lastly, the death receptor signaling pathway was significantly enriched only after *wt* infection. Death receptor induced apoptosis has been identified as an important pathway in West Nile virus pathogenesis (Clarke et al., 2014). Moreover, caspase 8, an initiator of the caspase cascade and a protein whose cognate transcript was upregulated after FV3 infection, promotes apoptotic signaling after infection. Collectively, the data suggest that these pathways may be involved in the pathogenesis of FV3 virus.

The ability of FV3 to markedly upregulate multiple immune-related functions while modestly downregulating others may reflect viral adaptations to the realities of life as an intracellular parasite. Controlling viral replication by balancing replicative needs on one hand and host survival and immunity on the other is thought to permit viral spread within a single organism and population. Although microarray studies do not provide a complete picture of metabolic events within virus-infected cells and require proof that cognate immune-related proteins are differentially expressed, they clearly indicate that host cells vigorously respond to ranavirus challenge. Moreover, since in vitro studies utilizing a single cell type may not accurately reflect events in vivo where multiple tissues and cell types interact and contribute to disease and resistance to disease, a better understanding of cellular responses to ranavirus infection can only be gained by infecting whole animals or specific subsets of immune cells, e.g., macrophages and T cells. Nevertheless, functional analysis of transcriptional data identified potential mechanisms involved in the differential pathogenesis of FV3 and FV3-induced mortality.

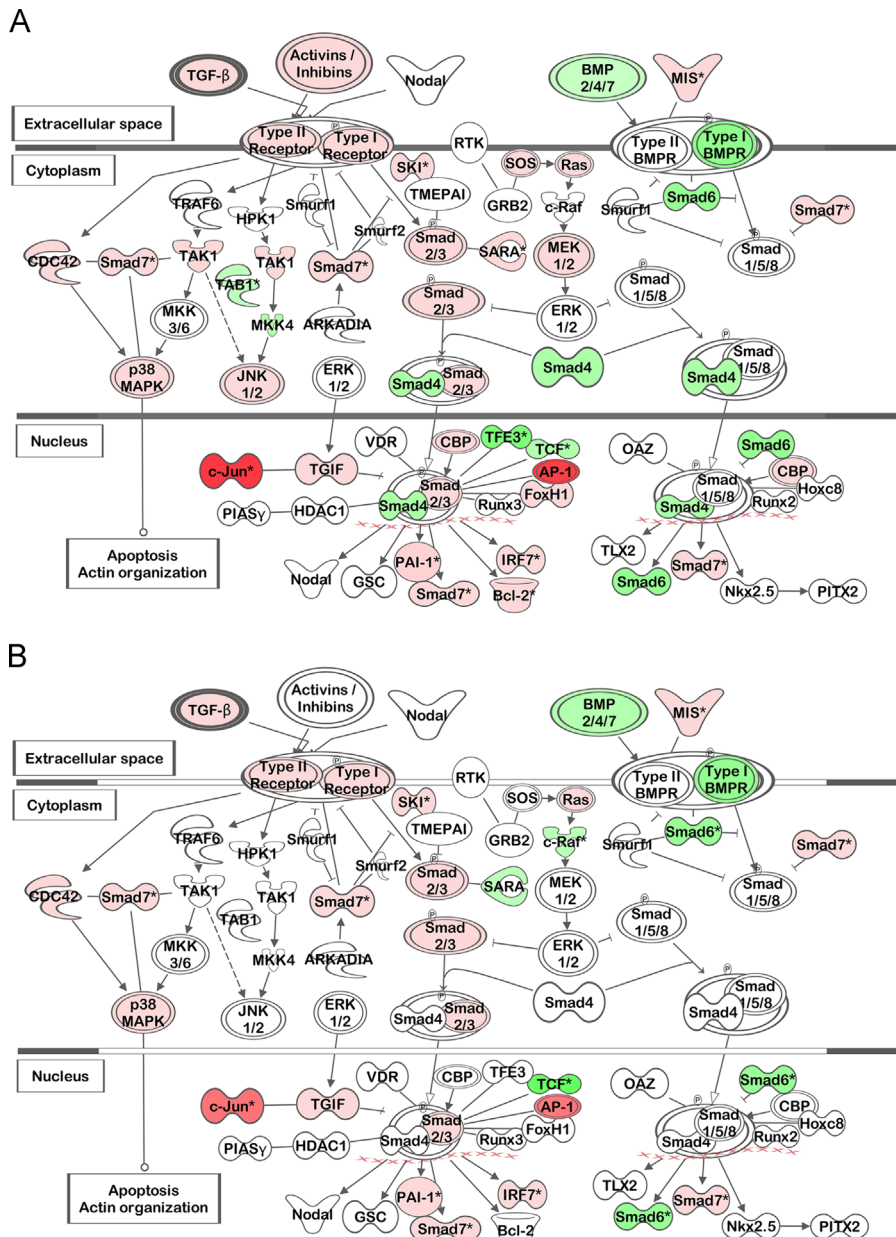
## Material and methods

### Cells and virus

For biochemical analyses, *wt* FV3 (ATCC VR-569) was grown in fathead minnow cells (ATCC No. CCL-42) in Dulbecco's modified Eagle's medium (DMEM) containing 4% fetal bovine serum (D4) at 26 °C in a humidified incubator in an environment of 95% air/5% CO<sub>2</sub> (Granoff et al., 1966). To prepare viral stocks, FV3 was propagated on monolayers of FHM cells grown in 150 cm<sup>2</sup> flasks and incubated in Eagle's minimum essential medium with Hank's salts (HMEM) supplemented with 4% fetal bovine serum (H4). Virus stocks were generated by infecting FHM cells at a multiplicity of infection (MOI) of 0.01 PFU/cell and harvesting when cytopathic effect was marked, i.e., ~5 days post infection (p.i.). Virions, released by three freeze-thaw cycles, were clarified by low speed centrifugation, and titers determined by plaque assay on FHM monolayers under an overlay of 0.75% methylcellulose. A knock out mutant ( $\Delta$ 18K) lacking the entire 18 kDa immediate early gene (Chen et al., 2011) was propagated as indicated above.

### RT-PCR analysis of viral and cellular gene expression

To confirm a productive infection and determine the kinetics of induction of immune-related genes, RNA obtained from mock- and virus-infected cultures was subjected to RT-PCR analysis. Confluent monolayers of FHM cells grown on 35 mm dishes were mock-infected or infected with *wt*- or  $\Delta$ 18K-FV3 at a MOI=5 PFU/cell. At the indicated times p.i. the cells were lysed with 1 ml Trizol (Invitrogen Life Technologies, Carlsbad, CA) and RNA isolated as directed by the manufacturer. cDNA was synthesized from 1  $\mu$ g of total RNA using random primers and SuperScript<sup>®</sup> III Reverse Transcriptase as directed by the supplier (Invitrogen Life Technologies). Amplification of cDNA was performed using primers specific for FHM 18S rRNA, Mx, and  $\beta$ -actin and FV3 transcripts



**Fig. 5.** TGF- $\beta$  pathway is differentially regulated in wt- and  $\Delta$ 18K-infected FHM cells. (Panel 5A) Expression levels of elements of the TGF- $\beta$  signaling pathway following infection with wt-FV3. (Panel 5B) Expression levels of elements of the TGF- $\beta$  signaling pathway following infection with  $\Delta$ 18K KO virus. Gene transcripts indicated in red were upregulated, whereas those in green were down regulated relative to control (i.e., mock-infected) cells. The intensity of the color reflects the level of up or down regulation.

encoding the 18K protein and the major capsid protein (MCP) (Table 1). PCR reactions (50  $\mu$ l) were performed using GoTaq Flexi DNA polymerase as directed by the manufacturer (Promega, Madison, WI). Reactions were incubated at 94  $^{\circ}$ C for 4 min followed by 30 cycles of 94  $^{\circ}$ C for 30 s, 55  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 90 s, and a final elongation cycle of 72  $^{\circ}$ C for 10 min. Amplified products were separated by electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide.

#### Protein synthesis in mock- and FV3-infected cells

Protein synthesis was monitored in 35 mm culture dishes containing confluent monolayers of FHM cells that were mock-infected or infected with  $\Delta$ 18K- or wt-FV3 at a MOI of 5 PFU/cell. Virus was allowed to adsorb for 1 h at 26  $^{\circ}$ C, at which time 2 ml DMEM4 was added and incubation continued at 26  $^{\circ}$ C. Using a

standard protocol, cells were radiolabeled from 6 to 8 h p.i. with methionine–cysteine free Eagle's minimum essential medium containing Earle's salts and 20  $\mu$ Ci/ml [ $^{35}$ S] methionine–cysteine (EasyTag Express Protein Labeling Mix, Perkin-Elmer) (Mao et al., 1997). At 8 h p.i., the labeling medium was removed, the cell monolayer lysed in 300  $\mu$ l 125 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.02% 2-mercaptoethanol, 0.01% bromophenol blue and the sample denatured by boiling for 1–2 min. Radiolabeled proteins were separated by electrophoresis on 10% SDS-PAGE gels (Laemmli, 1970) and visualized by phosphorimaging using a BioRad Personal Molecular Imager.

#### Microarray analysis

Replicate cultures of FHM cells grown on 35 mm dishes were mock-infected or infected with wt- or  $\Delta$ 18K-FV3 at a MOI of 10

**Table 1**  
Primers used for RT-PCR and qPCR.

Gene	Forward (F) and reverse (R) primers <sup>a</sup>	Use
FHM 18S rRNA	F GTAGACGTCAACAAGATGTGGATCC R CAGGAAGGTAATGTTCTTTGTGGCCG	RT-PCR
FHM $\beta$ actin	F CCGTGCTGCTGGAGGTA R AAGGAGCAAGGGAGGTGATTTC	RT-PCR
FHM Mx1	F TGGCATGGGAGAATCAGTTACAAG R TGCCCCAGCCATCTCATCC	RT-PCR
FV3 MCP	F TTATGGTGCAGAACGTCA R AGCCTTGTGGTGTTCGTA	RT-PCR
FV3 18K	F GCCAAGAATGCTTTTCCA R CGGTCAGTCCAGGTTTTTC	RT-PCR
FHM interleukin-8 (IL-8)	F CCCTCCTAGCCCTCACTGTAAA R GGATCTTCTCAATGACCTTCTT	qPCR
FHM heat shock protein 8B (HSPB8)	F CGAGCAGTACGCGTGGGAGTC R AGCGTGATGGGTAGCCGATGAAC	qPCR
FHM interferon (INF)	F CAACAACATCATGACCCGCTACCT R GTTCTCGCTCCGTTCTGTCCTT	qPCR
FHM interferon regulatory factor 3 (IRF3)	F AGCATGCTTTGAGACAGGAC R CACGAAGAGCGCTACGGAAGTT	qPCR

<sup>a</sup> Primer sequences were designed using PrimerSelect (DNASar, Madison, WI).

**Table 2**  
Differential expression of cellular genes following infection with wt FV3 or the  $\Delta$ 18K knock out mutant: upregulated transcripts.

Category	Gene	Fold change	
		wt	$\Delta$ 18K
IFN, IFN-related, IFN-induced	GILT	30	9
	IFN $\alpha$	11	4
	MXD	9	ND <sup>a</sup>
	IRF2	8	4
	IRF3	5	2
	IRF1	3	2
	ADAR	4	4
Cytokines & chemokines	IL8	145	70
	IL17C	20	18
	IL-12a	12	12
	SOCS3	12	7
	IL11	10	7
	IL1 $\beta$	9	4
	CXCR7	4	2
	RIG-I	3	3
Pathogen recognition and immune signaling	IL-12b	2	ND
	TNFRSF14	151	100
	NFKBIA	44	23
	TRAF3	20	12
	TLR18	7	5
	CASP9	6	3
Antigen presentation	TLR5B	5	ND
	ICLP2	63	11
	MHC class I	5	4
Metabolic	EIF2S2	96	24
	JUND	77	40
	CAV1	14	8
	JUNB	6	4

<sup>a</sup> ND, not differentially expressed.

**Table 3**  
Differential expression of cellular genes following infection with wt FV3 or the  $\Delta$ 18K knock out mutant: down-regulated transcripts.

Category	Gene	Fold change	
		wt	$\Delta$ 18K
Immune-related	ATG7	-5	-3
	NKEF	-4	-4
	C9	-3	-4
	TRIM 25	-3	-3
	TLR5A	-3	-2
	IL-6R	-3	-2
	EIF2AK	-3	ND <sup>a</sup>
Metabolic	SP1	-4	-3
	POLA2	-3	-2
	HSPB8	-4	-5
	POLB	-2	ND
	POLE	-3	-2

<sup>a</sup> ND, not differentially expressed.

analysis using an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA was stored at  $-80^{\circ}\text{C}$  until analyzed.

RNA from four ( $\Delta$ 18K FV3-infected) or five (mock- and wt FV3-infected) biological replicates was analyzed using custom fathead minnow 60,000 gene arrays (GPL17098) purchased from Agilent Technologies. cRNA labeling was performed using an Agilent Quick-AMP one color kit and hybridization was performed following the manufacturer's recommendations (One-color microarray hybridization protocol, v 6.5; Agilent). An Agilent high-resolution scanner (Model No. G2565CA) was used to acquire microarray images and data was processed using Agilent's Feature Extraction software v10.7 (Agilent). Raw microarray data from this study have been deposited at the Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>; GSE53739).

### Bioinformatics

Raw microarray data were imported into GeneSpring version GX11 (Agilent), and normalized using quantile normalization followed by median scaling across all samples. To identify differentially expressed genes (DEGs), one-way Analysis of Variance (ANOVA) was performed followed by pair-wise comparison and

PFU/cell. At 8 h p.i., total RNA was isolated from one set of replicates for RT-PCR and qPCR analysis using Trizol (Invitrogen Life Technologies, Carlsbad, CA). RNA was isolated from the remaining replicates using RNeasy (Qiagen, Valencia, CA, USA) and subjected to microarray analysis. RNA was DNase-treated, quantified using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE), and its quality assessed prior to microarray



Benjamini-Hochberg multiple testing correction ( $p < 0.05$ ). Hierarchical clustering was performed with GeneSpring (Agilent Technologies). Functional analysis and identification of upstream regulators of pathways were performed using the human ortholog genes of fathead minnow DEGs and Ingenuity Pathway Analysis software (IPA, Redwood City, CA).

#### Quantitative RT-PCR (qPCR)

To validate microarray results, qPCR was performed using primers (Table 1) targeting FHM interleukin-8 (IL-8), INF, heat shock protein 8B (HSP8B), IRF3, and 18S rRNA (control). To compare directly microarray and qPCR results, replicate RNA samples were prepared from mock- and virus-infected cells at 8 h p.i. as indicated above. Relative expression levels of putative immune-related genes were determined based on Ct (threshold crossing) values using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

Quadruplicate reactions were performed using cDNA templates prepared from RNA isolated from mock- or virus-infected FHM cells. Reactions were carried out in 96-well plates in a 25  $\mu$ l final reaction volume consisting of 12.5  $\mu$ l of RT2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, USA), 0.5  $\mu$ l each of forward and reverse primers (10  $\mu$ M each), 1  $\mu$ l cDNA, and sterile nuclease-free water to 25  $\mu$ l. Amplification conditions were 1 cycle at 95 °C for 1 min followed by 30 cycles of 95 °C for 20 s and 55 °C for 1 min. To verify that a single product was synthesized, melt curves were generated for each product and only primer pairs and cDNAs that generated a single peak were used in the final analyses. The PCR efficiencies for each primer set were  $\geq 90\%$ . The same threshold was used for each sample on a given plate; CT values  $\leq 31$  were considered positive.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2014.03.014>.

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