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USING RAINBOW TROUT CELL LINES AS A MODEL FOR UNDERSTANDING THE INNATE ANTI-FV3 IMMUNE RESPONSE

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By

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ABSTRACT

Ranavirus infections are on the rise and have been implicated in numerous species die-offs across the globe. Frog virus 3 (FV3) is the type-species of the genus, yet the immune mechanisms governing susceptibility remain poorly understood. Arguably the most important immune response to infection is the type I interferon (IFN) response. Type I IFNs trigger an "antiviral state" in host cells via the production of numerous interferon-stimulated genes (ISGs) that act to inhibit virus replication in various way, including the induction of apoptosis. Apoptosis is an important antiviral defense mechanism to limit virus replication within infected cells. This study employed the use of two rainbow trout cell lines, RTgutGC (intestinal origin) and RTG-2 (gonadal origin), previously shown to differ in susceptibility to FV3, thereby providing an excellent model to study innate anti-FV3 immune responses. Time-lapse infection videos and cell viability assays were used to quantify differences in the extent of cell death over time. RTG-2 exhibited greater cell death at a lower virus titre, compared with RTgutGC. The mechanism of cell death was investigated via DAPI staining and DNA laddering to observe nuclear condensation and intranucleosomal fragmentation, respectively, both hallmarks of apoptosis. Both cells underwent apoptosis in response to FV3. Moreover, UV-inactivated FV3 exhibited similar apoptotic cell death, suggesting that FV3-induced apoptosis is independent of productive virus replication. Likewise, poly I:C induction of IFN and ISGs inhibited virus replication, but had no effect on FV3-induced cell death. Using real-time RT-PCR IFN, ISG, and viral transcript expression was examined in both cell lines. Surprisingly FV3 elicited an equally poor IFN and ISG response in both cell lines, and was only detectable at 72h post-infection. Even when UV-inactivated, FV3 did not elicit a significant IFN response. However, viral transcript expression appears to be greater in the highly susceptible RTG-2 cell line. Further investigation into this difference in susceptibility between cell lines revealed

that RTG-2 exhibited greater viral entry and cellular metabolism, which may account for the enhanced level of infection. Thus, FV3 appears to exhibit virulence factors that are independent of replication, yet the mechanisms governing susceptibility appear to be the result of intrinsic cellular features that are IFN-independent.

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General Introduction

INTRODUCTION

1.1. Innate Antiviral Immunity

The ability to detect foreign invaders and mount an effective immune response is essential for the survival of any host organism. The immune system of jawed vertebrates is divided into adaptive and innate responses. Adaptive immunity develops after the innate response and is antigen-specific. Innate immunity, on the other hand, involves the rapid activation of the body's inherent and relatively non-specific defense mechanisms. Innate immunity represents the body's first line of defense against infection and is responsible for priming the adaptive response. Thus, the innate branch of immunity represents the cornerstone of any immune response. While adaptive immunity is unique to jawed vertebrates, innate immune mechanisms are present in nearly every multicellular organism, further emphasizing their important role in immune defense (Medzhitov and Janeway, 2000).

Innate immunity is based on the detection of pathogen-associated molecular patterns (PAMPs) via host-expressed pattern recognition receptors (PRRs; DeWitte-Orr and Mossman, 2010). PAMPs are simple molecules or molecular patterns exclusively associated with specific microbial groups, and are often essential for microbial survival (Medzhitov and Janeway, 2000). These serve as the primary targets of innate immune recognition. Common viral-associated PAMPs include viral proteins, genomic DNA, 5' triphosphate RNA, single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA; Takeuchi and Akira, 2009). During genomic replication and transcription, viruses produce nucleic acids, most of which are foreign to the host cell (Poynter et al., 2015). These viral nucleic acids reside in the endosome or cytoplasm of infected cells, or may be released into the extracellular space following lysis, where they may be sensed by neighbouring cells. Double-stranded RNA (dsRNA) is by far the most important PAMP, as it is exclusively expressed

by virtually all viral species at some point during their replication (DeWitte-Orr and Mossman, 2010). For all viral genome types: dsRNA is generated as genome fragments from dsRNA viruses, replicative intermediates from ssRNA viruses, and even as overlapping convergent transcripts from DNA viruses (Jacobs and Langland, 1996). Viral nucleic acids reside in the endosome or cytoplasm of infected cells, or may be released into the extracellular space following lysis, where they may be sensed by neighbouring cells.

Pattern recognition receptors (PRRs) are germline-encoded receptors involved in sensing a variety of microbial PAMPs. There are many PRRs for the detection of viral PAMPs, located at different cellular interfaces (surface, endosome, cytoplasm) to ensure appropriate detection. Common viral PRRs include toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and cytosolic DNA sensors (CDSs). When activated, these PRRs stimulate the production of numerous cytokines involved in mounting an antiviral immune response, namely, type I interferons (IFNs; Takeuchi and Akira, 2009). There are a number of specific PRRs involved in the induction of type I IFNs. TLRs are membrane–associated PRRs responsible for sensing extracellular viral PAMPs. Of the many TLRs that have been identified, TLR3, -7, -8, -9, and -22 are essential in virus recognition. TLR3 responds to dsRNA; TLR7, and -8 recognize ssRNA; and TLR9 is able to detect CpG DNA (Garcia-Sastre and Biron, 2006). TLR22 is specific to fish cells and is responsible for recognizing extracellular dsRNA (Poynter et al., 2015). On the other hand, RLRs reside in the cytoplasm and are capable of sensing a variety of intracellular viral PAMPs. Currently, two major RLRs have been described: RIG-I and melanoma differentiation-associated gene 5 (MDA5), both of which are activated by viral dsRNA (Poynter et al., 2015). In addition to the RLRs, another cytoplasmic PRR exists that is essential for the innate antiviral response: the dsRNA-dependent protein kinase, PKR. PKR is a serine-threonine kinase that also responds to

dsRNA and triggers signaling cascades that ultimately inhibit protein synthesis and induce programmed cell death (Gil and Esteban, 2000). Lastly, CDSs, as their name suggests, recognize and respond to foreign DNA within the cytosol. In mammals, a number of CDSs have been identified and shown to play key roles in the induction of IFN through different signaling pathways (Poynter et al. 2015). However, to date, only DEAD-box helicase 41 (DDX41) has been identified in teleosts (Quynh et al., 2015). DDX41 was identified in Japanese flounder and shown to exhibit similar antiviral activity to that of mammals, inducing the expression of antiviral and inflammatory cytokines. Of particular interest to this study, DDX41 was shown to be upregulated in response to ranavirus (a DNA virus) infection (Quynh et al., 2015).

1.1.2. Type I IFN Production and Response

The activation of host PRRs by viral PAMPs induces the production of type I IFNs through a multitude of pathways, depending upon the mechanism of viral entry and subcellular location (See Fig. 1.1; Garcia-Sastre and Biron, 2006; Poynter et al., 2015). Viruses entering the host cell via fusion at the surface or in endocytic vesicles are detected by surface and endosomal-associated TLRs, respectively. Those entering the cytoplasmic compartment through fusion are recognized by RLRs, NLRs, PKR, and CDSs. Following their activation, host PRRs activate different adaptor molecules, which interact with intracellular kinases, ultimately mediating the activation of key transcriptional regulators, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IFN regulatory factor 3/7 (IRF3/IRF7; Poynter et al., 2015). Once activated, these transcription factors translocate to the nucleus and bind to their corresponding regulatory domains to induce the expression of type I IFN (See Fig. 1.1).

Type I IFNs are important signaling molecules, named according to their ability to "interfere" with virus replication (Isaacs and Lindenmann, 1957). In mammals, these antiviral glycoproteins

are divided into the multigene IFN-α (13 in humans) and single gene IFN-β subtypes. In teleost fish, however, IFN genes have been divided into two groups, based on their cysteine composition (Fensterl and Sen, 2009). In rainbow trout (*Oncorhynchus mykiss*), there are at least 5 type I IFNs (IFN1-5; Chang et al., 2009; Purcell et al., 2009). Following expression, these cytokines are secreted in an autocrine or paracrine fashion, where they bind to their cell surface receptor, the type I IFN α/β receptor (IFNAR; Garcia-Sastre and Biron, 2006). Upon binding, the receptor dimerizes, initiating a well-characterized Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway of signal transduction, ultimately resulting in the expression of hundreds of interferon-stimulated genes (ISGs; See Fig. 1.2; Garcia-Sastre and Biron, 2006). The resultant ISGs initiate and maintain an "antiviral state" in neighbouring cells, while actively inhibiting virus replication in the infected host cell (Stetson and Medzhitov, 2006; DeWitte-Orr and Mossman, 2010).

Interferon-stimulated genes (ISGs) are the true effector proteins that limit virus replication, the expression of which, is stimulated by IFN signaling. There are numerous ISGs in different families, many with unknown functions. Many of the fish ISGs are homologous to mammalian ISGs and exhibit similar antiviral activity. One such family is the viral hemorrhagic septicemia virus (VHSV)-induced genes (vigs), a group of antiviral proteins, originally identified in rainbow trout, which are upregulated in response to virus infection. In fish, a number of vigs have been identified and found to inhibit virus replication analogous to their mammalian counterparts (Poynter and DeWitte-Orr, 2016).

One recently-identified vig, Vig4, exhibits high sequence similarity with mammalian ISG56/IFIT1, a member of the IFN-induced proteins with tetratricopeptide repeats (IFIT) protein family (Zhou et al., 2013). ISG56 represents the best-studied member of a family of genes that

exhibit tremendous responsiveness to IFN, as well as certain viral and bacterial PAMPs (Fensterl and Sen, 2011). As their name suggests, ISG56 and other IFIT family members possess repeating tetratricopeptide domains, which mediate protein-protein interactions (Cortajarena and Regan, 2006). In mammals, it was shown that ISG56 effectively inhibits parainfluenza type 5 (PAV5) and human papillomavirus (HPV) replication, thereby exhibiting significant antiviral activity (Andrejeva et al., 2013; Terenzi et al., 2008). While the exact mechanism of ISG56/IFIT1 viral inhibition remains poorly understood, a number of mechanisms have been suggested (Diamond and Farzan, 2013). Among the best-studied mechanisms is via translation inhibition. ISG56 directly interacts with eukaryotic initiation factor 3 (eIF3), preventing stabilization of the eIF2-GTP-Met-tRNA ternary complex required for translation initiation (Guo et al., 2000; Hui et al., 2003, 2005). Other mechanisms may be involved, including binding uncapped viral RNA, sequestering viral proteins, and modulation of IFN or inflammatory responses (Diamond and Farzan, 2013). Similar to mammals, fish Vig4 also contains tetratricopeptide repeats and is upregulated in response to viral infection (Poynter and DeWitte-Orr, 2016). Antiviral effects of Vig4 in fish have also been observed. In tongue sole (*Cynoglossus semilaevis*), knockdown of ISG56 resulted in enhanced *Megalocytivirus* infection, while overexpression caused replication inhibition (Long and Sun, 2014). Additionally, Vig4 has been identified in rainbow trout and induced by synthetic dsRNA, a potent inducer of type I IFN (Poynter and DeWitte-Orr, 2015). While fish Vig4 has been identified in several fish and shown to exhibit antiviral activity (Poynter and DeWitte-Orr, 2016), its mechanism of action remains poorly understood.

Among the best studied ISGs is the Myxovirus resistance gene (Mx), a GTPase that sequesters viral nucleocapsid structures to specific subcellular compartments (Garcia-Sastre and Biron, 2006). Mx proteins are dynamin-like high molecular weight GTPases, which help to facilitate

intracellular trafficking and membrane modulation (Haller et al., 2007). Both mammalian and fish Mx proteins have shown to alter viral transcription by interfering with viral RNA polymerases (Turan et al., 2004; Wu et al., 2010). While many isoforms have been identified in fish, teleost Mx proteins are also IFN-inducible, and exhibit broad antiviral activity against a diverse range of viruses (Poynter and DeWitte-Orr, 2016). For instance, Atlantic salmon Mx1 exhibits strong antiviral activity against infectious pancreatic necrosis virus (IPNV; Larson et al., 2004). Interestingly, while efficient in preventing replication of a broad range of viruses, Mx proteins of many fish species have proven ineffective in inhibiting infection by members of the family *Iridoviridae*. For instance, Japanese flounder Mx exhibits strong antiviral activity against two species of Rhabdovirus, but not Red seabream iridovirus (RSIV; Caipang et al., 2003). As well, Baramundi Mx inhibits replication of IPNV and viral nervous necrosis virus (VNNV), but not Taiwan grouper iridovirus (TGIV; Wu et al., 2012; Wu and Chi, 2007). Similarly, rainbow trout Mx1 exhibits antiviral activity against IPNV, viral hemorrhagic septicemia virus (VHSV), Salmonid alpha virus (SAV), but remains ineffective against infectious hematopoietic necrosis virus (IHNV; *Rhabdovirus*) and Epizootic hematopoietic necrosis virus (EHNV; *Ranavirus*) replication (Lester et al., 2012; Poynter and DeWitte-Orr, 2016; Trobridge et al., 1997). While the involvement of Mx proteins in protecting against many viruses is well understood, their role in protecting against infections from certain virus families remains in question.

In addition to its role as a PRR, PKR is also a well-known ISG. Consistent with its receptor function, PKR is constitutively expressed in an inactive form prior to stimulation with IFN (Balachandran, 2000). Unlike Mx3, which effectively inhibits virus replication at the transcription level, PKR acts at the translation level. Following the detection of dsRNA, PKR phosphorylates the translation initiation factor, eIF2α, thereby terminating host protein synthesis, and

consequently, viral replication. PKR homologs have been identified in a variety of fish species (Poynter at al., 2016). What's more, fish PKR, as in mammals, is IFN-inducible and exhibits antiviral activity toward a range of viruses (Liu et al., 2011). For instance, Crucian carp (*Carassius carassius*) and Japanese flounder (*Paralichthys olivaceus*) PKR was shown to inhibit the replication of grass carp hemorrhagic virus and Scopthalmux maximus rhabdovirus (SMRV), respectively (Liu et al., 2011; Zhu et al., 2008). Antiviral activity of PKR has not been demonstrated in the case of ranavirus infection. However, much research has been done regarding the ability of certain ranaviruses, particularly FV3, to encode a viral PKR inhibitor (Jancovich and Jacobs, 2011). FV3 encodes a viral homolog of eIF2α, designated vIF2α, which binds PKR and abrogates translation inhibition.

While the functions of many ISGs remain unknown, it is generally understood that these proteins work together to attack the virus at every stage by blocking protein synthesis, inhibiting viral replication, or inducing apoptosis. In this way, innate antiviral responses are often sufficient for limiting and controlling viral infection during our daily exposures to viruses.

1.1.3. Virus-Induced Apoptosis

Apoptosis is an energy-dependent mechanism of programmed cell death in which the cell dies in a controlled and orderly fashion. Apoptotic cell death is recognized as a normal mechanism for the removal of excess and/or potentially harmful cells from the body, and as such is an important aspect of innate immunity. The process of apoptosis is characterized by distinct morphological features and biochemical processes. Cells dying by apoptosis typically exhibit cell shrinkage and severe chromatin condensation (pyknosis) during early stages, which may be detected with DNA stains or electron microscopy (Elmore, 2007). However, late stages of apoptosis involve the activation of cellular endonucleases, which cleave genomic DNA between

nucleosomes, producing 180-200 base pair multimers which 'ladder' when subject to gel electrophoresis (Hardwick, 1997). Plasma membranes of apoptotic cells actively "bleb" or pinch outward, but remain intact, eventually forming small membrane-enclosed cellular packets, called apoptotic bodies. These apoptotic bodies are (*in vivo*) subsequently cleared by neighbouring cells and phagocytes, without inducing an inflammatory response (O'Brien, 1998). Unlike *in vivo* experiments, apoptotic bodies in cell culture are not phagocytosed and ultimately lyse, a phenomena known as secondary necrosis (Cejna et al., 1994).

Necrotic cell death, on the other hand, is an uncontrolled and passive process, typically the result of cell injury or a loss of cell energy supply. Necrosis is characterized by a loss of membrane integrity, lysis, and the release of cellular contents into the extracellular space. Ultimately, cytosolic spillage causes inflammation and damage to neighbouring cells and tissues (Elmore, 2007). During necrotic cell death, unlike apoptosis, DNA degrades randomly. With respect to virus infection, necrosis is often associated with the release of newly assembled virus particles (Hardwick, 1997). Virologists have traditionally presumed viral cell death to be necrotic, but recently, however, it has been suggested that apoptosis, rather than necrosis, is the common outcome of virus-infected cells (Chinchar et al., 2003).

Apoptosis is regarded as a defense mechanism to limit viral replication and infection of susceptible neighbouring cells. Viruses are obligate intracellular parasites that require the use of host cell machinery for replication. Thus, it is no surprise that the premature death of virus-infected cells may be a powerful antiviral mechanism. In fact, many of the innate antiviral immune responses, including the type I IFN response, actually involve the induction of apoptosis. In light of this, IFN-α and IFN-β have both been shown to induce apoptosis in a number of cell lines with a variety of histologies. In fact, a plethora of ISGs have been identified, which are responsible for

the induction of apoptosis, including PKR, various caspases, and many more (Chawla-Sarkar et al., 2003). A number of other innate antiviral responses may also culminate in the induction of apoptosis, echoing its importance as an important defense mechanism against virus infection. This view is further supported by the discovery that many viruses encode genes that inhibit the apoptotic pathway (Hardwick, 1997; Dai et al. 1999; Gillet and Brun, 1996; Liu and Cohen, 2014; Roulston et al. 1999). Viral inhibition of apoptosis may enhance infection in two ways. At the cellular level, preventing cell suicide would ensure maximal replication to achieve higher virus titres. Additionally, at the organismal level, blocking apoptosis would prevent the onset of essential antiviral immune responses to enhance viral spread and the likelihood of a successful transmission (Mossman et al., 1996; Nash et al., 1999; Tortorella et al., 2000).

The exact mechanisms by which viruses trigger apoptosis vary. Mechanisms of virus-induced apoptosis may be direct or indirect. Simple binding of virion-associated molecules to cell surface receptors may be enough to trigger the death pathway. For instance, certain avian leukosis virus subgroups are able to trigger apoptosis by simply binding target cells via a member of the TNF receptor family (Brojatsch et al., 2000; Diaz-Griffero et al., 2003). As well, some viruses may trigger apoptosis by direct action of viral proteins. This is evident from adenovirus E1A, which alone is sufficient to trigger apoptosis (Rao et al., 1992). Alternatively, some viruses may shutdown host macromolecular synthesis, resulting in apoptotic death of the host cell. Consistent with this pathway, certain viruses of the family *Iridovirdae*, including frog virus 3 (FV3), are known for shutting down host DNA, RNA, and protein synthesis, followed by rapid apoptotic cell death (Goorha and Granoff, 1979; Raghow and Granoff, 1979). Whether this inhibition of host macromolecular synthesis is directly responsible for apoptosis, or simply an outcome of virus infection, remains to be studied. However, recently, a virion-associated protein with PKR-like

activity has been identified in Chilo iridescent virus (CIV), another member of the *Iridoviridae* family (Chitnis et al., 2011). This protein was shown to be responsible for both inhibition of macromolecular synthesis, as well as the induction of apoptosis. Virus-host interactions, such as the induction of apoptosis, for a number of viral species remain poorly understood. Understanding whether this mechanism of cell death occurs following infection, and the specific mechanisms and inhibitory actions that take place, will provide insight into the ways in which host organisms protect themselves from different viral species, such as FV3.

1.2. Frog Virus 3 (FV3)

FV3 is a viral species that belongs to the family *Iridoviridae*, which include large dsDNA viruses with icosahedral symmetry, ranging in size from 120-200 nm (Chinchar, 2002). Currently, the *Iridoviridae* family is divided into five genera. Viruses belonging to the genera *Ranavirus, Megaolcytivirus,* and *Lymphocystivirus* are known to infect ectothermic vertebrates, whereas *Iridovirus* and *Chloriridovirus* require invertebrate hosts (primarily insects and crustaceans). *Lymphocystivirus* tend to only infect fish resulting in cutaneous lesions (Chinchar, 2002). In contrast, Ranaviruses (RVs) have a broad host range, infecting fish, amphibians, and reptiles, culminating in severe multi-organ systemic disease. FV3 is the type species and best characterized member of the Ranavirus genus. This viral species is recognized worldwide as a multi-host pathogen, capable of crossing multiple species barriers to infect a variety of new hosts (Jancovich et al., 2010; Grayfer et al., 2014). Interestingly, FV3 have previously been shown to infect, replicate, and induce apoptosis in mammalian cell cultures, albeit at lower temperatures (Willis et al., 1985; Chinchar et al., 2003). RVs were initially discovered in 1965 by Allan Grannof whilst attempting to generate a frog kidney cell line for the propagation of the oncogenic Lucke Herpesvirus (Granoff et al., 1966). Observation of virus-like cytopathic effects (CPEs) in some

cell cultures led to the subsequent isolation of FV1 and -2 from normal kidneys, and FV3, from the renal carcinoma of leopard frogs. This discovery sparked initial FV3 research that focused on the virus as a model for RV replication. However, FV3 research at that time soon became stagnant, due to the inability of RVs to cause cancer or serious disease in humans or economically important animals (Chinchar and Waltzek, 2014). This attitude soon changed in the 1980s when RVs were first associated with major die-offs among ecologically and economically important fish, amphibians, and reptiles (Lesbarreres et al., 2012; Chinchar and Waltzek, 2014).

Thorough understanding of FV3 replication is crucial to understanding the mechanisms of FV3 PAMP expression and detection. FV3 exists as both naked (unenveloped) and enveloped forms, gaining entry into the cell through fusion or receptor-mediated endocytosis, respectively (Gendrault et al, 1981; Braunwald et al 1985; Chinchar et al., 2002). Despite both naked and enveloped forms being infectious, infectivity of enveloped virions is much greater (Braunwald et al., 1979). Viral replication takes place in both the nucleus and the cytoplasm, utilizing both host and viral enzymes (See Fig. 1.3). Upon entry, the viral dsDNA genome enters the nucleus where viral transcription and genome replication take place (Chinchar et al., 2002). As viruses rely on the use of host cellular machinery for replication, there exists a clear link between cellular metabolism and virus replication. Early transcription is catalyzed by host RNA polymerase II, leading to the expression of immediate-early and delayed-early genes (Majji et al., 2009). Among the first expressed is the viral DNA polymerase, responsible for viral genome replication. Following its synthesis, viral DNA is transported to the cytoplasm where it is methylated and linked to large concatamers (Chinchar et al., 2011). Also in the cytoplasm, transcription of late genes, including the major capsid protein (MCP) occurs via a virus-encoded RNA polymerase (Majji et al 2009; Chinchar et al., 2011). Virion formation takes place in morphologically distinct

viral assembly sites (VAS), containing viral proteins and concatameric DNA. These sites, unique to *Iridoviridae*, permit efficient virion assembly without interference of host cell functions (Murti et al., 1985). The FV3 genome is large, consisting of 105,057bp, with 97 open-reading frames (ORFs), able to encode anywhere from 100-140 proteins (Chinchar et al., 2011). This tremendous coding potential is beneficial in order to increase replication efficiency and evade host immune responses. In fact, FV3 is known to encode a number of putative immune evasion proteins, some of which may inhibit IFN responses or delay apoptosis (Chinchar et al., 2011; Grayfer et al., 2012). While much of the molecular biology of FV3 replication has been elucidated, much remains to be discovered regarding the anti-FV3 immune response.

1.3. Impact of Ranaviruses on Teleosts

Initially, RVs were believed to have little impact on fish and amphibian populations (Chinchar et al. 2009; Williams et al. 2005). This view has drastically changed, however, as more and more evidence has implicated these viruses in widespread epidemics and mortality of several species of fish, amphibians, and reptiles (Ahne et al. 1997; Chinchar 2002; Williams et al. 2005). This viral genus has been implicated in mortality events on six continents across the globe, involving over 175 species across 52 ectothermic vertebrate families (Duffus et al. 2015; Miller et al. 2011). Additionally, RVs can be transferred between taxonomic classes of ectothermic vertebrates (Brenes et al., 2014) and are known to infect multiple species at a single outbreak site (Duffus et al., 2008; Mao et al., 1999). This staggering, ever-expanding distribution, coupled with the ability to cause rapidly-progressing mass die-off events, warrants their classification as an emerging infectious disease (Daszak et al. 1999; Grayfer et al. 2014).

With respect to teleosts, RVs have caused severe morbidity and mortality among both wild and captive fish populations (Whittington et al., 2010). The first RV associated with fish die-offs

was epizootic hematopoietic necrosis virus (EHNV), isolated in Australia in 1985 (Langdon et al., 1986). Fish were collected during an investigation into the death of a large number of red fin perch (*Perca fluviatilis L.*), and represents the first virus isolation from Australian fish. This viral species causes mass mortality in red fin perch and recurrent die-offs in captive rainbow trout (Whitington et al., 1994; 2010). Shortly after, European catfish virus (ECV) was isolated from terminal sheatfish (*Silurus glanis*) in Europe (Ahne et al., 1989). Another ranavirus isolate, Santee-cooper ranavirus (SCRV) has been isolated from large-mouth bass from six different reservoirs across southeastern USA (Plumb et al., 1996). Ranavirus isolates exhibit epizootics confined within particular global regions: EHNV, is unknown outside Australia; ECV, is largely confined to European countries; and, LMBV is mainly found in North American fish (Whittington et al., 2010).

While only a single case of FV3 infection in wild fish has been reported, where FV3 was isolated from a single diseased threespine stickleback (*Gasterosteus aculeatus*; Mao et al., 1999), there have been numerous cases of infections among captive fish (Whittington et al., 2010). For instance, an FV3-like ranavirus was isolated from a mortality event involving juvenile white sturgeon (*Acipenser transmontanus*) in 1998 (Waltzek et al., 2014). Furthermore, there have been repeated FV3 epizootics among pallid sturgeon (*Scaphirhynchus albus*) in the Missouri River Basin (Waltzek et al., 2014). Fish hatcheries in the USA have been raising juvenile pallid sturgeon, in attempt to replenish ever-decreasing wild stocks of this critically endangered species. However, there have been multiple mass mortality events, particularly among juveniles, caused by FV3, further hindering efforts to restore sturgeon populations (Chinchar and Waltzek, 2014; Waltzek et al., 2014). Regarding host lethality, an FV3 was isolated from captive Russian sturgeon (*Acipenser gueldenstaedtii*) and shown to be lethal to both Russian and Lake sturgeon (*Acipenser fulvescens*;

Waltzek et al., 2014). In fact, experimental transmission studies have demonstrated that FV3 isolated from numerous ectothermic vertebrate families are capable of infecting northern pike (*Pike esox lucius*), mosquito fish (*Gambusia affins*), bluegill (*Lepomis macrochirus*), and black bullhead (*Amerius melas*), however no mortality was observed (Bang-Jensen et el., 2009; 2011; Brenes et al., 2014; Gobbo et al., 2010). Consistent with this, a recent North American fish health survey lead to the isolation of FV3 from asymptomatic fathead minnow (*Pimephales promelas*), northern pike (*Esox lucius*), and walleye (*Sander viterus*; Waltzek et al., 2014). The ability of fish to serve as asymptomatic carriers for FV3 have been suggested in infection trials with northern pike, pike-perch and rainbow trout (Ariel et al., 2010; Jensen et al., 2009; 2011). Though FV3 and FV3-like RVs have impacted a number of fish populations, research in the field suggests an enhanced susceptibility of sturgeon to FV3, while other fish may simply act as asymptomatic FV3 reservoirs. The ability of fish to serve as asymptomatic carriers of certain viruses is important as they are key vectors for transmission to other susceptible species. While this is merely preliminary, further research is required to investigate the impact of FV3 across a broader range of wild and captive fish species. Yet, there remains a lack of detailed reports of specific tissues and cell types that are permissive or susceptible to FV3 infection, which may be essential to understanding susceptibility to FV3 at the organismal level.

1.4. Anti-FV3 Immunity

RVs, particularly FV3, are often considered to be major amphibian pathogens, and as such, much of their research has focused on their effect on global amphibian populations. Currently, we are facing a rapid decline in amphibian populations, with 32% of amphibian species currently facing extinction (Grayfer et al., 2014). While the cause of this decline is poorly understood, RVs are considered to be significant contributors (Chinchar, 2002; Grayfer et al., 2014). To date, most

research regarding immunity to RVs has been conducted using the African clawed frog, *Xenopus laevis*, amphibian model. Using this model organism, it has been shown that *Xenopus* adults can effectively clear FV3 infection by developing rapid innate immune responses (rapid upregulation of proinflammatory cytokines), followed by an efficient cytotoxic T lymphocyte response and the generation of potent anti-FV3 antibodies (Gantress et al., 2003; Maniero et al., 2006; Morales Robert, 2007). However, tadpoles, which lack MHC I expression and effective cellular immune responses, succumb to infection and exhibit significant morbidity and mortality (Gantress et al., 2003; Robert et al., 2005). In adults, FV3 infections are confined to the kidney and is resolved within weeks with little-to-no mortality observed (Gantress et al., 2003). However, in tadpoles and immunocompromised adults, infection begins in the kidney, but spreads systemically to multiple organs. Recent work has demonstrated that macrophages proliferate, followed by recruitment of natural killer (NK) cells, and finally by T cell activation (Morales et al., 2010). Despite their active role in anti-FV3 immune responses, macrophages are susceptible to infection and harbor FV3 for extended periods of time, albeit quiescent (Morales et al., 2010). Despite playing key roles in immunity, macrophages may enhance FV3 infection in two ways: by inhibiting their ability to present viral antigens to lymphocytes and serving as a reservoir of persistently infected cells. Most interesting, recent research has demonstrated, for the first time, the essential role of type I IFN in FV3 infection in *X. laevis*. Using recombinant IFN (r*Xl*-IFN), Grayfer et al., (2014) demonstrated that r*Xl*-IFN protected A6 kidney cells and tadpoles from FV3 infection, reducing the level of infection in both cases. As well, highly susceptible tadpoles exhibited weaker IFN responses, and enhanced viral burdens, as compared to adults (Grayfer et al., 2014). While r*Xl*-IFN treatment protected adults from infection, and reduced viral burdens in tadpoles, it only transiently protected tadpoles, which eventually succumbed to infection.

While several studies have investigated the amphibian immune response to RVs, very little research has been conducted in piscine hosts, particularly with respect to the type I IFN response. As the type I IFN response represents the first line of defense against infection, it is likely that numerous cellular genes, especially ISGs, play a critical role in antiviral immunity to FV3, and other RVs. Infection of epithelioma papulosum cyprinid (EPC) with four distinct ranaviruses, including FV3, stimulated expression of inflammatory genes in distinct expression profiles (Holopainen et al., 2012). Very recently, the role of various immune-related genes in FV3 infection of fathead minnow cells has been described (Cheng et al., 2014). Following infection, a number of genes were upregulated, including IFN, IFN regulatory factors (IRFs), ISGs such as Mx and MHC I, and interleukins IL-1β, IL-8, IL-2 and IL-17C. While Mx proteins are key ISGs upregulated in response to infection, they appear to be ineffective in protecting against infection by viruses of the *Iridoviridae* family. Thus, it appears that the role of IFN and ISGs in FV3 infections of fish is complex and further examination into the protective role of the type I IFN response in other fish species is required.

1.5. Cell Lines

Cell culture is arguably one of the most important approaches to understanding key virus-host interactions. Cell culture is frequently used in the study of virology, and offers a number of benefits to *in vivo* virus research and include the ability to investigate virus-host interactions without the need to sacrifice whole animals. Importantly, as viruses are intracellular pathogens, the cell represents the initial interaction between the virus and the host organism. For this reason, the initial defense mechanisms take place at the cellular level. Understanding the cell types that are susceptible or permissive to virus infection, as well as the mechanisms that are effective in viral inhibition, is essential in understanding the fate of an infected animal and the mechanisms that

determine whether an organism serves as asymptomatic carriers or succumb to infection.

Accordingly, two rainbow trout (*Oncorhynchus mykiss*) cell lines were used in this study: RTgutGC, an epithelial cell line of intestinal origin, and RTG-2, a fibroblastic cell line of gonadal origin (Wolf and Quimby, 1962; Kawano et al., 2010). Interestingly, preliminary observations suggested that there were susceptibility differences between these two rainbow trout cell lines: RTG-2 appeared to be more susceptible to FV3 infection than RTgutGC (Soares, unpublished results). Due to these differences in susceptibility, between individual cell types of the same species, these two cell lines provide an excellent model to study innate antiviral responses to FV3.

RTG-2 is a well-characterized fibroblastic rainbow trout cell line derived from both male and female gonadal tissue (Wolf and Quimby, 1962). Many *in vitro* rainbow trout studies have been performed using this cell line. Previous studies have shown that RTG-2 constitutively expresses IFN2, but not IFN1 (Zou et al., 2007). However, both IFN1 and IFN2, as well as common ISGs (including Mx 1-3 and Vig1) are strongly upregulated in response to poly I:C, as well as virus infection (DeWitte-Orr and Bols, 2007; Tafalla et al., 2008; Zou et al., 2007). As well, a recent report has demonstrated that both poly I:C (synthetic dsRNA), as well as *in vitro* viral dsRNA, were capable of inducing robust IFN and ISG expression in RTG-2 in a length-dependent manner (Poynter and DeWitte-Orr, 2015). The ability of RTG-2 to undergo apoptotic cell death has been demonstrated using a number of toxic compounds, including gliotoxin, polybrominated diphenyl ethers (PBDEs), butylated hydroxyanisole, as well as virus (chum salmon reovirus; CSV) infection (DeWitte-Orr and Bols, 2005; 2007; Jin et al., 2010; Jos et al., 2005).

RTgutGC is an adherent epithelial cell line derived from the intestine of rainbow trout (Kawano et al., 2010). Few studies have been conducted using this cell line, and as such, very little is known regarding the innate antiviral response. However, similar to that of RTG-2, RTgutGC

was shown to constitutively express IFN2 (Zou et al., 2007). As well, RTgutGC was shown to strongly upregulate IFN1, Mx1, Mx3, and Vig3 in response to extracellular dsRNA (poly I:C; Poynter et al., 2015). With respect to FV3 infection, a recent study investigated the ability of rainbow trout cell lines to support FV3 infection (Pham et al., 2015). It was shown that, for both cell lines, FV3 entered and caused cell death. However, RTG-2 exhibited much greater virus production, as compared to RTgutGC and other rainbow trout cell lines, which is consistent with the fore mentioned difference in susceptibility.

1.6. Research Objectives and Hypotheses

The overarching goal of this research was to investigate the innate antiviral response to FV3 in RTgutGC and RTG-2 cell lines, and evaluate any differences there may be. This study will test the hypothesis that RTgutGC is more resistant to FV3 than RTG-2, exhibiting a stronger type I IFN response and less cell death. The objectives of this study are:

(1) Quantification of cell death differences between the two cell lines.

(2) Investigation of the ability of FV3 to induce apoptosis.

(3) Quantifying IFN/ISG expression following FV3 infection.

(4) Investigation of intrinsic cellular features that may determine differences in susceptibility.

Figure 1.1: Type I IFN production and PRR signaling pathways in fish. There are numerous PRRs for the detection of viral nucleic acids in the extracellular space, endosome, or cytosol. The activation of host PRRs by viral PAMPs induces the production of type I IFN through different pathways, depending on the mechanism of entry and subcellular location. Following their activation, host PRRs (red) activate different adaptor molecules (green), which interact with numerous intracellular kinases to ultimately mediate the activation of key transcription factors (orange), NF-κB, IRF3 and IRF7. Once activated, these transcription factors translocate to the nucleus where they mediate the expression of type I IFN and pro-inflammatory cytokines, which exhibit antiviral function. Note: Endosomal dsRNA can escape (unknown mechanism), which may be recognized by cytosolic RLRs to activate the downstream signaling pathway. DNA within the cytosol is recognized by cytosolic DNA sensors (CDSs). To date, DDX41 remains the only CDS described in fish. Whether additional CDSs exist in fish remains to be elucidated. CDS = cytosolic DNA sensor; CpG DNA = cytosine-phosphate-guanosine deoxyribonucleic acids (DNA); dsRNA = double-stranded ribonucleic acids (RNA); IFN = interferon; IKK = IκB kinase; IPS-1 = IFN-β promoter stimulator 1; IRF = interferon regulatory factor; LGP2 = laboratory of genetics and physiology 2; MDA5 = melanoma differentiation-associated gene 5; MyD88 = myeloid differentiation primary response protein 88; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; RIG-I = retinoic acid-inducible gene I; single-stranded RNA = ssRNA; STING = stimulator of interferon genes; SR-A = class A scavenger receptor; TBKI = tank-binding kinase-1; TLR = toll-like receptor; TRIF = TIR domain-containing adaptor-inducing IFN-β. Adapted from Poynter et al., 2015.

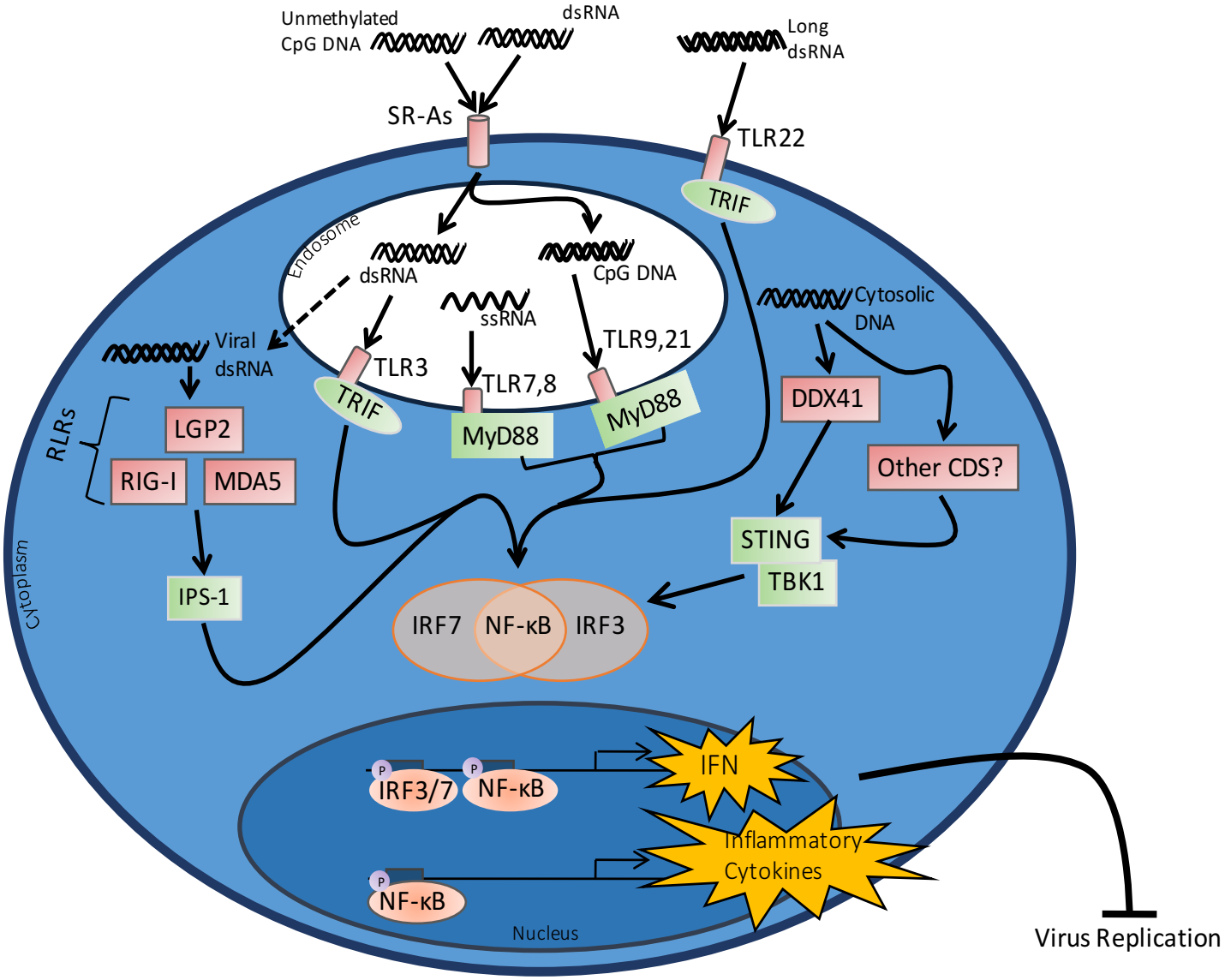

Unmethylated
CpG DNA
dsRNA
Long
dsRNA
SR-As
TLR22
TRIF
Endosome
dsRNA
CpG DNA
ssRNA
TLR9,21
TLR3
TLR7,8
TRIF
MyD88
MyD88
Cytosolic
DNA
Viral
dsRNA
RLRs
LGP2
RIG-I
MDA5
DDX41
Other CDS?
STING
TBK1
IPS-1
IRF7
NF-κB
IRF3
Cytoplasm
IRF3/7
NF-κB
IFN
Inflammatory
Cytokines
NF-κB
Nucleus
Virus Replication

Figure 1.2: ISG production by type I IFN. The binding of type I IFNs to the IFNα/β receptor (IFNAR) results in receptor dimerization and activation of JAK1 and TYK2 associated with the receptor cytoplasmic tails. JAK1 and TYK2 then phosphorylate STAT1 and STAT2, which form a trimeric complex with IRF9 referred to as ISGF3. Alternatively, STAT1 may form a homodimer complex called GAF. ISGF3 and GAF translocate to the nucleus to bind DNA regulatory sequences including ISREs and GAS, respectively. This leads to the production of numerous ISGs. IFN = interferon; IFNAR = IFN α/β receptor; JAK1 = Janus-activated kinase 1; TYK2 = tyrosine kinase 2; STAT = signal transducers and activators of transcription; ISGF3 = IFN-stimulated gene factor 3; GAF = IFN-gamma-activated factor; ISRE = IFN-stimulated response elements; GAS = IFN-gamma-activated site. Adapted from Garcia-Sastre and Biron, 2006.

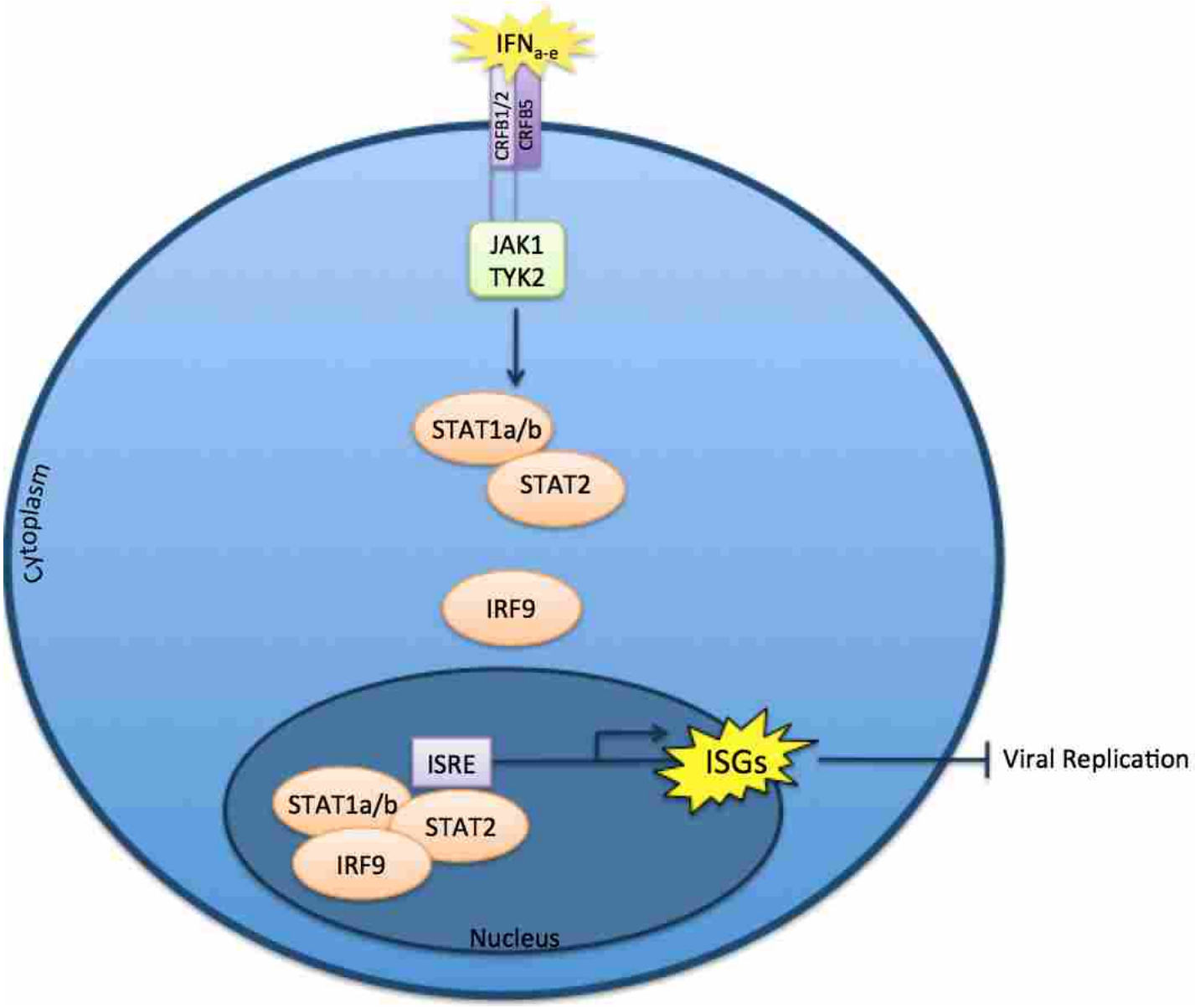
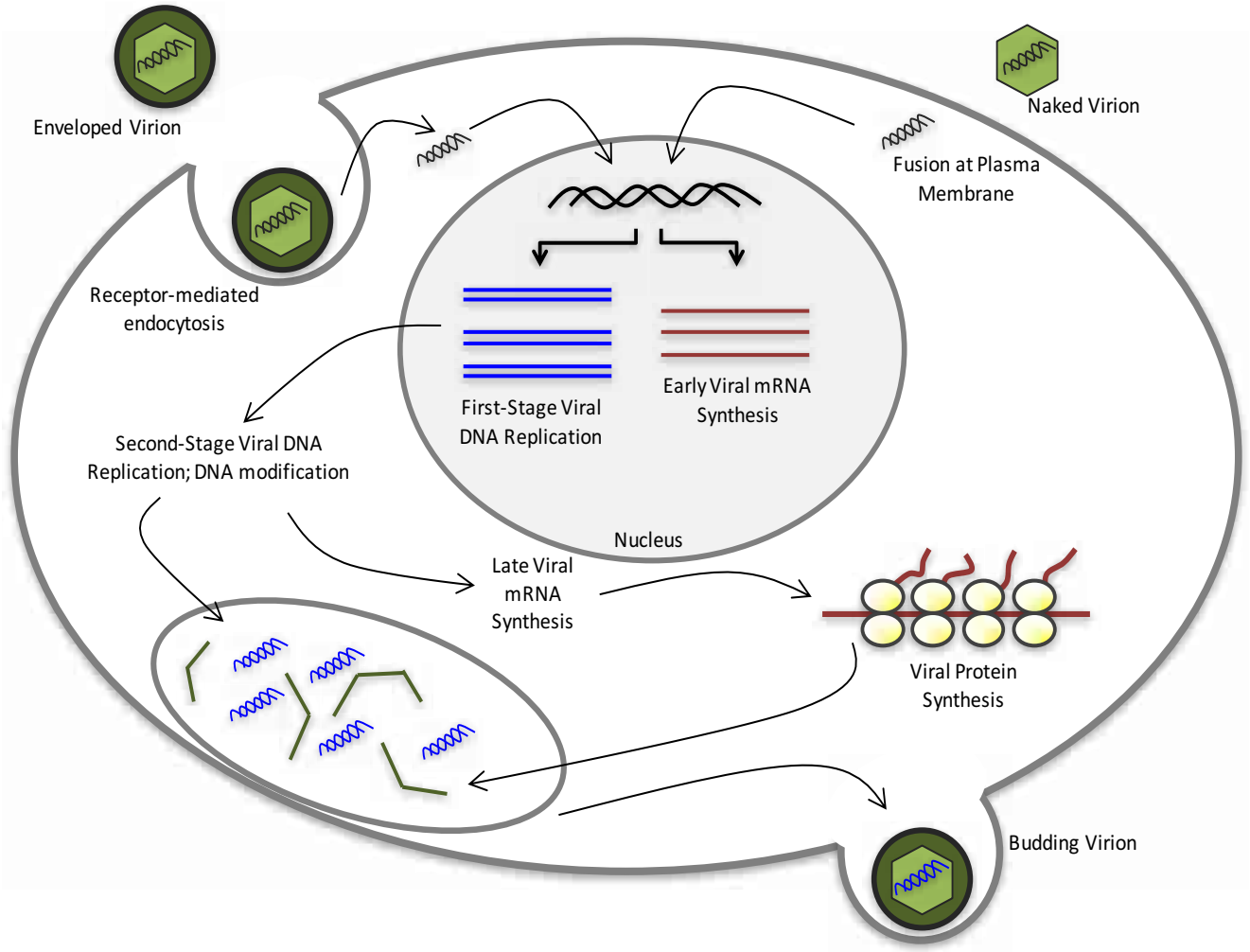

IFNa-e
CRFB1/2
CRFB5
JAK1
TYK2
STAT1a/b
STAT2
IRF9
Cytoplasm
ISRE
ISGs
Viral Replication
STAT1a/b
STAT2
IRF9
Nucleus

Figure 1.3: FV3 Life Cycle. FV3 exists as both naked and enveloped forms, gaining entry into the cell through fusion or endocytosis, respectively. The virion is uncoated and the viral dsDNA genome translocates to the nucleus where viral transcription and genome replication take place. Early transcription is catalyzed by host RNA polymerase II leading to the expression of immediate-early and delayed-early genes. One of the first genes expressed is the viral DNA polymerase, responsible for viral genome synthesis, and a host of other proteins involved in immune evasion, nucleic acid synthesis, and growth *in vivo*. Following its synthesis, viral DNA is transported to the cytoplasm where it is methylated and linked to large concatamers. Also in the cytoplasm, transcription of late genes, including the major capsid protein (MCP) occurs via a virus-encoded homolog of RNA pol II. Virion formation takes place in specific viral assembly sites (VAS), containing viral proteins and concatameric DNA. Assembled FV3 particles may be seen as part of paracrystalline arrays or budding from the host cell membrane. Adapted from Chinchar, 2002.


Enveloped Virion
Naked Virion
Fusion at Plasma Membrane
Receptor-mediated endocytosis
First-Stage Viral DNA Replication
Early Viral mRNA Synthesis
Second-Stage Viral DNA Replication; DNA modification
Nucleus
Late Viral mRNA Synthesis
Viral Protein Synthesis
Budding Virion

Chapter 2

Using Rainbow Trout Cell Lines as a Model for Understanding the Innate Anti-FV3 Immune Response

1. Introduction

At the time of their discovery in northern leopard frogs (*Rana pipiens*), ranaviruses (RVs) were believed to have little impact on fish and amphibian populations (Granoff et al., 1966; Chinchar et al., 2009; Williams et al., 2005). This view has drastically changed, however, as more and more evidence has implicated these viruses in widespread epidemics and mortality of several species of fish, as well as amphibians, and reptiles (Ahne et al., 1997; Chinchar, 2002; Williams et al., 2005). This virus genus has been implicated in mortality events on six continents across the globe, involving over 175 species across 52 ectothermic vertebrate families (Duffus et al., 2015; Miller et al., 2011). This staggering distribution, coupled with the ability to cause mass die-off events, warrants their classification as emerging infectious diseases (Chinchar et al., 2009; Daszak et al., 1999). While the majority of RV species have, to date, been restricted to their respective natural hosts, frog virus 3 (FV3) exhibits a broader host range, capable of crossing multiple species barriers to infect a variety of new hosts (Becker et al. 2003; Haislip et al., 2011; Hyatt et al., 2000; Jancovich et al., 2010).

FV3 is the type species and best-characterized member of the *Ranavirus* genus (family *Iridoviridae*), which consists of large icosahedral viruses possessing a single, linear, double-stranded DNA genome (Chinchar et al., 2002; 2009; 2011). FV3, as well as other members of the family *Iridoviridae*, possess large genomes able to encode anywhere from 100-140 proteins (Chinchar et al., 2011; Pham et al., 2015). This tremendous coding potential is beneficial in order to enhance host range, replication efficiency and evade host immune responses. Characteristic of *in vitro* FV3 infection is the rapid inhibition of host cell macromolecular synthesis, followed by extensive cytopathic effect (CPE). Previous reports have suggested that phosphorylation of eukaryotic initiation factor 2 (eIF2-α) by FV3 is responsible, and likely reflects its ability to take

over the translational capacity of the host cell (Chinchar and Dholakia, 1989; Chinchar and Yu, 1992). While these events may be involved in FV3-induced cell death, the exact mechanism of death, and the pathways responsible, require further investigation.

A common outcome of virus infection is cell death, either as a necrotic event, resulting from excessive virus production and release, or an apoptotic event, involving activation of the cellular death program. Recently, there is a growing understanding among virologists that apoptosis, rather than necrosis, is the common outcome of virus-infected cells (Chinchar et al., 2003). Viruses rely heavily on host cell machinery for replication. As such, it is no surprise that premature death of the host cell can be a powerful antiviral mechanism. In light of this, viruses have developed strategies to inhibit apoptosis to permit continued replication and dissemination to neighboring cells (Dai et al., 1999; Gillet and Brun, 1996; Liu and Cohen, 2014; Roulston et al., 1999). Previous studies have suggested that productive ranavirus infections typically culminate in the induction of apoptosis (Chinchar et al., 2003; Pham et al., 2012, 2015; Ring et al., 2013). As well, previous work with FV3 has suggested that virus-encoded proteins play a role in blocking or delaying apoptosis (Chinchar et al., 2003; Andino et al., 2015).

While apoptosis is an effective antiviral response, it is typically the outcome of another innate antiviral immune response, the type I interferon (IFN) response. This response is considered the first line of defense against virus infection, and as such, represents the cornerstone of all antiviral responses. Following the detection of viral products, innate immune sensors trigger the expression of IFNs, which signal the expression of hundreds of antiviral effector proteins, collectively referred to as IFN-stimulated genes (ISGs; Baum and Garcia-Sastre, 2010; Garcia-Sastre and Biron, 2006). While the IFN systems of warm-blooded vertebrates are well characterized, much remains to be understood regarding the IFN system of ectothermic vertebrates

that serve as hosts for RV infections. Previous work has demonstrated the ability of immunocompetent adult frogs to successfully clear RV infection, while tadpoles are unable to recover and succumb (Gantress et al., 2003; Grayfer et al., 2014; Maniero et al., 2006; Morales Robert, 2007). Responsible for this enhanced susceptibility, tadpoles lack full expression of MHC class I molecules and possess deficient cellular and cytokine immune responses (Grayfer et al., 2014; Robert and Ohta, 2009). IFN specifically has been shown to limit FV3 replication in amphibian cells (Grayfer et al., 2014) and induce IFN responses in fish cells (Cheng et al., 2014). However, despite their powerful roles in limiting infection to most viruses, numerous reports have demonstrated an inability of certain ISGs in inhibiting RV infections (Alvarez-Torres et al., 2013; Caipang et al., 2003; Lester et al., 2012; Trobridge et al., 1997; Wu et al., 2012; Wu and Chi, 2007). In addition to the role of cellular immune proteins in limiting infection, FV3-encoded proteins are believed to play key roles as immune antagonists. In fact, FV3 is known to express a number of putative immune evasion genes, which have been shown to be essential in pathogenicity and virulence (Andino et al., 2015; Grayfer et al., 2012). For example, FV3 encodes a caspase activation and recruitment domain-containing protein (vCARD), which is believed to modulate interactions with cellular CARD-containing proteins, typically associated with IFN immune sensors or pro-apoptotic molecules (Andino et al., 2015). Other immune evasion proteins have been identified and shown to be essential for FV3 replication in certain hosts (Andino et al., 2015; Grayfer et al., 2012). Clearly, the precise role of IFN and ISGs in limiting FV3 replication and the counter immune evasion strategies of FV3 requires further investigation.

Consequently, two rainbow trout cell lines were utilized in this study: RTgutGC (epithelial; intestinal origin) and RTG-2 (fibroblastic; gonadal origin). Preliminary observations suggested a susceptibility difference between the two cell lines, with RTG-2 being more susceptible to FV3-

induced cell death compared to RTgutGC. As such, these two cell lines serve as an excellent model to study susceptibility differences, which we postulated was due to differences in innate anti-FV3 responses. In this study, these susceptibility differences were investigated by a number of approaches. First, FV3-induced cell death was quantified in both cell lines, and method of cell death determined. IFN and ISG induction following FV3 infection was measured, as well as the ability of IFN to limit virus-induced cell death. FV3 was inactivated by UV, and the ability of replication deficient-virus to induce cell death and IFN/ISG induction was determined. Finally, differences in virus susceptibility between RTgutGC and RTG-2 were determined by comparing virus entry and cellular metabolism between the two cell lines. Interestingly, IFN and ISGs were not induced during FV3 infection in either cell line, and poly I:C-induced IFN limited virus replication, but not cell death. Additionally, UV-FV3 induced cell death similar to infectious FV3 (*wt*-FV3); and IFNs were also not induced. These data suggest that FV3 has potent cytotoxic effects that are not limited by IFN and do not require virus replication.

2. Materials and Methods

2.1 Cell culture

This study employed the use of two rainbow trout cell lines: RTG-2, a fibroblastic cell line of gonadal origin, and RTgutGC, an epithelial cell line of intestinal origin. Both of these cell lines were obtained from Dr. N. Bols (University of Waterloo). Both cell lines were maintained at 21°C in L-15 supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin G/streptomycin sulphate (P/S; 10,000 U/mL penicillin; 10 mg/mL streptomycin). As well, both cell lines were grown in 75-cm^2 plastic tissue culture flasks (BD Falcon, Bedford, MA, USA). For experimental use, both cell lines were seeded at 2×10^4 cells/well for 96-well plates and 3×10^5

cells/well for 6-well plates, unless otherwise described. Cells were incubated 24h before experiments were performed.

2.2 FV3 Propagation and Infection

Frog virus 3 (FV3) was propagated in confluent monolayers of *epithelioma papulosum cyprinid* (EPC) cells, shown to support high titres of the virus (Pearman et al., 2004; Pham et al., 2015). EPC cells were grown in Leibovitz's L-15 supplemented with 10% FBS and 1% P/S at 21°C. FV3 propagation occurred in L-15 with 5% FBS and 1% P/S at 21°C for 5 days or until complete cytopathic effect (CPE) was observed. FV3-containing medium was collected 5 days post-infection (PI) and freeze-thawed three times at -80°C, vortexing intermittently. The virus-containing media was then filtered through a 0.2 μm filter, and kept frozen at -20°C until required. The virus titre was determined by serially diluting viral suspensions (10^{-1} to 10^{-11}) and infecting confluent monolayers of EPC cells seeded into 96-well plates. The EPC cells were incubated for 7 days at 21°C. Following this infection period, the infected EPC cells were scored for the presence of CPE and a final titre, expressed as a $TCID_{50}$/mL (50% Tissue Culture Infective Dose), was estimated using the Karber method (Karber, 1931). $TCID_{50}$/mL values were converted to multiplicity of infection (MOI), based on the cell density of each assay:

$$MOI = \frac{(TCID_{50}/mL \times 0.7)(Volume)}{(Cell\ Density)}$$

UV-inactivated FV3 (UV-FV3) was generated by exposing working dilutions of FV3 in 5% FBS media (total volume 1 mL) to 150 mJ of UV light in a UVP HL-2000 HybriLinker™ UV crosslinker, a method previously shown to successfully inactivate the virus (Chinchar et al., 2003).

2.3 Time-lapse FV3 Infection Videos

RTgutGC and RTG-2 cells were seeded into 6-well plates and left to incubate for 24h at normal growth conditions. Following incubation, cells were infected with FV3 in 5% FBS at an MOI of 1.5 for 48h. Infected cultures were then viewed with a Nikon Eclipse Ti-S microscope (Japan) at 4× magnification. Photographs were taken every 10 min over the 48h infection period, upon which time-lapse videos were created with the images stitched together at 50 frames per second (fps). Included are videos representative of at least three independent experiments.

2.4 Cell Viability Assay

2.4.1 Treatments

For killing curves, RTgutGC and RTG-2 cell lines were infected with serial dilutions of FV3, from 10^{-1} to 10^{-6} (MOI 55 to 5.5×10^{-4}), in 96-well plates with 5% FBS media. Uninfected cultures (control) received 5% FBS media alone. Following infection, cells were left to incubate for 48h, 72h, and 96h at normal growing temperatures, after which, cell viability assays were performed (2.4.2).

For UV-FV3 treatments, RTG-2 cultures were infected with infectious FV3 (*wt*-FV3) or UV-FV3 at an MOI of 1.5 for 48h in 96-well plates. Following a 48h infection period, cell viability assays were performed as described below.

For the poly I:C antiviral assays, RTG-2 cells were plated in 96-well plates; cells were pretreated with 40 μg/mL poly I:C in 5% FBS media for 18h, while control wells received 5% FBS media alone. Following 18h, both pretreated and non-pretreated cells were infected with FV3 at an MOI

of 1.5, 3, and 4.5, with the exception of uninfected control cultures which, again, received 5% FBS media alone. Infection proceeded 48h, after which, cell viability assays were performed (2.4.2).

For the metabolic assays, RTgutGC and RTG-2 cells were plated in 96-well plates. Following 24h in culture, both cell lines received 5% FBS media. Cell viability assays were carried out 24h, 48h, and 72h post-plating. In order to determine basal levels of metabolic activity at the time of plating (designated "1h"), 1.2×10^5 cells (2×10^4 x 6 wells) of each cell line were transferred to 1.5mL microcentrifuge tubes and pelleted by centrifugation at 2400×*g* for 6 minutes. The cells were then resuspended in 600 μL (100 μL/well × 6 wells) of a diluted alamarBlue® solution (in 1× PBS; Hyclone) and transferred to 6 wells of a 96-well plate, from which a cell viability assay was performed (2.4.2).

2.4.2 Alamar Blue/CFDA-AM assays

Cells were then washed once with 100 μL 1× PBS/well (with Ca^{2+} and Mg^{2+}; Hyclone). A solution containing 4mM 5-CFDA-AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester; ThermoFisher Scientific) and 5% v/v alamarBlue® (ThermoFisher Scientific) diluted in 1× PBS (Hyclone) was added to the cells at 100 μL/well of a 96-well plate and left to incubate 1h in the dark. AlamarBlue® is a redox indicator dye which fluoresces in response to metabolic activity. Viable cells reduce alamarBlue® from non-fluorescent blue resazurin to fluorescent pink resorufin (Bopp and Lettieri, 2008). 5-CFDA-AM is also taken up by viable cells where it is hydrolyzed by intracellular esterases into fluorescent carboxyfluorescein. As these esterases are only active within intact cells, 5-CFDA-AM serves as a measure of membrane integrity (Bopp and Lettieri, 2008). Fluorescence was measured using a Synergy HT plate reader (Bio-Tek). Relative

fluorescent units (RFUs) for treated or infected samples was expressed as a percentage of the uninfected control sample. Data represents the average of at least three independent experiments.

2.5 Fluorescence Microscopy: DAPI Staining

RTgutGC and RTG-2 cells were seeded into 6-well plates. Subsequently, cells were infected with FV3 in 5% FBS media at an MOI of 1.5 for 7h, 20h, 36h, and, for RTgutGC only, 60h PI. Uninfected control cells received 5% FBS media alone for 36h (RTG-2) or 60h (RTgutGC). Cells were fixed with 10% neutral buffered formalin (Fisher Scientific) for 12 min followed by 3× rinse with PBS. Cells were then stained by adding 400 μL DAPI/well (10 μg/mL), diluted in PBS, for 5 min in the dark. Upon staining, the cells were rinsed twice with PBS, twice with milliQ water, and viewed with a Nikon Eclipse Ti-S microscope (Japan). Three representative photographs were taken for each treatment and the number of apoptotic/affected nuclei were counted manually and expressed as a percentage of the total nuclei in the field of view. Data represents the average of at least three independent experiments.

2.6 DNA Ladder Assays

RTgutGC and RTG-2 cells were seeded at 3.2×10^6 cells in 25-cm^2 tissue culture flasks and left to incubate for 24h. Cultures were infected with *wt*-FV3 or UV-FV3 (MOI 1.5). At the indicated time post-infection, cells were collected by the addition of trypLE dissociation reagent and centrifugation (1400×*g* for 6 min) and genomic DNA (gDNA) was extracted using a GenElute mammalian genomic DNA miniprep kit (Sigma). The genomic DNA was eluted from the column using 75 μL elution solution (Sigma), and 4 μg of this DNA was resolved by electrophoresis on a

2% (w/v) agarose gel for 4h at 60 V. The DNA ladders were visualized by staining gels with 0.5 μg/mL ethidium bromide and imaged under a UV transilluminator.

2.7 qRT-PCR: IFN/ISG/Viral Transcript Expression Analysis

2.7.1 Cell Infections and Treatments

RTgutGC and RTG-2 cells were seeded at a cell density of 6×10^5 cells/well of a 6-well plate and left to incubate 24h in normal growth medium and temperature. Both cell lines were infected with FV3 in 5% FBS media at an MOI of 1.5 for 4, 8, and 18h. Additionally, cells were infected with *wt*-FV3 and UV-FV3 in 5% FBS media at an MOI of 0.15 for 72h. In either case, uninfected (control) cells were included, which received 5% FBS media alone at the time of infection. As well, RTG-2 cells were pretreated with 40 μg/mL poly I:C in 5% FBS media for 18h prior to infection with FV3 at an MOI of 0.15 for 72h. In these experiments, poly I:C controls were included in which cells received 40 μg/mL poly I:C alone at the time of pretreatment for the duration of the 72h infection period. Following the indicated length of infection, treatment, or incubation, RNA was extracted from the cells as described below.

2.7.2 RNA Extraction and cDNA synthesis

Total RNA was extracted from both cell lines by use of TRIzol (Life Technologies) according to the manufacturer's instructions. RNA was treated with DNAse I to remove any contaminating genomic DNA. RNA was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific) and stored at -80°C.

cDNA was synthesized from 1μg of RNA, which was combined with iSCRIPT reverse transcription supermix (BioRAD) to make a 20μL reaction mixture. The reaction mixture was incubated at 25°C for 5min, 46°C for 20 min, 95°C for 1 min, and held at 4°C. Following amplification, all of the resulting cDNA was diluted 1:10 in molecular biology grade water and stored at -20°C.

2.7.3 qPCR Reactions

Individual qPCR reactions included 4 μL of template cDNA and 6 μL of qPCR mastermix, containing: 10 μM forward/reverse primer (Table 2.1; 200 nM final concentration), molecular biology grade water, and SsoFast EvaGreen Supermix (BioRad; 2× concentration). The qPCR reactions were carried out using a BioRAD CFX Connect real-time system at the following cycling conditions: 95°C for 2 min, 40 cycles of 95°C for 5s and 55°C for 10s, with plate reads between each cycle. Following PCR amplification, the relative normalized expression of all genes was calculated using the ΔΔCt method. Gene expression was normalized to the endogenous control (β-actin) and expressed as a fold change over the uninfected control, the expression of which was set to 1. For viral transcripts, in which no threshold value is given for uninfected control samples, the control values were set to a threshold (C_t) of 40. Single PCR melting peaks were employed to determine product specificity.

2.8 Viral Entry Assay

RTgutGC and RTG-2 cells were seeded at a cell density of 8×10^5 cells/well of the same 6-well plate and left to incubate 24h under normal growth conditions. Different lengths of infection were separated on different 6-well plates, while both cell lines of the same infection period were seeded

in the same 6-well plate. Both cell lines were simultaneously infected with FV3 at an MOI of 1.5 in 400 μL of 5% FBS media, with the exception of the uninfected control, which received 5% FBS media alone. Following the addition of FV3 to each well, always from the same virus prep, plates were left to incubate 1h, rocking every 12 min. At 1h PI, gDNA was extracted from the 1h infection wells of both cell lines by use of GenElute mammalian genomic DNA miniprep kit (Sigma). At the same time, FV3-containing media was removed from the 2h and 3h infection wells of both cell lines and replaced with fresh 5% FBS media for an additional 1h and 2h, respectively. Following the 2h and 3h infection periods, gDNA was extracted as described previously. gDNA was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific) and individual 12.5 ng/μL dilutions were made from each sample. The level of MCP in each of the fore mentioned samples was measured by qPCR (2.7.3). Data represents the average of four independent experiments.

2.9 Statistics and Quantitative Analysis

LD_{50} values reported in FV3 killing curves were generated using Prism software (GraphPad Software, Version 6). All statistical analyses were performed using KaleidaGraph (version 4.1.0, Synergy software). Specific analysis details are included in the figure headings for each Figure.

Table 2.1: qPCR Primers: Primer sequences used in real-time PCR. Included are product sizes, annealing temperatures (T_A), and references or accession numbers.

Gene or Fragment	*Primer Sequence 5'-3'*	*Product Size (bp)*	*T_A (°C)*	*Reference or Accession Number*
β-actin	**F**-GTCACCAACTGGGACGACAT **R**-GTACATGGCAGGGGTGTTG	174	55	Poynter and DeWitte-Orr, 2015
IFN1	**F**-AAAACTGTTTGATGGGAATATGAAA **R**-CGTTTCAGTCTCCTCTCAGGTT	141	55	Chaves-Pozo et al., 2010
Vig4	**F**-GGGCTATGCCATTGTCCTGT **R**-AAGCTTCAGGGCTAGGAGGA	151	55	Poynter and DeWitte-Orr, 2015
Mx3	**F**-TGAGGCCATTAAGCAGGTGA **R**-TGGTAAGGGTCGGTCGTCT	151	55	Poynter and DeWitte-Orr, 2015
FV3 MCP	**F**-CGTCGGCTCCAATTACACCT **R**-CTCTTGACGGGATCTACCGC	90	55	KM114252.1
FV3 vCARD	**F**-ATGCAAAACTTTGGAGCACA **R**-TGGTGACGCTGTCTATCAGG	196	55	Majji et al., 2009

3. Results

3.1 RTG-2 is more susceptible than RTgutGC to FV3-induced cell death

FV3-infected RTgutGC cultures exhibited low to moderate cell death over a 48h infection period (Video 1A). FV3-induced cell death began at around 20h PI and progressed slowly. However, under the same infection conditions, RTG-2 exhibited rapid, extensive cell death; beginning at around 7h PI (Video 1B). Following 48h, very few RTG-2 cells remained in culture. In order to quantify differences in FV3 susceptibility between different rainbow trout cell lines, RTG-2 and RTgutGC cultures were infected with serial dilutions of FV3 to generate a killing curve, from which LD_{50} values could be calculated. Both cell lines included mock-infected cultures, which received 5% FBS media alone. Both cell lines exhibited a virus titre-dependent decline in viability, when infected with the virus (Figure 2.1). At a high MOI of 55, both cell lines exhibit extensive cell death compared to the uninfected control at 48h, 72h and 96h PI (Figure 2.1). However, at an

MOI of 5.5, the differences in susceptibility between the two cell lines became very prominent. RTG-2 cells exhibited viability of only ~10%, whereas RTgutGC exhibited viability of ~65% by 72h PI. As the FV3 titre decreased with serial dilutions of the virus, the differences in susceptibility became less apparent. LD_{50} values were generated from the fore mentioned killing curves and RTG-2 demonstrated significantly lower LD_{50} values, as compared to RTgutGC (Figure 2.1). While this trend was consistent over the 48h, 72h, and 96h infection periods, the data became less variable and more statistically significant with a longer infection.

3.2 Mechanism of FV3-induced cell death is apoptosis

To determine the mechanism of FV3-induced cell death, both cell lines were infected with FV3 and examined for characteristic features of apoptosis. One such hallmark of apoptosis is chromatin condensation and fragmentation into apoptotic bodies, as measured by DAPI staining and fluorescence microscopy (Hardwick et al., 1997). Mock-infected RTgutGC and RTG-2 possessed round, uniformly-staining nuclei (Figure 2.2A). Beginning at 20h PI, RTgutGC cultures began to exhibit significant numbers of apoptotic bodies (~10%). This percentage of apoptotic nuclei remained relatively the same over 36h and 60h PI. Surprisingly, in FV3-infected RTG-2 cultures there was an absence of "traditional" apoptotic bodies, in which the nuclei are highly fragmented into small nuclear packets; instead, many irregular and highly condensed nuclei were observed (Figure 2.2A). While a very small percentage of these condensed nuclei were observed in control cultures, the percentage of these increased with both the length of infection, as well as the MOI of virus (Figure 2.2B and C). A second hallmark of apoptosis is the presence of gDNA laddering, i.e., the fragmentation of cellular DNA into ~180bp oligomers (Hardwick et al., 1997). When gDNA from mock-infected cultures was run on a 2% agarose gel, no evidence of DNA degradation

was observed for either cell line (Figure 2.2D). However, gDNA laddering was detected in FV3-infected cultures from both cell lines. DNA fragmentation was detected as early as 20h PI for RTgutGC. However, for RTG-2, laddering was only detectable at 36h PI. Important to note, due to the extensive cell death in the more susceptible RTG-2 cell line, there were very few cells remaining for analysis following 36h PI. For this reason, only RTgutGC cultures were examined at 60h PI.

3.3 Differences in FV3-induced cell death may be due to constitutive, but not induced, type I IFN production

As IFN is a key regulator of the innate antiviral state, its expression and expression of its downstream interferon stimulated genes (ISGs) were measured either constitutively in the cell lines or induced by FV3. Levels of IFN1, as well as Vig4, and Mx3, were measured at the transcript level using qRT-PCR. To determine whether constitutive levels of IFN or ISGs differ between the two cell lines, RTG-2 and RTgutGC, control (uninfected) cultures were examined for mRNA levels of IFN1, Vig4, and Mx3. Threshold (C_t) levels for each gene, including actin, were recorded and a ratio of the target gene C_t to actin C_t was generated. Average actin C_t values for RTG-2 and RTgutGC were similar values of 19.45 and 20.66, respectively. RTgutGC exhibited significantly lower C_t ratios for each gene, thus higher transcript levels, as compared to RTG-2 (Figure 2.3A). In addition, IFN and ISG transcripts were measured following FV3 infection in both RTgutGC and RTG-2 (Figure 2.3B). Significant upregulation of IFN or ISG transcripts could not be detected in either cell line within the first 18h of infection. Even at 72h PI, IFN and ISG expression was absent, with the exception being RTG-2 at 72h PI, where there was a 4-fold increase in IFN1 and an 8-fold increase in Mx3. Also detected was a subtle upregulation in Mx3 for RTgutGC at 72h

PI, albeit very modest. MCP transcript levels were also measured to determine the level of FV3 replication over an 18h infection period (Figure 2.3C). RTG-2 supported significantly more virus replication compared to RTgutGC at 18h PI.

3.4 Poly I:C inhibits FV3 replication, but not FV3-induced cell death

The importance of type I IFN in FV3 infection was suggested by the observation that the highly-susceptible RTG-2 cell line, exhibited a lower constitutive expression of IFN and ISGs (Figure 3A). For this reason, the ability of poly I:C pretreatment to protect against FV3 infection was investigated in RTG-2 cultures, the more susceptible cell line with poorer constitutive IFN and ISG expression. Poly I:C induced IFN1 and ISGs (Vig4 and Mx3) at significantly high levels in RTG-2 18h post-treatment (Figure 2.4A). When pretreated with 40μg/mL poly I:C for 18h, FV3 replication was significantly inhibited, as measured by virus transcript expression (MCP and vCARD; Figure 2.4B). MCP transcript expression is ~ 3 log fold lower, and vCARD expression ~ 2 log fold lower, when cultures were pretreated with poly I:C prior to infection. However, cell death, as measured by cell viability assays, was not affected by poly I:C pretreatment (Figure 2.4C). Despite IFN presence, RTG-2-infected cultures exhibited similar levels of FV3-induced cell death, as compared to FV3 infection alone. This was consistent over multiple MOIs of virus (Figure 2.4C).

3.5 UV- inactivated FV3 kills, but does not induce type I IFNs

Next, UV-FV3 (FV3 inactivated by UV irradiation) was utilized to determine whether virus replication was required for virus-induced cell death, and whether IFN and ISG expression was altered when virus replication is inhibited. UV irradiation provided an effective mechanism for

FV3 inactivation, as FV3 transcripts (MCP and vCARD) were significantly reduced in RTG-2 following UV exposure (Figure 2.5A). Interestingly, UV-FV3 was able to induce cell death in RTG-2 with the same efficacy as infectious (*wt*) virus (Figure 2.5B). Both *wt*-FV3 and UV-FV3 caused ~50% reduction in viability following 48h of infection. In addition to the extent of cell death, the mechanism of cell death induced by UV-FV3 was investigated by monitoring the presence of gDNA laddering. Following infection with UV-FV3, both RTG-2 and RTgutGC exhibited gDNA laddering at 36h PI, absent in mock-infected cultures (Figure 2.5C). Additionally, IFN and ISG (Vig4 and Mx3) expression was measured in RTG-2 and RTgutGC infected with UV-FV3 for 72h. Following infection, neither cell line expressed IFN or ISGs significantly different between WT and UV-FV3 (Figure 2.3B and Figure 2.5D).

3.6 Greater FV3 susceptibility may be due to enhanced viral entry and metabolism

In addition to differences in constitutive IFN expression observed (Figure 2.3A), other possible explanations for differences in FV3 susceptibility between RTgutGC and RTG-2 were pursued. Among the first steps in FV3 replication is the entry of FV3 virions into host cells. Accordingly, the rates of viral entry in both cell lines were quantified by measuring the level of viral DNA (MCP) at 1h, 2h, and 3h PI. Despite both cultures being synchronously infected with an MOI of 1.5, RTG-2 exhibited significantly greater levels of MCP DNA at all three time-points compared to RTgutGC (Figure 2.6A). As viruses require the use of host cell machinery for replication, virus replication and cellular metabolism are closely associated. In light of this, we set out to determine whether the difference in FV3 susceptibility between RTG-2 and RTgutGC was the result of differences in cellular metabolism/virus replication efficiency. Cellular metabolism, as measured by alamarBlue® reduction, was evaluated over 3d in both cell lines. RTG-2 exhibited a higher

metabolic/replication capacity at 1d, 2d and 3d post plating, as compared to RTgutGC despite having similar basal metabolic rates at 1h (Figure 2.6B).

4. Discussion

FV3 was lethal to both rainbow trout cell lines, RTgutGC and RTG-2. FV3 infection lead to a decline in metabolism and impairment of membrane integrity, as measured by alamarBlue® and 5-CFDA-AM fluorescent indicator dyes. At a relatively high MOI of 55, both cell lines demonstrated extensive cell death as measured by low relative fluorescence compared to uninfected control cultures. As expected, cell viability increased with a decrease in MOI, demonstrating that cell death is proportional to both the length of infection and the MOI of virus. Of particular interest was the drastic difference in FV3 susceptibility between the two rainbow trout cell lines. As observed in the time-lapse infection videos, RTG-2 exhibited much more extensive cell death following FV3 infection, as compared to RTgutGC. Over the 48h infection period, RTgutGC exhibits low to moderate cell death, which begins at ~20h PI and progresses slowly. Conversely, under the same conditions, RTG-2 exhibits extensive cell death, beginning at ~7h PI, with very few cells remaining at the end of the infection period. This difference in susceptibility to FV3 was confirmed with killing curves, in which LD_{50} values were generated for each cell line. As expected, RTG-2 reported a lower LD_{50}, compared to that of RTgutGC, indicating its greater susceptibility. What's more, this differential FV3 susceptibility was observed over 48h, 72h, and 96h of infection. While this difference in susceptibility was consistent over various infection periods, the 48h infection course had greater variability. The significance of the data increased in proportion the length of infection. Susceptibility also correlated with virus replication, as RTG-2 supported more FV3 transcription compared with RTgutGC. The difference

in FV3 susceptibility between RTgutGC and RTG-2 provided an excellent model to study the mechanisms that may enable protection from FV3-induced cell death. While some studies have suggested that certain teleost fish may act as asymptomatic carriers of FV3 (Ariel et al., 2010; Jensen et al., 2009; 2011; Robert et al., 2007), there remains a lack of comprehensive reports of cell types that are susceptible or permissive to FV3 infection, essential to understanding their ability to serve as potential reservoir hosts of FV3.

In attempt to prevent viral replication, dissemination, and persistent infection, host organisms have developed an impressive integrated network of defense mechanisms, many of which ultimately involve the induction of apoptosis. In an effort to determine the mechanism of FV3-induced cell death, and any differences there may be between cell lines, the ability of FV3 to induce apoptosis was investigated. Nuclear condensation and fragmentation are hallmarks of apoptotic cell death (Hardwick et al., 1997; Elmore, 2007), and these characteristic features were observed in FV3-infected cultures under a variety of conditions. FV3-induced cell death appears to be apoptotic as significant numbers of apoptotic nuclei were observed in both cell lines within 20h of FV3 infection. As expected, the highly susceptible RTG-2 cell line exhibited a greater percentage of apoptotic nuclei, as compared to RTgutGC, albeit a different nuclear morphology. While FV3-infected RTG-2 cultures lacked the traditional apoptotic bodies observed in RTgutGC, in which the nucleus is highly fragmented into small apoptotic bodies, many nuclei displayed highly condensed, shrunken, and irregular morphology. The presence of these highly irregular nuclei in FV3-infected RTG-2 cultures not only increased with the length of infection, but also with the titre of virus, suggesting that this nuclear morphology was virus-induced. Given that apoptosis, particularly early-stage apoptosis, is associated with a high degree of nuclear condensation and shrinkage, these nuclei could also be considered apoptotic (Tone et al., 2007).

The presence of traditional apoptotic bodies in RTG-2 cultures has been reported in a previous study involving treatment with gliotoxin, a lethal fungal metabolite (DeWitte-Orr et al., 2005). Possibly, the lack of traditional apoptotic bodies in FV3-infected RTG-2 cultures is due to viral interruption of the normal progression of apoptosis in this cell line.

To confirm this mode of cell death, DNA laddering was also investigated. Following FV3 exposure, DNA laddering was detected in both cell lines, further supporting apoptosis as the death mechanism. DNA laddering was detectable in FV3-infected RTgutGC and RTG-2 cultures within 20h and 36h, respectively. Why DNA fragmentation is detected later in FV3-infected RTG-2, despite showing significantly greater numbers of apoptotic nuclei earlier, remains unclear. Perhaps the lack of DNA laddering at 20h PI, despite the significant number of apoptotic nuclei is due to the fact that the fore mentioned nuclear morphology is that of early-stage apoptosis prior to DNA degradation. The ability of Ranaviruses to trigger apoptosis in host cells has been observed previously in numerous cell types from fish, amphibians, and even mammals (Chinchar et al., 2003; Lai et al, 2008; Chiou et al., 2009; Morales et al., 2010; Pham et al., 2012; Ring et al., 2013; Yuan et al., 2013). FV3 specifically has been shown to induce apoptosis in fathead minnow (FHM), rainbow trout macrophages (RTS11), *Xenopus laevis* peritoneal leukocytes, baby hamster kidney (BHK), and baby green monkey kidney (BGMK) cells (Chinchar et al., 2003; Pham et al., 2015; Morales et al., 2010; Ring et al., 2013). While virus activation of the caspase cascade has been reported (Chinchar et al., 2003), the exact mechanism of FV3-induced apoptosis requires further investigation. Apoptosis is an essential defense mechanism by which viral replication is limited by premature death of the host cell. However, programmed cell death represents only one of many antiviral responses.

Among the first responses to virus infection involves the induction of a family of cytokines, called IFNs. Viral infection triggers the expression of IFN, which signals in an autocrine or paracrine fashion to trigger the expression of hundreds of ISGs that directly inhibit virus replication or even facilitate apoptosis. As the type I IFN response, represents the cornerstone of antiviral immune responses, IFN/ISG expression in response to FV3 was investigated to uncover potential differences between the two cell lines that would enable RTgutGC to control infection. To accomplish this, transcript levels of IFN1, as well as Vig4, and Mx3, common fish ISGs (Poynter and DeWitte-Orr, 2016), were measured at 4, 8, and 18h PI by qPCR. Surprisingly, no significant IFN or ISG expression was detected in either cell line within 18h of infection. In order to ensure an effective infection and the initiation of virus replication within both cell cultures at the time of measurement, the expression of viral MCP was also quantified. MCP is an abundant late viral gene product, required to create the viral capsid, and thus serves as a measure of the level of infection (Devauchelle et al., 1985; Chinchar et al., 2002). MCP was detected in both cell lines at 4h, 8h, and 18h PI, with levels increasing over time, thereby indicating a successful infection and support of virus replication. As well, despite being infected with an equal MOI of virus, RTG-2 exhibited a three log-fold greater level of infection than RTgutGC at 18h PI. This confirms previous observations regarding differences in susceptibility, in which RTgutGC appears to control viral replication in some way. In attempt to observe significant IFN and ISG expression to FV3 at some point during the course of infection, cultures were infected with FV3 at a lower MOI of 0.15 for 72h. Even after 72h of infection, IFN and ISG expression was absent, with the exception being RTG-2 at 72h PI, where there was a 4-fold increase in IFN1 and an 8-fold increase in Mx3. Also detected was a subtle upregulation in Mx3 for RTgutGC at 72h PI, though very modest. Thus, subtle IFN/ISG expression was detected, albeit at very low levels.

This meagre IFN response may be the result of active or passive inhibition by the virus. With respect to active inhibition, FV3 is known to express a number of putative immune evasion genes (Grayfer et al., 2012). Among these is a caspase activation and recruitment domain-containing protein (vCARD), which, through the use of FV3 knock-out (KO) studies, has been shown to be involved in the impairment of IFN induction, as well as in the inhibition of apoptosis (Chen et al., 2015; Andino et al., 2015). Perhaps virus-encoded immune evasion proteins are inhibiting the FV3-infected cultures, blocking their ability to express IFN and ISGs. Alternatively, there may be passive inhibition of IFN/ISG expression taking place. This is based on the potential lack of recognition by innate immune sensors, which are essential in initiating type I IFN responses. While, the presence of cytosolic RNA sensors in teleost fish, particularly rainbow trout, have been well characterized, cytosolic DNA sensors (CDSs) in teleost fish remain to be identified. While the presence of dsRNA, a common intermediary by-product of virus infection, has recently been demonstrated during the course of FV3 infection (Doherty et al., 2016), the primary abundant nucleic acid would be the dsDNA genome. With the exception of the newly characterized DDX41 in Japanese flounder (Quynh et al., 2015), no other CDSs have been identified in teleost fish (Poynter et al., 2015). Possibly, a lack of CDSs in rainbow trout cells is leading to a low immunogenicity of FV3 and, in turn, a diminished IFN response. Additionally, RVs encode an RNAse III-like protein, suggested to be involved in degrading viral dsRNA, thereby inhibiting viral recognition by the cell (Grayfer et al., 2015).

To explore this further, UV-FV3 was utilized in IFN/ISG expression analysis studies. UV-FV3 is replication-deficient, unable to express any immunosuppressive proteins, while conserving the ability to bind and infect host cells. Successful inactivation of FV3 was determined by demonstrating significantly decreased expression of viral MCP and vCARD, by qPCR.

Surprisingly, exposure of UV-FV3 to both cell lines resulted in an equally modest IFN response. Thus, while FV3 expresses a number of immune evasion proteins, UV-FV3 did not elicit a strong type I IFN response; suggesting *de novo* viral protein synthesis was not required for IFN modulation. Previous studies have demonstrated that FV3 mutants lacking putative immune evasion proteins replicate as well as *wt*-FV3 in non-host (non-amphibian) cell lines (Andino et al., 2015). As well, previous work in FHM cultures demonstrated an inability of FV3 to block or delay apoptosis, a potential immune evasion mechanism associated with FV3 vCARD (Chinchar et al., 2003). Thus, it is possible that these immune evasion proteins function only in certain host species. Taken together, this data supports the hypothesis of passive immune evasion by FV3.

Given that the type I IFN response to FV3 is equally poor in both cell lines, further investigation into the difference in FV3 susceptibility was required. A previous study demonstrated that both cell lines constitutively express IFN2 (Zou et al., 2007). In this light, constitutive IFN/ISG expression was investigated in both cell lines. Thus, uninfected control cultures were analyzed for the expression of IFN1, Vig4, and Mx3 by qPCR. Interestingly, RTgutGC exhibited lower threshold ratios for each gene, thereby indicating greater constitutive expression, compared to RTG-2. While the virus-induced IFN response to FV3 may be lacking in both cell lines, perhaps the basal levels of these genes, prior to infection, is sufficient to provide some protection against FV3-induced cell death.

As the highly susceptible RTG-2 cell line exhibits inferior constitutive expression of IFN and ISGs, it was hypothesized that pretreating RTG-2 cultures with poly I:C, a potent inducer of type I IFN, would reduce the effects of FV3. RTG-2 demonstrated exceptionally high expression of IFN1, Vig4, and Mx3 when treated with poly I:C for 18h. Accordingly, RTG-2 cultures were pretreated with poly I:C for 18h prior to FV3 infection, and examined for changes in virus

replication. When pretreated with poly I:C, FV3 replication in RTG-2 was markedly inhibited. This is evident from a drastic reduction in MCP and vCARD expression in pretreated cultures, as compared to FV3 alone. Previous reports on *X. laevis* kidney implicate the role of type I IFN in the inhibition of FV3 replication both *in vivo* and *in vitro* (Grayfer et al., 2014). Thus, the ability of the IFN response to limit FV3-induced cell death was investigated. Despite this drastic reduction in virus replication, poly I:C pretreatment had no effect on FV3-induced cell death in RTG-2. Thus, there appears to be, not only a lack of protection by IFN, but also an apparent disconnect between virus replication and virus-induced cell death. To further support this hypothesis, UV-FV3 induced cell death similarly to *wt*-FV3. This further confirms a dissociation between virus replication and cell death. Previous reports have demonstrated the ability of FV3 to induce apoptotic cell death in FHM and BHK cells in the absence of productive virus replication (Chinchar et al., 2003). For this reason, the ability of UV-FV3 to induce apoptosis in both RTG-2 and RTgutGC was investigated. As observed in other cell lines, exposure to UV-FV3 resulted in DNA fragmentation at 36h PI in both rainbow trout cell lines. Thus, the ability of UV-FV3 to induce apoptosis indicates that *de novo* viral protein synthesis is not required for virus-induced cell death, via apoptosis, in rainbow trout cell lines.

As the type I IFN response, specifically the induced response, is equally absent in both cell lines, the mechanisms responsible for this difference in susceptibility remained unknown. While constitutive IFN/ISG expression was greater in more resistant cell line, the poly I:C-induced IFN response was unable to protect against FV3-induced cell death. Thus, greater constitutive expression of IFN/ISGs is unlikely the primary mechanism accounting for this difference in susceptibility. In order to further investigate the mechanisms responsible for this difference in susceptibility, intrinsic cellular features were investigated, including virus entry and cellular

metabolism. While the ability of FV3 to infect and enter rainbow trout cell lines has been investigated previously (Pham et al., 2015), differences in the rates of viral entry between cell lines has not been reported, yet may provide important insight into mechanisms governing FV3 susceptibility. Based on the detection of viral MCP DNA by qPCR, FV3-infected RTG-2 cultures exhibited greater levels infection at 1h, 2h and 3h PI, compared to the less susceptible cell line RTgutGC. Owing to the very short infection period, as well as the detection of viral DNA, rather than transcript mRNA, the greater level of infection observed in RTG-2 can reasonably be attributed to enhanced entry of FV3 virions. FV3 preparations contain both enveloped particles and non-enveloped (naked) particles (Chinchar et al., 2011). While receptor-mediated endocytosis is one of two possible modes of entry, the other being fusion, it is likely the primary route (Gendrault et al., 1981; Braunwald et al., 1985; Chinchar et al., 2002). Thus, RTG-2 may express more surface receptors, to date of unknown identity, to enhance virus entry, and in turn, susceptibility.

Following entry, the viral genome translocates to the nucleus where viral transcription takes place, catalyzed by host RNA polymerase II (Chinchar et al., 2011). Viruses rely on the use of host cell machinery for replication at some stage of their replicative cycle. One of the first products of early viral transcription is a viral DNA polymerase, which replicates the viral DNA genome (Goorha, 1982). Thus, while the greater level of viral DNA between cell lines at earlier time-points can reasonably be attributed to viral entry alone, later infection periods (such as 3h) may also be the result of enhanced cellular metabolism. As such, the metabolic rate, and thus the replicative capacity of both cell lines, was monitored over 3 days. Despite both cell lines exhibiting similar basal metabolic levels, RTG-2 exhibited greater metabolic and/or replicative capacity over time. As mentioned previously, RTG-2 also supported a greater level of FV3 replication as

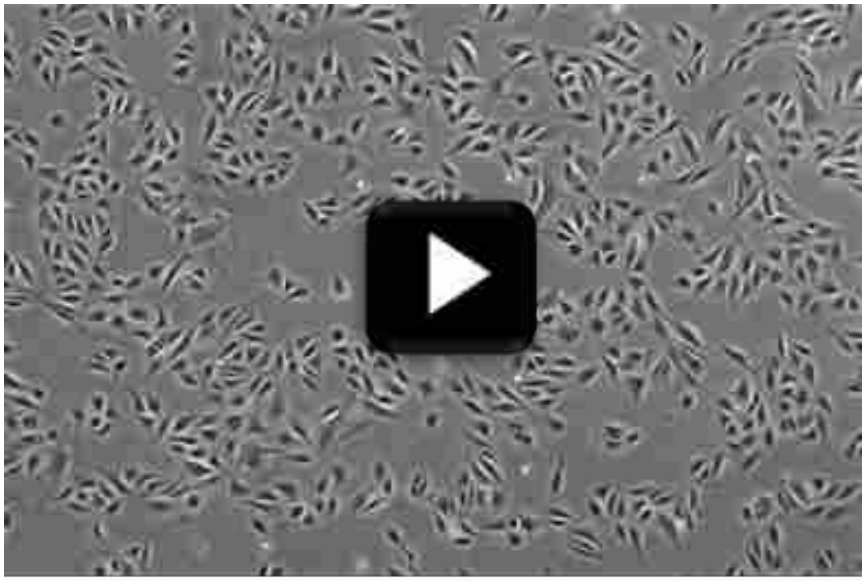
measured by changes in MCP expression over 4h, 8h, and 18h PI. Indeed, RTG-2 displayed a three-fold greater level of MCP expression compared to RTgutGC at 18h PI. The greater metabolic activity of RTG-2 enables FV3 to replicate at a faster rate. Taken together, these results suggest that FV3 susceptibility may be due to enhanced viral entry, metabolic capacity, and constitutive interferon expression, as opposed to any *induced* innate antiviral immune response.

5. Conclusion

The above results demonstrate that FV3 induced apoptotic cell death in both rainbow trout cell lines. RTG-2 appears to be significantly more susceptible to FV3-induced cell death compared to RTgutGC. Intrinsic cellular features of RTgutGC, including constitutive IFN/ISG levels, as well as reduced rates of viral entry and metabolism, may be responsible for the observed differences in susceptibility. However, this susceptibility difference does not appear to be due to any differences in the *induced* type I IFN response, as this response is surprisingly deficient. While IFN and ISG induction and UV-irradiation drastically inhibited FV3 replication, apoptosis remained the outcome, suggesting apoptotic cell death does not require *de novo* viral protein synthesis. These data highlight the many factors influencing host cell susceptibility to FV3, and the possible disconnect between the IFN response and cell death in rainbow trout cells infected with this ecologically important pathogen.

6. Figures and Figure Legends

Video 1: FV3-infected RTgutGC and RTG-2 time-lapse. (**A**) RTgutGC and (**B**) RTG-2 cultures were infected with FV3 at an MOI of 1.5 for 48h. Photographs were taken every 10 minutes for 48h. Images were stitched together and played at 50 fps. Videos are representative of at least three independent experiments (external media access provided).



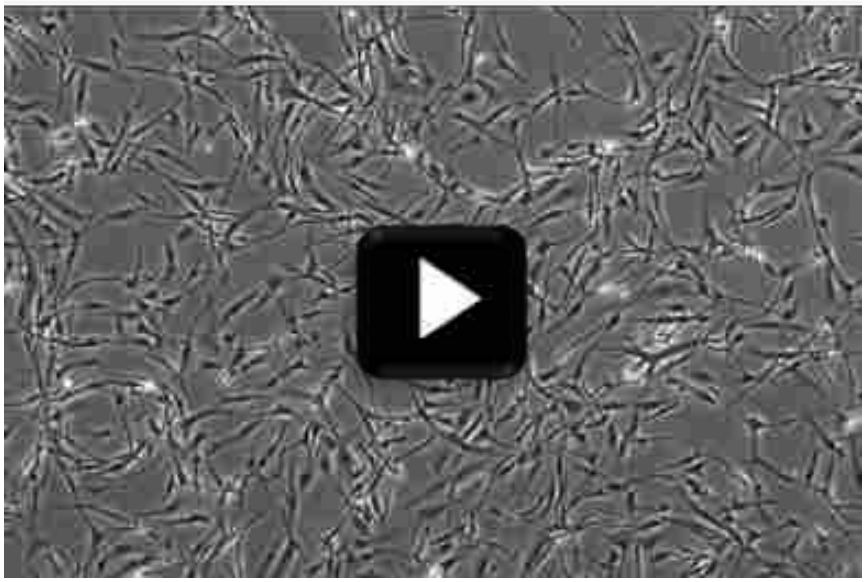


Figure 2.1: Susceptibility differences of RTG-2 and RTgutGC to FV3. Cells were infected with serial dilutions of FV3 (MOI of 55 to $5.5x10^{-4}$) for 48h, 72h, and 96h. Cell viability was measured on a fluorescence plate reader using alamarBlue® (AB) and 5-CFDA-AM (CFDA) fluorescent indicator dyes, expressed on the y-axis as a percent of control (uninfected) cultures. For each MOI, six wells were infected (technical replicates), and data represents mean ± SEM. These data include three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey post-test (*$p<0.05$, **$p<0.01$). *represents statistically significant differences between RTG-2 and RTgutGC at the corresponding measurement.

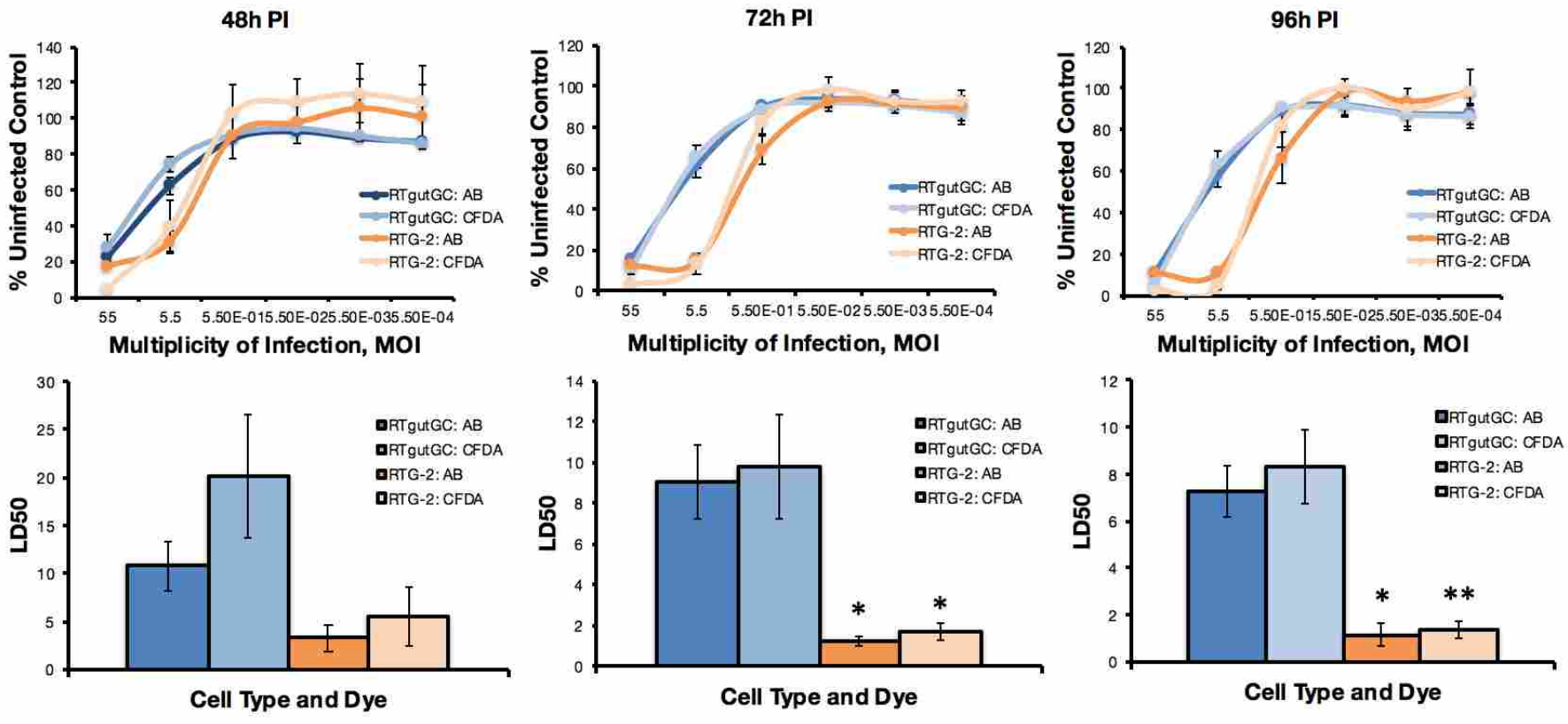
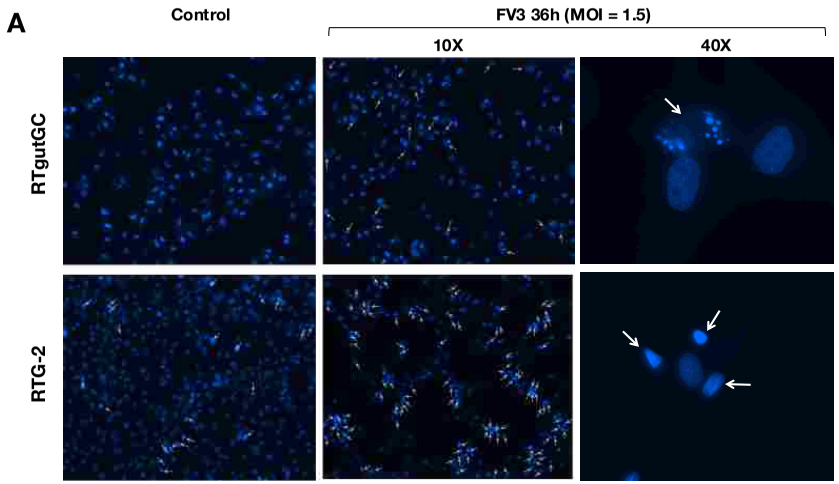
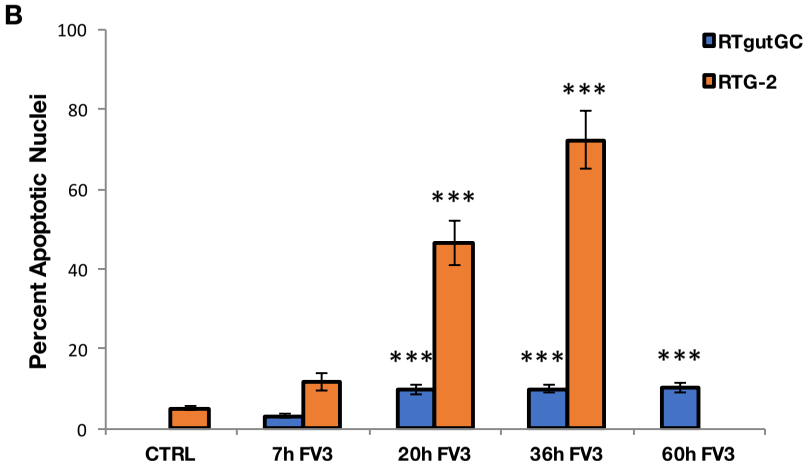
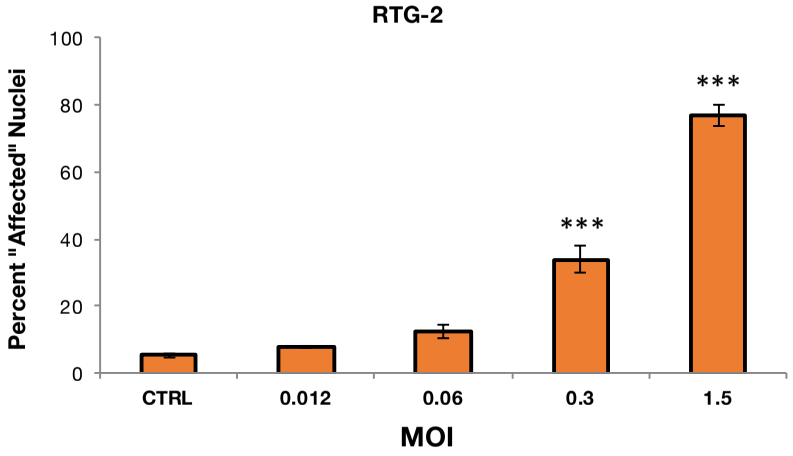

48h PI
% Uninfected Control
Multiplicity of Infection, MOI
RTgutGC: AB
RTgutGC: CFDA
RTG-2: AB
RTG-2: CFDA
72h PI
% Uninfected Control
Multiplicity of Infection, MOI
96h PI
% Uninfected Control
Multiplicity of Infection, MOI
LD50
Cell Type and Dye
*
**

Figure 2.2: Mechanism of FV3-induced cell death in RTG-2 and RTgutGC. Following infection with FV3 at an MOI of 1.5, RTG-2 and RTgutGC cultures were examined for nuclear fragmentation, as well as gDNA laddering, two hallmarks of apoptosis. RTG-2 and RTgutGC cells were mock-infected (uninfected controls) or infected with FV3 at an MOI of 1.5 for 7h, 20h, 36h, and in the case of RTgutGC, 60h. (**A**) Following the indicated length of infection, cultures were fixed and stained with DAPI, which stains nuclear DNA, and examined by fluorescence microscopy. Shown are representative images of mock- and FV3-infected cultures for both cell lines at 36h PI. (**B**) The percentage of apoptotic nuclei in DAPI-stained images were scored. (**C**) Additionally, RTG-2 cultures were infected with FV3 at MOIs ranging from 0.012 to 1.5 for 36h and examined for nuclear condensation/fragmentation. The percentage of apoptotic nuclei in DAPI-stained cultures were scored. This data represents means ± SEM, and is representative of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Dunnett's post-test (***$p<0.001$). *indicates statistically significant difference from control. **(D**) Following the indicated infection period, DNA was extracted and subject to gel electrophoresis. Shown are representative images of two independent experiments.


A
Control
FV3 36h (MOI = 1.5)
10X
40X
RTgutGC
RTG-2


B
Percent Apoptotic Nuclei
100
80
60
40
20
0
RTgutGC
RTG-2
***
CTRL
7h FV3
20h FV3
36h FV3
60h FV3


C
RTG-2
Percent "Affected" Nuclei
100
80
60
40
20
0
***
CTRL
0.012
0.06
0.3
1.5
MOI

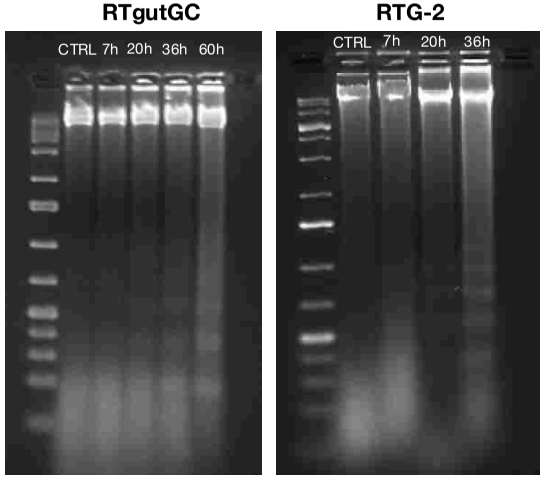

D
RTgutGC
RTG-2
CTRL 7h 20h 36h 60h
CTRL 7h 20h 36h

Figure 2.3: IFN and ISG expression in RTG-2 and RTgutGC, constitutive and virus-induced. (**A**) Constitutive expression of IFN1, Vig4, and Mx3 were measured by qRT-PCR with primers specific for these genes. Threshold (C_t) values of the fore mentioned genes were gathered. A ratio of actin C_t to gene-of-interest C_t was generated for each experiment. Shown are means ± SEM from fourteen independent experiments. Data was compared by an unpaired t-test (***$p<0.0001$). * represents statistically significant differences between RTG-2 and RTgutGC gene expression. The expression of IFN1, Vig4, Mx3, (**B**) and MCP (**C**) was measured by qPCR in RTG-2 and RTgutGC cells, either mock infected or infected with FV3 at an MOI of 1.5 for 4, 8, and 18h and MOI of 0.15 for 72h. All gene expression was normalized to the housekeeping gene, actin. The results are means ± SEM of gene expression from three independent experiments. Statistical analysis was performed by a one-way ANOVA with a Dunnett post-test (*$p<0.05$; **$p<0.01$). * represents statistical difference between FV3-infected cultures and uninfected (control) cultures.

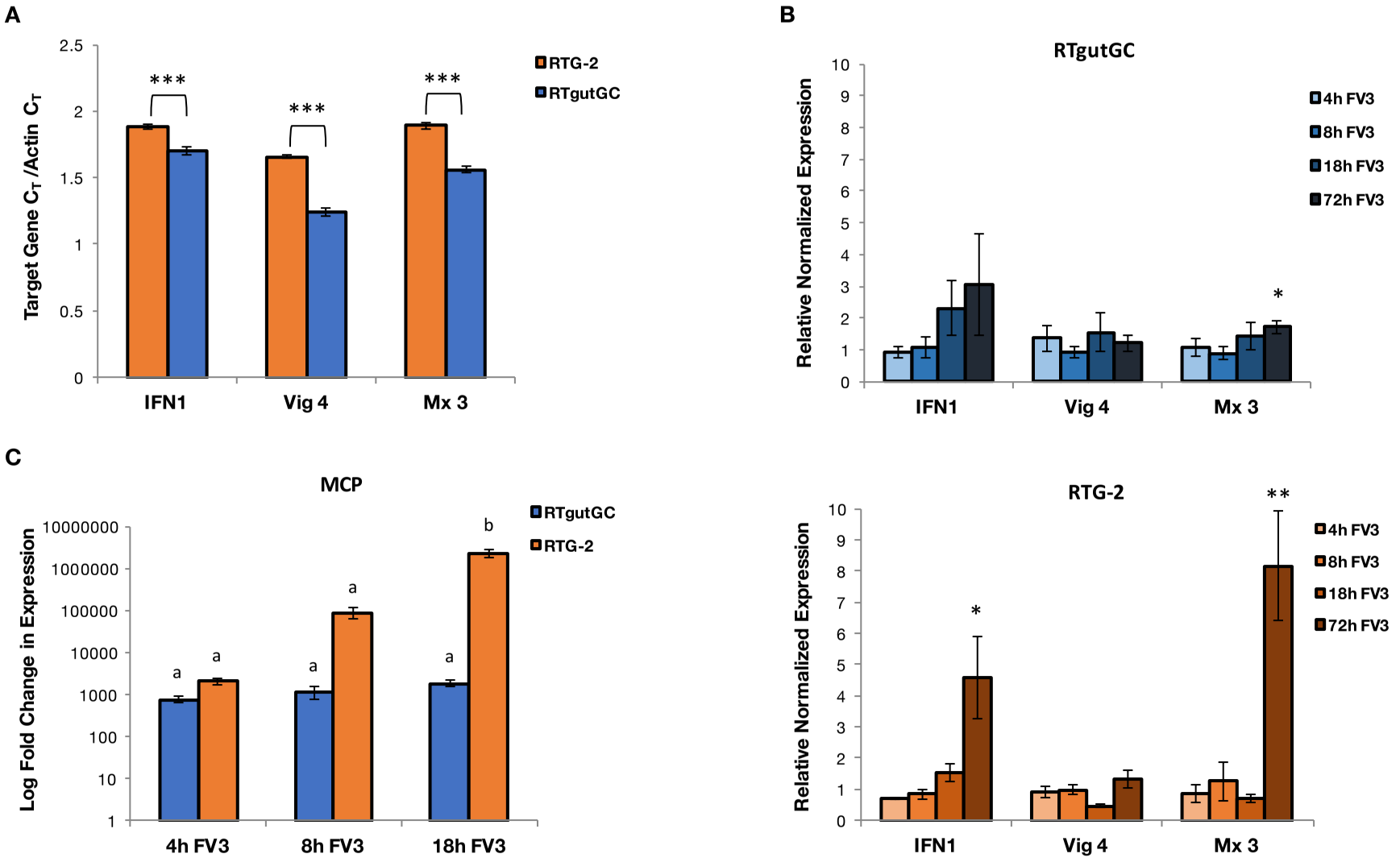

A
Target Gene C_T /Actin C_T
2.5
2
1.5
1
0.5
0
***
***
***
IFN1
Vig 4
Mx 3
RTG-2
RTgutGC
B
RTgutGC
Relative Normalized Expression
10
9
8
7
6
5
4
3
2
1
0
*
IFN1
Vig 4
Mx 3
4h FV3
8h FV3
18h FV3
72h FV3
C
MCP
Log Fold Change in Expression
10000000
1000000
100000
10000
1000
100
10
1
a
a
a
a
a
b
4h FV3
8h FV3
18h FV3
RTgutGC
RTG-2
RTG-2
Relative Normalized Expression
10
9
8
7
6
5
4
3
2
1
0
*
**
IFN1
Vig 4
Mx 3
4h FV3
8h FV3
18h FV3
72h FV3

Figure 2.4: Poly I:C inhibits FV3 replication, but not cell death in RTG-2. (**A**) Transcript levels of IFN1, Vig4 and Mx3 were measured in RTG-2 cultures treated with 40 μg/mL poly I:C for 18h by qRT-PCR. All gene expression was normalized to the housekeeping gene, actin. Results represent means±SEM of gene expression from three independent experiments. Statistical analysis was performed by comparing treated samples for each gene to uninfected control samples by an unpaired *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$). (**B**) MCP and vCARD transcript levels were measured by qRT-PCR in either mock-treated or 18h poly I:C (PIC)-treated RTG-2 cells followed by either mock-infection or infection with FV3 at an MOI of 0.15 for 72h. All gene expression was normalized to the housekeeping gene, actin. The results are means ± SEM of gene expression from four independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey's post-test between treated and/or infected samples and uninfected control samples (not shown) for each gene (**$p<0.01$). *represents statistically significant differences between PIC+FV3 and FV3 alone. (**C**) Cell viability was measured in either mock-treated or poly I:C-treated RTG-2 cells for 18h followed by a mock-infection or FV3 infection (MOI of 1.5). At 48h PI, cell viability was measured using alamarBlue® (AB) and 5-CFDA-AM (CFDA) fluorescent dyes, expressed as a percent of the uninfected control cultures. Statistical analysis was performed by a paired t-test. "n.s." represents data that is not statistically significant in comparison to one another.

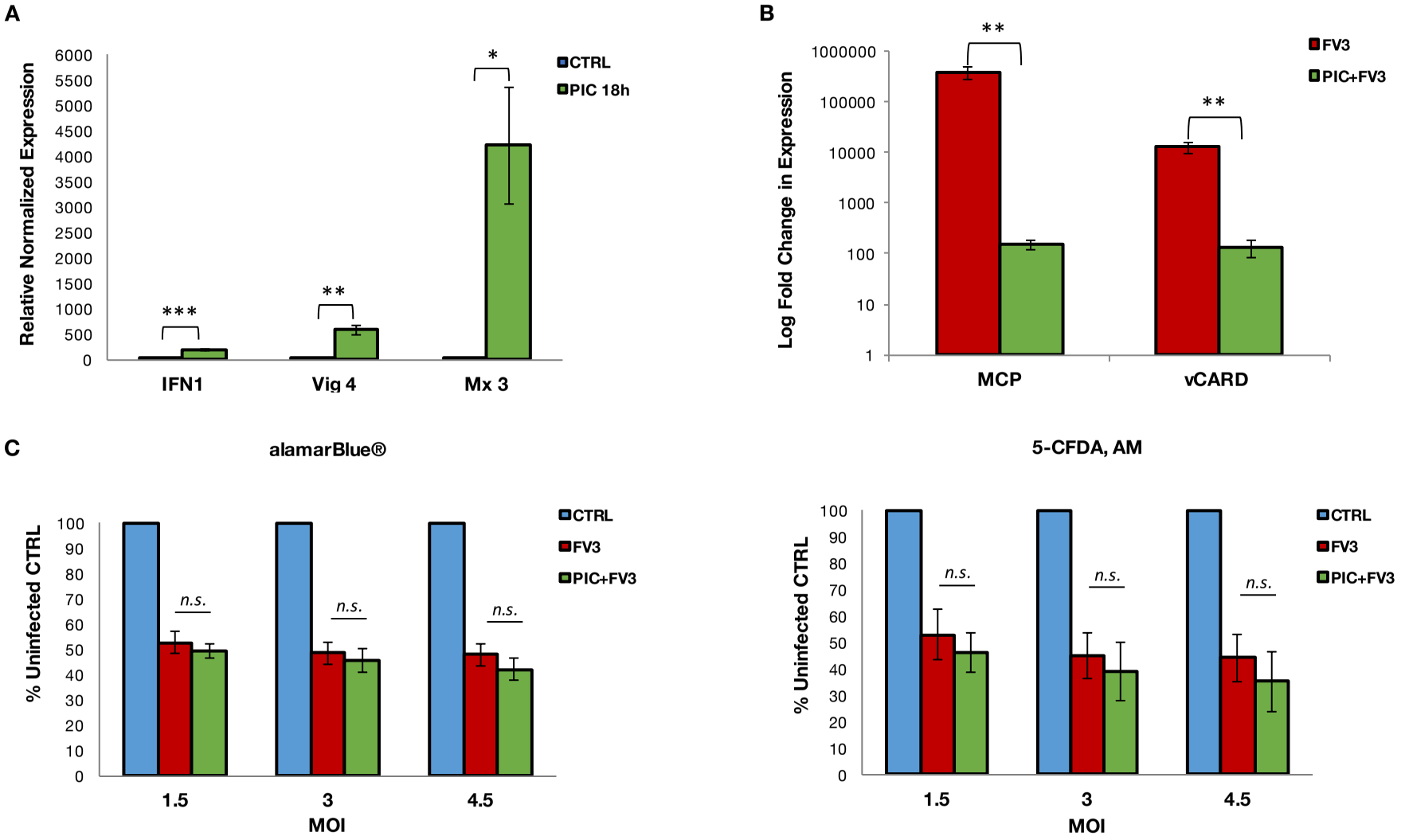
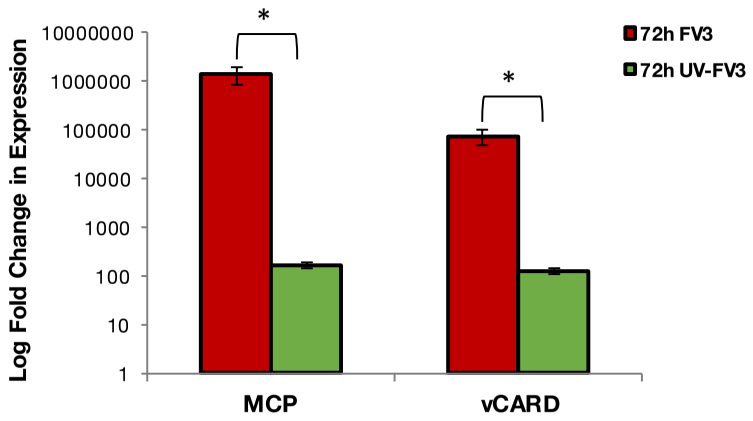
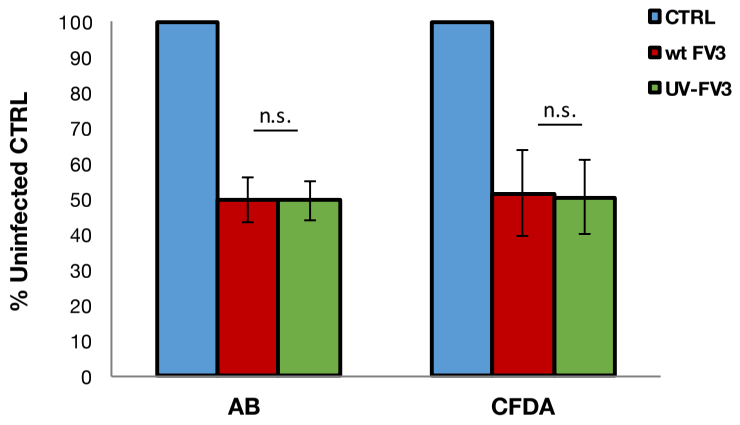

A
Relative Normalized Expression
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
***
**
*
IFN1
Vig 4
Mx 3
CTRL
PIC 18h
B
Log Fold Change in Expression
1000000
100000
10000
1000
100
10
1
**
**
MCP
vCARD
FV3
PIC+FV3
C
alamarBlue®
% Uninfected CTRL
100
90
80
70
60
50
40
30
20
10
0
n.s.
n.s.
n.s.
1.5
3
4.5
MOI
CTRL
FV3
PIC+FV3
5-CFDA, AM
% Uninfected CTRL
100
90
80
70
60
50
40
30
20
10
0
n.s.
n.s.
n.s.
1.5
3
4.5
MOI
CTRL
FV3
PIC+FV3

Figure 2.5: UV-inactivated FV3 induces apoptosis, but does not induce significant IFN expression compared with wildtype. (**A**) MCP and vCARD expression was measured by qRT-PCR in RTG-2 cultures that were mock-infected or infected with FV3 and UV-FV3 at an MOI of 0.15 for 72h. Gene expression was normalized to rainbow trout actin. Results are means±SEM of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey's post-test within a single cell line, including uninfected control samples (not shown; *$p<0.05$). (**B**) Cell viability was measured in mock, *wt*-FV3 or UV-FV3 (MOI=1.5 for 48h) -infected RTG-2 cells, using two fluorescent indicator dyes, alamarBlue® (AB) and 5-CFDA-AM (CFDA), which was expressed as a percent of the uninfected control cultures along the y-axis. Statistical analysis was performed by a paired t-test. "n.s." represents data that is not statistically significant in comparison to one another. (**C**) Intranucleosomal fragmentation was observed in RTG-2 and RTgutGC cells infected with UV-FV3 at an MOI of 1.5 for 36h. Shown is a representative image of two independent experiments. (**D**) IFN1, Vig4 and Mx3 expression was measured by qRT-PCR in RTG-2 and RTgutGC cells infected with UV-FV3 at an MOI of 0.15 for 72h. All gene expression was normalized to the rainbow trout actin endogenous control. The results are means ± SEM of gene expression from three independent experiments. Statistical analysis was performed by a one-way ANOVA with a Tukey's post-test comparing IFN/ISG expression induced by UV-FV3 to that induced by *wt*-FV3 at 72h PI.

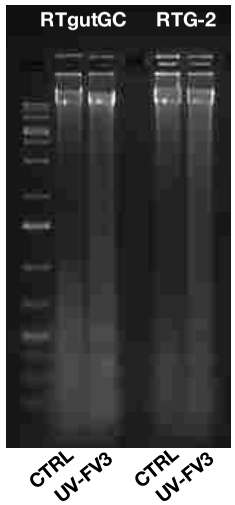
A


Log Fold Change in Expression
10000000
1000000
100000
10000
1000
100
10
1
*
*
MCP
vCARD
72h FV3
72h UV-FV3

B


% Uninfected CTRL
100
90
80
70
60
50
40
30
20
10
0
n.s.
n.s.
AB
CFDA
CTRL
wt FV3
UV-FV3

C


RTgutGC
RTG-2
CTRL
UV-FV3
CTRL
UV-FV3

D

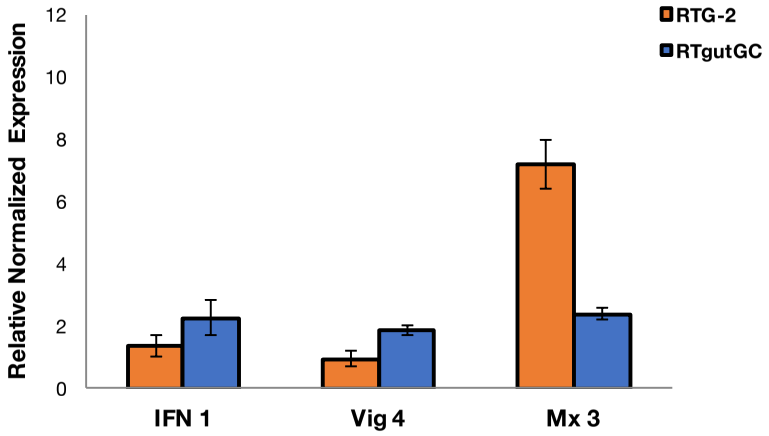
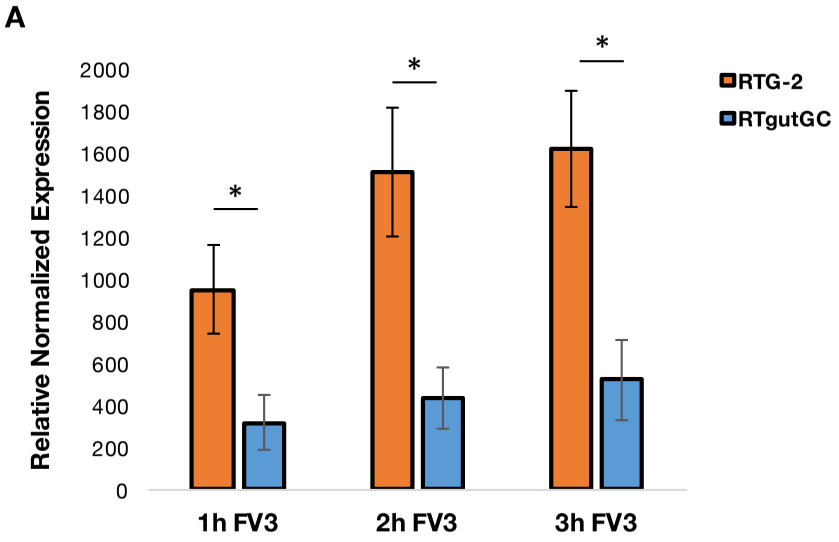
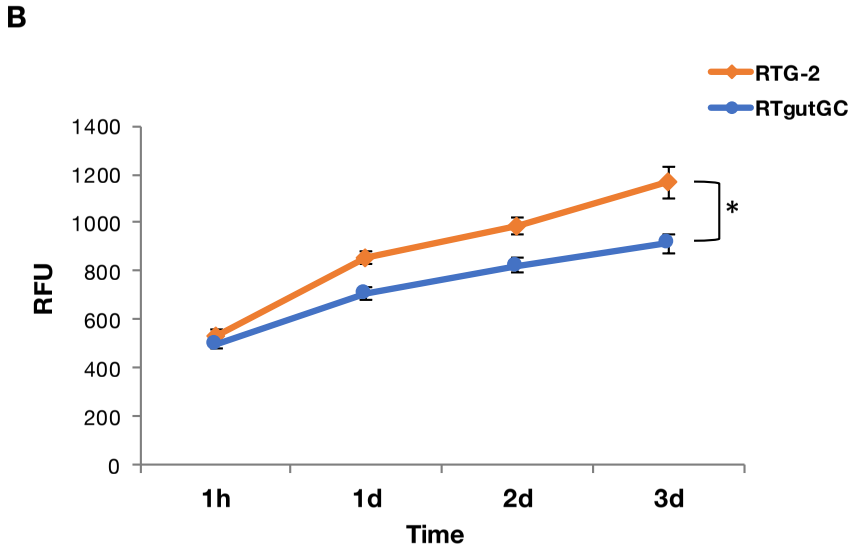

Relative Normalized Expression
12
10
8
6
4
2
0
IFN 1
Vig 4
Mx 3
RTG-2
RTgutGC

Figure 2.6: Differential rates of viral entry and metabolism between cell lines. (**A**) MCP DNA levels were measured by qPCR in both RTG-2 and RTgutGC following FV3 infection (MOI=1.5; 1h, 2h and 3h PI). MCP expression was normalized to rainbow trout actin endogenous control. This data represents means±SEM of gene expression from four independent experiments. * represents statistically significant difference between RTG-2 and RTgutGC at the corresponding time-point (*$p<0.05$). (**B**) Cell metabolism was measured in RTG-2 and RTgutGC cells using alamarBlue®, at 1h, 24h, 48h, and 72h post-plating. This data represents means ± SEM of relative fluorescence units (RFU) for four independent experiments. Cultures were compared using a paired t-test. *indicates statistically significant difference between each corresponding time point (except 1h) in RTgutGC and RTG-2 (1d $p<0.001$; 2d $p<0.01$; 3d $p<0.05$).


A
Relative Normalized Expression
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
1h FV3
2h FV3
3h FV3
RTG-2
RTgutGC


B
RFU
1400
1200
1000
800
600
400
200
0
1h
1d
2d
3d
Time
*
RTG-2
RTgutGC

Chapter 3

Major Research Findings and Contributions to the Field of Anti-Ranaviral Immunology

General Discussion

Ranaviruses (RVs) are emerging pathogens of fish, amphibians, and reptiles, responsible for numerous species die-offs and outbreaks across the globe. RV infections of wild and captive ectothermic vertebrates are on the rise and are of considerable concern to the future of these ecologically and economically important organisms (Duffus et al. 2015; Miller et al. 2011). While it appears that susceptibility to these pathogens differs between individual host species, the immune and viral determinants governing disease remain ambiguous. Due to the rapid increase in the prevalence of RV infections and their capacity to cause mass die-offs in a multitude of new hosts, RVs such as Frog virus 3 (FV3), are considered to be potential global threats to ectothermic vertebrate populations (Chinchar et al., 2002; 2011; Jancovich et al., 2010; Miller et al. 2011). As such, there is an urgent need to determine whether susceptibility of a given species reflects an inability to mount appropriate antiviral immune responses or the ability of the virus to overcome otherwise effective immune mechanisms. RVs are known to possess a number of immune evasion and host modulation mechanisms, which may circumvent host efforts to clear infection. As a result, a more thorough understanding of virus-host interactions at the cellular and molecular level is required in order to develop antiviral strategies for susceptible hosts, and to minimize potential future consequences to new off-target hosts. Accordingly, we investigated the innate antiviral immune response to FV3, with a focus on potential strategies of immune evasion by the virus, in attempt to determine the mechanisms that dictate susceptibility.

This study identified and quantified a major susceptibility difference between two rainbow trout cell lines: RTgutGC, an epithelial cell line of intestinal origin; and RTG-2, a fibroblastic cell line of gonadal origin. RTG-2 exhibited greater susceptibility to FV3-induced cell death, compared to RTgutGC. This provided an excellent model to study the innate antiviral immune response,

which governs susceptibility to the virus. As apoptosis is an important innate antiviral defense mechanism, the ability of both cell lines to undergo apoptosis following infection was investigated. We demonstrated that FV3 was able to induce apoptosis in both cell lines, despite the difference in susceptibility. One of the first lines of defense against virus infection is the type I IFN response. As such, this response was investigated in both cell lines following infection. Surprisingly, the IFN response was equally absent in both cell lines, again, despite differences in susceptibility. As FV3 is known to express putative immune evasion proteins, some of which have been shown to shutdown host type I IFN responses, this response was measured following infection with UV-inactivated FV3 (UV-FV3). Much to our surprise, the IFN response was equally absent following UV-FV3 exposure. Despite prior induction, IFNs and ISGs were able to significantly hinder virus replication, but had no effect on cell death. Likewise, UV-inactivated FV3 (UV-FV3) exhibited impaired replication, but again, virus-induced apoptotic cell death was not affected. This highlights an apparent disconnect between virus replication and cell death. Ultimately, we determined that FV3-induced cell death does not require productive FV3 replication, and the mechanisms protecting RTgutGC are IFN-independent. To uncover the mechanisms responsible for the observed susceptibility differences between the two cell lines, intrinsic cellular features were investigated, including constitutive IFN expression, viral entry, and cellular metabolism. We demonstrated that constitutive expression of IFN and ISGs is lower in the more susceptible RTG-2 cell line. This, coupled with enhanced viral entry and cellular metabolism may account for the observed susceptibility differences.

Based on the realization that virus replication is not required to trigger apoptosis, apoptotic induction may reasonably be the result of two mechanisms. Firstly, simple binding of FV3 to a specific transmembrane protein may be sufficient to trigger apoptosis. For instance, subgroups of

avian leukosis virus have been shown to bind host cells and trigger apoptosis via interactions with a TNF-family receptor (Brojatsch et al., 2000; Diaz-Griffero et al., 2003). While FV3 virion entry has been well-studied (Chinchar et al., 2011), the identity of the FV3 binding receptor remains unknown, complicating further investigation into this mechanism of apoptosis induction. As well, the existence of both enveloped and naked (unenveloped) FV3 virions, which enter cells by simple fusion (not receptor-mediated), suggests this pathway may be less likely. On the other hand, FV3-induced apoptosis may be triggered by one or more virion-associated proteins that are released during viral uncoating. Precedent for this pathway is seen in previous studies with Chilo iridescent virus (CIV), another member of the *iridoviridae* family, in which a virion-associated protein kinase was shown to induce apoptosis in the absence of virus replication (Chitnis et al., 2011). Characteristic of FV3 infections is the rapid inhibition of host cell protein synthesis and the activation of eIF2-α (Chinchar and Dholakia, 1989). Responsible for eIF2-α phosphorylation, as well as apoptosis induction, is PKR, a serine-threonine kinase that recognizes and responds to viral dsRNA (Gil and Esteban, 2000). In the fore mentioned CIV study, this protein kinase, termed iridoptin, was shown to possess serine/threonine kinase activity, and was responsible for both translational shutoff, as well as apoptosis induction. Possibly, FV3 possesses a virion-associated PKR-like molecule, which induces apoptosis in host cells.

While apoptosis, particularly the early onset of apoptosis, is well known as an important antiviral defense mechanism, this death mechanism may provide several advantages for the virus. It is understood that the early onset of apoptosis, and thus premature death of the host cell, may severely inhibits virus replication. However, following a sufficient infectious cycle, apoptosis may also be a mechanism that may enhance virus replication and persistence within a host organism. Following the induction of apoptosis, cellular contents are packaged into membrane-enclosed

apoptotic bodies, which are readily taken up by neighboring cells. As the virus is not exposed to the extracellular environment, the virus may spread undetected to neighbouring cells without triggering a significant inflammatory response (Roulston et al., 1999). Furthermore, virions enclosed within apoptotic bodies would be protected from host antibodies, proteases, and antimicrobial peptides. For this reason, *in vitro* studies of virus-induced apoptosis may not be entirely applicable in comprehending the role of apoptosis in whole organisms. The ability of FV3 to both induce and inhibit apoptosis suggests a complex relationship between virus replication and cell death. Perhaps, the virus is able to inhibit apoptosis momentarily, until a sufficient level of virus replication has taken place, upon which apoptotic cell death occurs, facilitating its safe transmission to neighbouring cells. Possibly, the ability of FV3 to induce apoptosis, rather than necrosis, prevents disease within the animal, enabling the virus to coexist with its host. This would explain the ability of these fish to serve as asymptomatic carriers, with no clinical signs of disease.

A multitude of research has been conducted investigating the ability of RVs to induce apoptosis in host cells. Earlier work has demonstrated the ability of RVs to induce apoptosis within a variety of cell types from fish, amphibians, and even mammals (Chichar et al., 2003; Essbauer and Ahne, 2002; Lai et al, 2008; Chiou et al., 2009; Morales et al., 2010; Pham et al., 2012; Ring et al., 2013; Yuan et al., 2013). FV3, in particular, has been shown to induce apoptosis in fathead minnow (FHM), rainbow trout macrophages (RTS11), *Xenopus laevis* peritoneal leukocytes, baby hamster kidney (BHK), and baby green monkey kidney (BGMK) cells (Chinchar et al., 2003; Pham et al., 2015; Morales et al., 2010; Ring et al., 2013). This study further confirms apoptosis as a characteristic outcome of RV infection, specifically by FV3. However, FV3-induced apoptosis was investigated in novel cell lines. Few reports have demonstrated the ability of apoptosis to occur in the absence of productive infection (Chinchar et al., 2003; Chitnis et al., 2011). Our

research adds to this by demonstrating the induction of apoptosis, which was independent of productive virus replication, in new cell lines. However, further research is essential in determining the mechanisms involved in virus-induced apoptosis.

With respect to antiviral immunity, some work has been performed on the immune mechanisms against RV infections. The ability of immunocompetent adult frogs to clear infection within weeks, while tadpoles succumb to infection, highlights a key role of immune mechanisms in governing susceptibility (Gantress et al., 2003; Maniero et al., 2006; Morales and Robert, 2007). Adults can develop rapid innate immune responses (upregulation of proinflammatory cytokines), followed by an efficient cytotoxic T cell response and the generation of potent anti-FV3 antibodies. Additionally, the protective role of cytotoxic T lymphocytes (CTLs) and antibodies in FV3 infections has been previously been demonstrated in *X. laevis* (Caipang et al., 2006; Morales and Robert, 2007; Ou-yang et al., 2012). While their role in survival is presently unknown, numerous antiviral immune-related genes have been shown to be upregulated during FV3 infection. Cheng et al. (2014) have reported the upregulation of numerous immune-related genes, including IFN, following FV3 infection of fish cells. As well, the protective role of type I IFN has been suggested in amphibian cells (Grayfer et al., 2014). IFN was shown to protect frog kidney cells, as well as induce an antiviral state in adults and tadpoles, which conferred protection against FV3 (Grayfer et al. 2014). Interestingly, while recombinant *X. laevis* IFN (r*Xl*IFN) provided protection, FV3-infected tadpoles eventually succumb (Grayfer et al., 2014). Our research is novel in that we demonstrate, not only a lack of induction of IFN and ISGs by the virus, but also a lack of protection by IFN and ISGs. Consistent with the fore mentioned studies, IFN/ISGs were found to significantly inhibit virus replication. However, IFN responses had no effect on cell death, similar to observations with r*Xl*IFN-treated tadpoles. This observation further suggests that RVs, such as

FV3, may exhibit virulence factors independent of virus replication. While the ability of FV3 to induce IFN and ISGs in fish cells has been investigated previously (Chen et al., 2014), a protective role of these genes in infection has been poorly investigated in fish. Furthermore, the ability of certain ISGs, such as Vig4, to protect against RV infection had not been previously addressed. This research emphasizes novel insights into the FV3-host immune interaction in fish cell lines, and suggests that immune protection to FV3 may be cell type specific.

Novel findings of this report are derived from further investigation into the mechanisms governing susceptibility differences. Our study, for the first time, investigated potential differences in the rates of FV3 virion entry between cell lines. We demonstrated that the more susceptible cell line, RTG-2, exhibited greater viral entry. The mechanisms of ranavirus entry have been previously characterized (Braunwald et al., 1979; Ma et al., 2014). Additionally, the ability of FV3 to enter rainbow trout cell lines has previously been studied (Pham et al., 2015). However, our research is novel in that the rates of virion entry were quantified in different cell lines. This differential rate of virion entry is suggested to account for differences in susceptibility, and may be due to enhanced FV3 receptor expression. In addition to viral entry, cellular metabolism was also investigated as a potential determinant of susceptibility. It is well understood that virus replication and host metabolic machinery are closely connected, as viruses utilize host enzymes for replication. However, the relationship between virus replication and cellular metabolism has been poorly investigated. In this light, cellular metabolism/replication capacity was measured in both cell lines. As expected, the more susceptible cell lines, RTG-2, exhibited a greater metabolism. Thus, it is reasonable to attribute this greater metabolic capacity to the greater level of FV3 replication, and thus susceptibility, observed in RTG-2.

Biological research, by its very nature, utilizes and relies on multiple areas of study in attempt to understand nature. Biology is so intrinsically integrative that it is difficult to classify this area of research into just a single field. This study investigated the innate antiviral immune response of rainbow trout cells to a relatively new and understudied virus, FV3. This research employs knowledge and techniques that draw heavily on concepts in immunology, virology, cell and molecular biology, physiology, genomics, and ecology. Not only is the virus itself being studied, but also the biological response of the organism (rainbow trout) to the virus, the genes upregulated in response, and the environmental contributions to infection. While at first it may not be evident, all biological research incorporates different subdisciplines, thus demonstrating the truly integrative nature of biology.

This interrelatedness is important because it provides alternative perspectives, enabling discoveries to be more widely applicable and have more dimensionality. The impact of RV infections on numerous species has been described. These viruses have been implicated in mortality events on six continents across the globe, involving over 175 species across 52 ectothermic vertebrate families (Duffus et al. 2015; Miller et al. 2011). This can have applications on an ecological level by providing answers as to why a particular animal population is declining, and what effects this may have on the ecosystem overall. While rainbow trout are merely asymptomatic carriers of the virus, a number of species have shown to be particularly susceptible, exhibiting mass mortality to the virus. Additionally, asymptomatic carriers of the virus serve as important vectors for transmission to other highly susceptible organisms within a particular environment. Furthermore, these viruses continue to demonstrate an ability to infect a variety of new hosts more and more. In this light, this research becomes essential from a conservation biology perspective. Among other species affected by RVs, amphibians in particular, are facing a serious

threat of extinction. RVs have been implicated as major causative agents in the global amphibian decline (Robert, 2010). Thorough understanding of the mechanisms that govern susceptibility may enable the development of effective antiviral strategies to protect certain species from die-off events. Complete loss of a particular species within a community, not only threatens the concept of biodiversity, but can have detrimental impacts on the ecosystem as a whole.

Additionally, as many antiviral immune responses, particular innate responses, are highly conserved among vertebrates, these findings in rainbow trout may be applied to virus interactions in a number of host organisms. Viruses are continually developing new strategies to circumvent host antiviral immune responses. This is true of all viruses, even those currently plaguing human populations. For instance, previous work with human immunodeficiency virus (HIV) has demonstrated the ability of HIV viral proteins to disrupt the IFN response by degrading interferon regulatory factor 3 (IRF3) in target T cells (Doehle et al., 2009; Okumura et al., 2008). By understanding these strategies in a number of viruses, even ranaviruses, the ability to develop successful antiviral therapeutics is improved. In this light, this research may hold potential biomedical implications. While it is difficult to specialize so much so that you incorporate only a single discipline, the result would be an overly narrow investigation, thus limiting the applicability of one's results.

While there have been significant advances regarding FV3-host interactions and antiviral immunity, much remains to be elucidated in order to fully comprehend these responses. Based on the literature discussed, it is clear that anti-FV3 immunity is very complex and may be species- and developmental stage-specific. Much remains to be understood regarding the immune responses that dictate susceptibility. The present study demonstrated the ability of FV3 to induce apoptosis in host cells, independent of virus replication. Whether FV3-induced apoptosis is a

consequence of PKR activation and translational shutoff or independent of these two events remains to be determined. To accomplish this, the ability of FV3 to shutdown host macromolecular synthesis in rainbow trout cell lines should be investigated with both infectious and inactivated FV3. As well, further investigation into whether FV3 virions possess the fore mentioned virion-associated PKR-like molecule, or a structurally and/or functionally similar molecule, is essential to understanding and characterizing FV3-induced cell death. Additionally, this study observed a particularly poor IFN or ISG response to FV3 in both cell lines. UV-inactivated FV3 did not show any difference in IFN or ISG induction, suggesting a lack of viral inhibition by immune evasion proteins. Whether these immune evasion proteins function similarly in rainbow trout cells would be an important future area of research. This may be accomplished by *in vitro* studies with FV3 knock-out mutants, lacking putative immune evasion genes. Observations of replication efficiencies and cell death in these cell lines in comparison to wild-type virus would benefit this area of research. As well, it was hypothesized that the meagre IFN response to FV3 may be due to a lack of appropriate recognition by the cell. With the exception of the recently-identified DDX41 receptor in Japanese flounder (Quynh et al., 2015), no other CDSs have been identified in teleost fish. Thus, investigation into whether these rainbow trout cell lines possess functional CDSs is essential into understanding why these cell lines are unable to mount appropriate IFN responses to this DNA virus.

Summary

1. Subject.

Ranavirus infections are becoming increasingly prevalent worldwide and are responsible for numerous species die-offs across the globe. Frog virus 3 (FV3) is a large ranavirus of the family *Iridoviridae* that possess a dsDNA genome. FV3 is considered to be the type species of the genus, yet its virus-host interactions are poorly understood. Essential virus-host interactions include the innate antiviral immune response, particularly the type I interferon (IFN) response. Following infection, cellular immune receptors detect the virus and trigger the expression of antiviral IFN molecules. These signaling molecules trigger the expression of more antiviral effector proteins, collectively referred to as interferon-stimulated genes (ISGSs). These proteins work together to cripple the virus at different stages, by inhibiting replication, blocking protein synthesis, or inducing apoptosis. Apoptosis, or cellular suicide, is regarded as an important innate antiviral defense mechanism, as premature death of virus-infected cells prevents further virus replication. Two rainbow trout cell lines, with differing susceptibility to FV3, were utilized as a model to study the innate antiviral immune responses that provide protection.

2. Findings.

2.1. *RTG-2 is more susceptible than RTgutGC to FV3-induced cell death*

FV3-induced cell death was much more extensive, and began at an earlier time-point, in RTG-2. RTG-2 exhibited lower LD_{50} values, compared to RTgutGC, indicating a greater susceptibility. Additionally, as measured by viral transcript expression, RTG-2 supported greater FV3 replication than RTgutGC.

2.2. *Mechanism of FV3-induced cell death is apoptosis*

Both FV3-infected RTG-2 and RTgutGC cultures exhibited significant apoptotic nuclei and DNA fragmentation, typical hallmarks of apoptosis.

2.3. *FV3 induces apoptosis independent of virus replication*

Pretreatment of RTG-2 cells with poly I:C significantly inhibited FV3 replication, but had no effect on FV3-induced cell death. Likewise, FV3 was successfully inactivated by UV irradiation (as measured by viral transcript expression), but UV-FV3 exhibited equal amount of cell death as wild-type (*wt*) FV3. As well, UV-FV3 induced apoptosis in both cell lines.

2.4. *FV3-induced IFN response was equally absent in both cell lines*

Despite the presence of actively replicating FV3 (as measured by viral transcript expression), no IFN or ISG response was detected in either cell lines within 18h of infection. Even at 72h, IFNs and ISGs were only detected at very low levels. As well, UV-FV3 did not induce a significantly greater IFN response in either cell line.

2.5. *Greater FV3 susceptibility may be due to intrinsic cellular features*

RTG-2 exhibited greater viral entry (as measure by levels viral DNA), than the less susceptible RTgutGC. As well, the highly susceptible RTG-2 cell line exhibits a greater metabolic/replication capacity, as compared to RTgutGC. Both features would enable RTG-2 to have a greater level of infection. As well, RTgutGC exhibited a greater level of constitutive expression of IFN and ISGs, perhaps enabling some initial protection from the virus.

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