Ranavirus Replication: Molecular, Cellular, and Immunological Events

James K. Jancovich, Qiwei Qin, Qi-Ya Zhang, and V. Gregory Chinchar

1 Introduction

Since their discovery nearly 50 years ago, our understanding of ranavirus biology has developed in two distinct stages. The first, driven by the work of Allan Granoff, his collaborators, and other investigators in Europe and the USA, took place between 1965 and 1985. During this initial period, the principal events in ranavirus replication in cell culture were elucidated through the study of *Frog virus 3* (FV3), the type species of the genus *Ranavirus* (family *Iridoviridae*). The second wave of ranavirus research began in the 1990s and continues to the present day. These studies, conducted in the USA and, increasingly, in Asia and Europe, are focused on ranavirus genes and genomes and have employed a variety of contemporary molecular approaches to ascertain the role of specific genes in viral replication. Moreover, current work has expanded beyond FV3 and has utilized additional ranavirus species as well as iridoviruses from other genera, especially the genus *Megalocytivirus*. Recent studies have identified viral genes that not only play direct structural and enzymatic roles in ranavirus replication, but also genes that likely enhance virus replication in

J.K. Jancovich
Department of Biological Sciences, California State University, San Marcos, CA 92096, USA

Q. Qin
Key Laboratory of Tropical Marine Bio-Resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China

Q.-Y. Zhang
State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China

V.G. Chinchar (✉)
Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216, USA
e-mail: vchinchar@umc.edu

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particular cellular and host environments, evade antiviral immune responses, and contribute to virulence. Ongoing studies involving the "knock out" of viral genes, "knock down" of gene function, and analysis of recombinant ranavirus proteins are providing a more complete understanding of viral gene function. Moreover, by identifying viral genes that play critical roles in virulence, these studies will provide a better understanding of protective innate and acquired immune responses in lower vertebrates and facilitate the development of effective anti-ranavirus vaccines. In addition to the biochemical and genetic studies that are the focus of this chapter, there has been an explosion of information describing the adverse impact that ranaviruses, and other vertebrate iridoviruses, have on wild and cultured ectothermic vertebrates (Duffus et al. 2015; Brunner et al. 2015). To provide a basis for understanding the molecular mechanisms of ranaviral disease, we describe below the salient events in ranavirus replication and the role of specific viral genes in this process. Although focused mainly on viral replication strategies as well as classical and contemporary methods for determining viral gene function, we also touch briefly on viral taxonomy and antiviral immune responses, two topics covered at length in other chapters within this book (Jancovich et al. 2015; Grayfer et al. 2015).

2   Ranavirus Taxonomy and Genomes

The genus *Ranavirus* is one of five genera within the family *Iridoviridae* (Table 1). The genera that comprise the family include two that infect only invertebrates, *Iridovirus* and *Chloriridovirus*, and three that target cold-blooded vertebrates, *Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus* (Jancovich et al. 2012). Whereas megalocyti- and lymphocystiviruses infect only fish, ranaviruses, despite their eponymous designation, target fish, amphibians, and reptiles. Moreover, indicative of their broad host range, some ranaviruses infect hosts from different vertebrate classes. For example, *Bohle iridovirus* (BIV) is capable of infecting both amphibians and fish (Moody and Owens 1994). Viral promiscuity is especially evident *in vitro* where ranaviruses infect cells from multiple vertebrate species, including mammals. Iridovirids, a generic designation for all members of the family, possess an icosahedral capsid that encloses a dsDNA genome. A summary of iridovirid genomic features, including abbreviated species and isolate designations, is found in Table 1. As shown therein, genomes vary in size depending upon the specific virus, and contain between 92 and 211 putative open reading frames (ORFs) (Jancovich et al. 2012). Phylogenetic analysis of a set of 26 genes conserved among all members of the family support the division of the family into four distinct groups: Ranavirus, Megalocytivirus, Lymphocystivirus, and Iridovirus/Chloriridovirus (Eaton et al. 2007). Although not distinguished phylogenetically, iridoviruses and chloriridoviruses display marked differences in the percentage of guanine and cytosine (G+C) residues, virion size, and host range. Whether these features provide sufficient grounds for the current division into two genera remain to be determined.

Early studies on ranavirus genomes focused on FV3 and showed that it possessed a linear, double-stranded DNA genome that was circularly permutated and termi-

Table 1 Iridovirus taxonomy: viral genera and species

Genus	Species[a]	Size (bp)	No. ORFs	% G+C	GenBank accession number
Iridovirus	*IIV-9*	206,791	191	31	GQ918152
	IIV-6	212,482	211	29	AF303741
Chloriridovirus	*IIV-3*	191,132	126	48	DQ643392
Lymphocystivirus	*LCDV-1*	102,653	108	29	L63545
	LCDV-C	186,250	178	27	AY380826
Ranavirus	*ATV*	106,332	92	54	AY150217
	FV3	105,903	97	55	AY548484
	EHNV	127,011	100	54	FJ433873
	ADRV	106,734	101	55	KC865735
	STIV	105,890	103	55	EU627010
	CMTV	106,878	104	55	JQ231222
	TFV	105,057	105	55	AF389451
	RGV	105,791	106	55	JQ654586
	ESV	127,732	136	54	JQ724856
	SGIV	140,131	139	49	AY521625
	GIV	139,793	139	49	AY666015
Megalocytivirus	*ISKNV*	111,362	117	55	AF371960
	RBIV	112,080	116	53	AY532606
	RSIV	112,414	93	53	BD143114
	OSGIV	112,636	116	54	AY894343
	TRBIV	110,104	115	55	GQ273492
	LYCIV	111,760	ND	ND	AY779031

[a]*IIV-9 invertebrate iridovirus type 9*, *IIV-6 invertebrate iridovirus type 6* (Chilo iridovirus), *IIV-3 invertebrate iridovirus type 3*, *LCDV-1 lymphocystis disease virus 1*, *LCDV-C lymphocystis disease virus*—China, *TFV tiger frog virus*, *ATV Ambystoma tigrinum virus*, *FV3 Frog virus 3*, *RGV Rana grylio virus*, *CMTV common midwife toad virus*, *STIV soft-shelled turtle iridovirus*, *ADRV Andrias davidianus ranavirus*, *EHNV epizootic haematopoietic necrosis virus*, *ESV European sheatfish virus*, *SGIV Singapore grouper iridovirus*, *GIV grouper iridovirus*, *ISKNV infectious spleen and kidney necrosis virus*, *RBIV rock bream iridovirus*, *RSIV red seabream iridovirus*, *OSGIV orange spotted grouper iridovirus*, *TRBIV turbot reddish body iridovirus*, *LYCIV large yellow croaker iridovirus*. Viral names in *bold italic* type indicate species recognized by the International Committee on Taxonomy of Viruses; those in standard type are either tentative species or isolates of recognized species, *ND* not determined

nally redundant, a characteristic of all viral genomes within the family *Iridoviridae* (Goorha and Murti 1982). In addition, the FV3 genome, as well as all other vertebrate iridoviruses with the exception of Singapore grouper iridovirus (SGIV), were shown to be highly methylated with each cytosine within every CpG dinucleotide methylated (Willis and Granoff 1980). While these early studies gave insight into the overall structure of ranavirus genomes, little was known about the genetic composition, coding capacity, and variations among ranavirus genomes.

As the second wave of ranavirus research built momentum, our understanding of ranavirus genomes quickly expanded. Beginning in 1997 with the sequencing of the genome for lymphocystis disease virus (Tidona and Darai 1997), complete genomic sequences were determined for a number of iridovirids, including multiple ranavirus species and isolates infecting a variety of hosts (Table 1). The availability of complete

genomic sequence information permitted analysis of overall genomic organization, protein sequence variation, and polymorphic regions among ranaviruses.

Ranavirus genomes range in size from 105 to 140 kbp, display a G+C content between 49 and 55 %, and are predicted to encode 92–139 viral proteins (Table 1). Currently, four unique genomic organizations, divisible into two groups, have been identified by dot plot and phylogenetic analyses of completely sequenced ranavirus genomes (Chen et al. 2013). Grouper iridovirus (GIV)-like ranaviruses (GIV and SGIV) comprise one group, while amphibian-like ranaviruses (ALRV) make up the second. GIV-like viruses display only short segments of genomic colinearity when compared to other members of the genus, whereas members of the ALRV group (i.e., ATV-, CMTV-, and FV3-like ranaviruses) share longer regions of colinearity (Jancovich et al. 2010; Chen et al. 2013; Mavian et al. 2012). However, among the three ALRV subgroups, inversion, deletions, and additions, have occurred that distinguish one from the other (Fig. 1).

Ranavirus genomes encode between 92 and 139 putative gene products that have been identified by detection of ORFs longer than 50 amino acids, SDS-
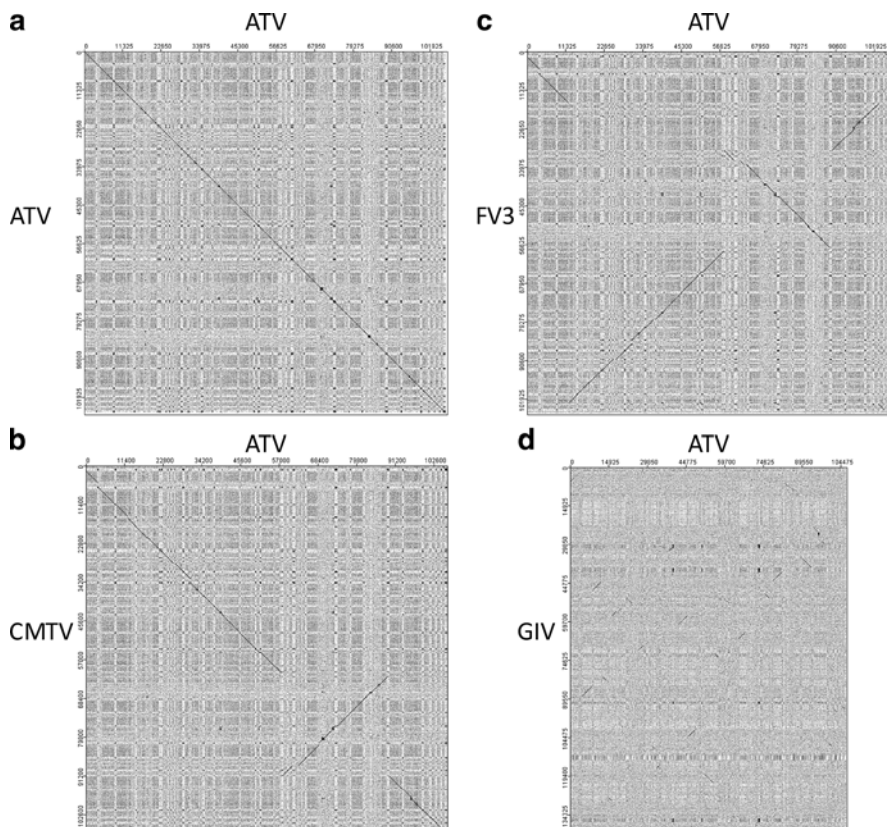

Fig. 1 Ranavirus genomic organization: Dotplot analysis of the genomic organization of representative members of the genus *Ranavirus*. The genomic sequence of ATV was compared to: (**a**) ATV; (**b**) CMTV; (**c**) FV3; (**d**) GIV. Dot plots were generated using JDotter (Brodie et al., 2004). *Lines* on the plot indicate regions of sequence similarity/colinearity

polyacrylamide gel analysis of purified virions and virus-infected cells, microarray analysis of viral transcripts, and proteomic analysis of virions (Eaton et al. 2007; Majji et al. 2009; Song et al. 2006). The functions of about a third of these genes have been inferred by similarity to other known proteins or genes. Although the roles of the remaining genes are unknown, most are homologous to genes within the family *Iridoviridae* indicating that they play important roles in viral biogenesis. All ranaviruses contain 26 core iridovirus genes as well as an additional 72 genes that are common to all members of the genus (Eaton et al. 2007). Since the latter are found only among ranaviruses, we hypothesize that by identifying their various roles in replication we may be able to identify genes that function in unique host environments and cause disease in a wide variety of hosts.

In addition to coding regions, ranavirus genomes contain palindromes, microsatellites, repeat regions, and areas of inter- and intragenic variation (Eaton et al. 2010; Jancovich et al. 2003; Lei et al. 2012b; Mavian et al. 2012; Morrison et al. 2014; Tan et al. 2004). Repeat and variable regions may serve as sites that facilitate recombination or regulate gene expression, and palindromic sequences at the 3′ end of viral messages may act as transcriptional termination signals. In addition, comparisons of closely related FV3-like viruses that vary in virulence suggest that intragenic differences, as well as variation within repeated sequences, may influence viral pathogenesis (Morrison et al. 2014).

There is growing evidence that ranaviruses encode microRNAs (miRNAs) that regulate host and viral gene expression and play a role in the evasion of host antiviral immunity. For example, 11 of the 16 novel SGIV-encoded miRNAs identified by Illumina/Solexa deep-sequencing were present and functional in SGIV-infected grouper cells when examined by stem-loop quantitative RT-PCR and luciferase reporter assays (Yan et al. 2011). One miRNA, SGIV miR-homoHSV attenuated SGIV-induced apoptosis thereby enhancing virus replication (Guo et al. 2013). Taken together, these data suggest that sequence variations among ranavirus genomes may significantly influence ranavirus host range and pathogenesis. To that end, our understanding of ranavirus genomics will expand as additional ranavirus genomes are sequenced.

3   Ranavirus Replication Strategy

In this section, we discuss virus-encoded events that play direct roles in the production of infectious virus particles, and in the following sections the impact of virus infection on host cells and the interaction between the virus and host immune system. For the most part, ranavirus replication will be reviewed using FV3 as the model, but where appropriate other ranaviruses, or even viruses from other genera within the family, will be discussed. Key events in ranavirus replication are shown schematically in Fig. 2. While the events depicted in Fig. 2 are based, for the most part, on work with FV3, it appears that all vertebrate iridoviruses replicate using essentially the same general strategy. Differences among ranaviruses, megalocytiviruses, and lymphocystiviruses may reside in how they interact with their hosts at the cellular
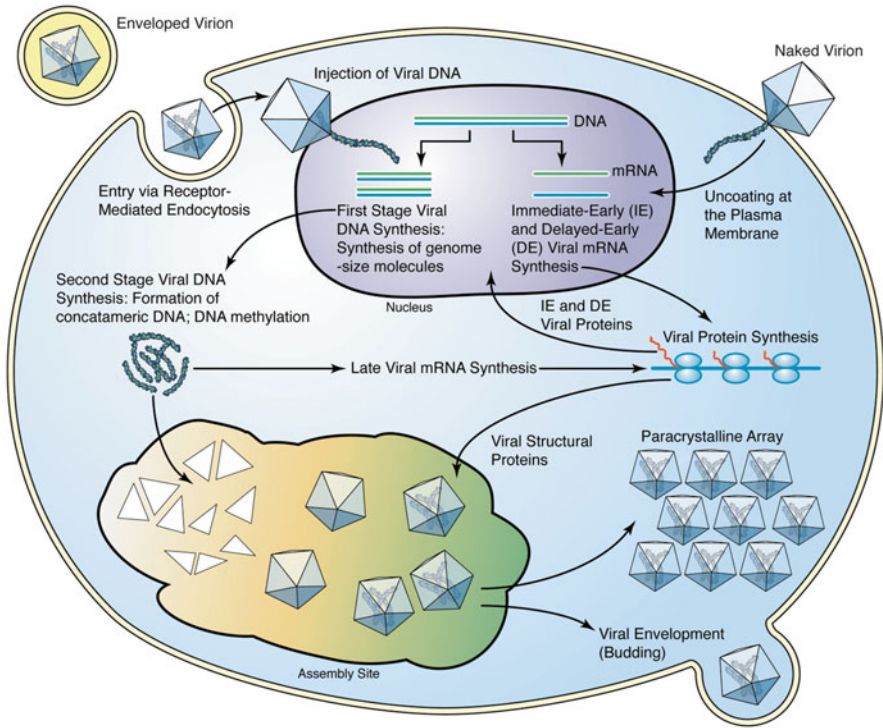
Fig. 2 Schematic diagram of ranavirus replication. Virions enter cells by one of two possible routes and initial events in virus replication (early viral transcription and the synthesis of unit length genomes) take place within the nucleus. Viral genomes are subsequently transported into the cytoplasm where they are methylated and serve as templates for concatemer formation. Viral assembly sites contain viral DNA and a number of virus-encoded proteins and serve as the loci of virion formation. Newly synthesized virions are found free within the cytoplasm or within paracrystalline arrays, and, a minority, at least *in vitro*, bud from the plasma membrane and in the process acquire an envelope

and immunological levels. Additional information on ranavirus replication strategies can be found in several comprehensive reviews (Chinchar et al. 2009, 2011; Williams 1996; Williams et al. 2005; Willis et al. 1985).

3.1 Viral Entry

Ranavirus particles are complex multilayered structures consisting, from inside out, of a core composed of the viral dsDNA genome associated with one or more virus-encoded proteins, an internal lipid membrane containing several intramembrane proteins, an icosahedral capsid composed almost entirely of a ~48 kDa major capsid protein (MCP), and (in those virions that are released by budding) a viral envelope

derived from the aforementioned plasma membrane and containing one or more virus-encoded proteins (Darcy-Tripier et al. 1984). In appearance, ranavirus particles show marked similarity to the virions of African swine fever virus (ASFV) and members of the family *Phycodnaviridae* (Tulman et al. 2009; Wilson et al. 2009). In contrast to members of most other virus families whose infectious forms consist of either enveloped virions or non-enveloped viral particles, both non-enveloped and enveloped ranavirus particles are infectious. However, the infectivity of enveloped virions appears to be greater and may be a reflection of their entry via receptor-mediated endocytosis (Braunwald et al. 1979; Ma et al. 2014). Although ranaviruses possess broad *in vitro* and *in vivo* host ranges, the identities of the viral and cellular receptor proteins are not known. Non-enveloped virions interact with the plasma membrane and uncoating is thought to take place by release of the viral DNA core into the cytoplasm. In contrast, enveloped viruses are thought to enter cells by receptor-mediated endocytosis, followed by release of non-enveloped virions into the cytoplasm. Virions are transported to the nuclear membrane and viral DNA is subsequently injected into the nucleus (Braunwald et al. 1985; Gendrault et al. 1981). The enhanced infectivity of enveloped virions may reflect the more efficient binding of viral envelope protein(s) to the cellular receptor or more efficient uptake and release achieved by receptor-mediated endocytosis. In addition to these mechanisms, entry may also involve interaction between virions and caveolae (Guo et al. 2011b, 2012; Jia et al. 2013; Wang et al. 2014).

3.2 Nuclear Events

The entry of viral cores into the nucleus sets the stage for the opening acts of ranavirus replication: the synthesis of early viral transcripts and the generation of unit-length copies of the viral genome. As with other DNA viruses, such as herpesviruses, ranaviruses utilize host RNA polymerase II to transcribe viral messages (Goorha 1981). However, in contrast to herpesviruses, ranavirus transcription requires the presence of one or more virion-associated proteins and, as a result, deproteinized viral genomic DNA cannot be transcribed and is not infectious (Willis et al. 1990; Willis and Granoff 1985; Willis and Thompson 1986). The first viral transcripts synthesized are termed "immediate-early" (IE) and among their gene products are one or more proteins that are required for the synthesis of a second class of early transcripts, designated "delayed early" (DE) (Willis and Granoff 1978). As a group, IE and DE transcripts likely encode regulatory and virulence proteins as well as key catalytic proteins such as the large and small subunits of the viral homolog of RNA polymerase II (vPOL-II) and the viral DNA polymerase (Majji et al. 2009). Following microarray analysis of FV3 gene expression, 33 IE and 22 DE transcripts, corresponding to approximately half of the FV3 coding potential, were identified (Majji et al. 2009). Similar levels of IE and DE gene products were seen with other ranaviruses (Chen et al. 2006; Teng et al. 2008). Host POL-II is responsible for the transcription of IE (and perhaps DE) viral mRNAs, whereas, as described below, vPOL-II

directs transcription of late viral messages. As with host transcripts, viral transcripts are capped and methylated, but unlike cellular messages ranavirus mRNAs lack poly[A] tails and introns.

3.3 Cytoplasmic Events

After its transport into the cytoplasm, viral DNA is methylated by a virus-encoded cytosine-specific DNA methyltransferase (DMTase) (Willis et al. 1984; Willis and Granoff 1980). Each cytosine within CpG dinucleotides is targeted leading to the methylation of 20–25 % of cytosines and resulting in the highest level of DNA methylation seen among vertebrate viruses. However, despite this extraordinary level of methylation, the precise role that methylation plays in the viral life cycle is not known. Methylation has been suggested to protect viral genomic DNA from attack by a virus-encoded restriction-modification enzyme that targets unmethylated host DNA (Kaur et al. 1995). Alternatively, methylation is thought to prevent recognition of viral genomic DNA by pattern recognition receptors such as TLR-9 and thus block activation of an immune response (Hoelzer et al. 2008; Krug et al. 2001, 2004). FV3 infection in the presence of 5′-azacytidine (azaC), a methylation inhibitor, does not affect viral transcription or translation, but results in modest decreases in DNA synthesis and marked reductions in viral yields (Goorha et al. 1984). In support of a protective role for DNA methylation, gradient analysis detected single-stranded DNA breaks in viral DNA synthesized in the presence of azaC. These breaks were thought to block DNA packaging and the development of infectious virions.

Within the cytoplasm, unit-length genomes serve as templates for the second stage of viral DNA synthesis which results in the formation of large concatemers containing ten or more interlaced copies of the viral genome (Goorha 1982). Using a collection of temperature-sensitive (*ts*) mutants, two complementation groups, involving first stage and second stage DNA synthesis, have been linked to viral DNA synthesis (Chinchar and Granoff 1986; Goorha and Dixit 1984; Goorha et al. 1981). Since sequence analysis identified only a single viral DNA polymerase gene, it is likely that one complementation group encodes a viral DNA polymerase that functions in both events, whereas the second may encode a viral protein needed for the transport of viral DNA from the nucleus to the cytoplasm or some other function related to concatemer formation.

3.4 Virus Assembly

Virion formation takes place within morphologically distinct areas of the cytoplasm referred to as virus assembly sites or virus factories. Assembly sites are electron lucent areas of the cytoplasm that are devoid of cellular organelles (Murti et al. 1985, 1988; Zhang and Gui 2012). Unlike autophagosomes, ranavirus
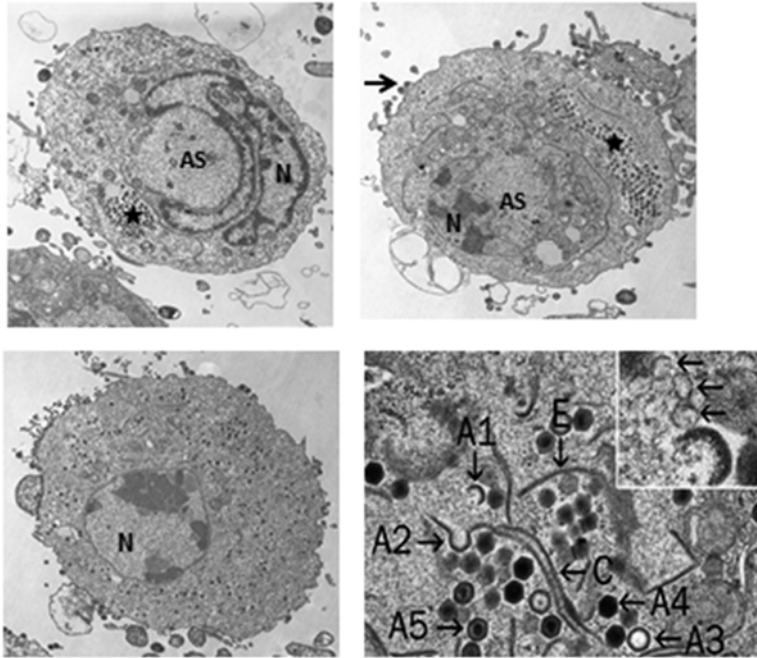
Fig. 3 Transmission electron micrographs of FV3-infected FHM cells. *Upper left* and *right panels* show typical virus-infected cells with nuclei (N) showing evidence of chromatin condensation, well-defined viral assembly sites (AS); intracytoplasmic paracrystalline arrays (*asterisk*), and virions budding from the plasma membrane (*arrow*). *Lower left panel* shows a virus-infected cell in which virions are scattered throughout the cytoplasm. *Lower right panel* is an enlargement of a viral assembly site showing virions in various stages of assembly. Full (A4 and A5) and empty (A3) viral particles are shown as well as two possible intermediates (A1 and A2) and two aberrant forms (C and E). The *inset* indicates membranes (*arrows*), possible originating from the ER, that play a role in virion morphogenesis

assembly sites are not enclosed within membranes, but are encased by intermediate filaments and surrounded by mitochondria and ribosomes (Fig. 3). Both early and late viral proteins are associated with assembly sites as well as presumably concatemeric viral DNA. Viral assembly sites were detected in the presence of an antisense morpholino oligonucleotide (asMO) that blocked late gene expression (Sample 2010; Sample et al. 2007). These results suggest that late viral protein expression is not required for assembly site development and strengthen an earlier study suggesting that early proteins are sufficient for assembly site formation (Chinchar et al. 1984; Sample 2010).

The specific steps required for the formation of infectious virus particles are still poorly understood. Genetic analysis of *ts* mutants identified 12 complementation groups that synthesized ostensibly all viral proteins and viral DNA yet were unable to generate infectious particles (Chinchar and Granoff 1986). Transmission electron microscopic (TEM) analysis of these complementation groups identified several *ts*

mutants in which virion structures did not form, whereas in others apparently complete, but noninfectious, particles were present (Purifoy et al. 1973; Sample 2010). Clearly multiple structural and catalytic proteins must be involved in the formation of infectious particles. By analogy to ASFV (Rouiller et al. 1998; Tulman et al. 2009), virion assembly may involve initial binding of a myristoylated viral protein (e.g., FV3 ORF53R) to fragments of the endoplasmic reticulum, followed by binding of the MCP on its opposite face (Whitley et al. 2010). Continued addition of 53R and MCP to membrane fragments is thought to result in the formation of crescent-shaped structures that eventually associate with viral DNA to form the virion. Consistent with this model, intermediates along the pathway to virion formation can be detected by transmission electron microscopy (Fig. 3).

The process by which viral DNA is encapsidated has not yet been determined. Although encapsidation of a "headful" of viral DNA explains the existence of circularly permuted, terminally redundant DNA genomes (Goorha and Murti 1982), it is not known if ranavirus DNA enters through a unique virion portal as seen with some dsDNA viruses or by engulfment of viral DNA by the enlarging icosahedron (Cardone et al. 2007; Chang et al. 2007). Moreover, although concatemeric DNA is thought to serve as the source of the DNA that is ultimately packaged into virions, how complete copies of the viral genome are resolved from concatemeric DNA is not known. Complete viral particles are present within viral assembly sites, the cytoplasm, paracrystalline arrays, and in association with the plasma membrane (Fig. 3). In cultured cells, most virions remain cell-associated and are released as naked particles following cell lysis. However, a variable number of particles bud from the plasma membrane and in the process acquire an envelope. The factors that determine whether a given particle remains cell-associated or enveloped are not known. Freeze-fracture electron microscopy indicates that mature FV3 particles display a Russian doll-like structure with a knobby, spherical core composed of viral DNA and associated proteins enclosed within a capsid composed primarily of a 48 kDa MCP (Darcy-Tripier et al. 1984; Devauchelle et al. 1985). Electron microscopy of Chilo iridescent virus (genus *Iridovirus*) has identified at least three minor proteins (designated finger, zip, and anchor) associated with the virion, but has not linked them with specific viral ORFs (Yan et al. 2009). In addition to the major and minor structural proteins, it is thought that other viral proteins, e.g., proteins responsible for translational shut-off and the transactivation of IE transcription, are also associated with mature virions. As described in Grayfer et al. (2015), these latter proteins may also contribute to virulence

In contrast to early viral messages that are, at least initially, transcribed in the nucleus by host POL-II, late viral messages such as those encoding the MCP and other virion-associated proteins may be transcribed within the cytoplasm by vPOL-II, a virus-encoded RNA polymerase whose two largest subunits are homologous to the corresponding units of cellular RNA polymerase II (Tan et al. 2004; Sample et al. 2007). As discussed below, knock down studies using an asMO targeted to vPOL-IIα resulted in a marked reduction in the synthesis of all

late proteins. At this time, it is not known if vPOL-IIα and vPOL-IIβ function as a minimal, two-subunit RNA polymerase, or whether these two viral subunits associate with other cellular or viral proteins to form the functional enzyme. In addition to the requirement for functional vPOL-II, late transcription is also dependent upon ongoing viral DNA synthesis as *ts* mutants defective in viral DNA synthesis or treatment of FV3-infected cells with DNA synthesis inhibitors such as phosphonoacetic acid (PAA) or cytosine arabinoside (araC) also inhibit late viral gene expression (Chinchar and Granoff 1984, 1986). In cell culture, FV3 replication takes place within a 12–24 h period. Early messages are detectable within the first 4 h; late transcripts and proteins, e.g., the MCP, by 8 h post infection; and virus particles by 8–12 h. However, the kinetics of viral replication are influenced by the multiplicity of infection, temperature, and metabolic state of the host cell. As stated above, although all vertebrate iridoviruses are thought to replicate using the same general strategy, differences among them likely influence how they interact with their hosts at the cellular and immunological levels and impact replication *in vivo*.

4 Impact of Virus Infection on the Host Cell

4.1 Cell Death: Necrosis, Apoptosis, and Parapoptosis

Ranavirus infections result in the rapid inhibition of host DNA, RNA, and protein synthesis and culminate in rapid cell death (Goorha and Granoff 1979; Raghow and Granoff 1979). Interestingly, both infectious and noninfectious virions (i.e., heat- or UV-inactivated virus) trigger the turn off of cellular transcription and translation indicating that the "shut-off protein" is virion-associated (Cordier et al. 1981; Raghow and Granoff 1979). Furthermore, virus-infected cells undergo apoptosis as indicated by chromatin condensation and the fragmentation of cellular DNA (Chinchar et al. 2003). Apoptosis appears following either productive infection or infection by inactivated virions and may result from the prior inhibition of host cell macromolecular synthesis or the activation of protein kinase R (PKR). Ranavirus-induced apoptosis is dependent upon caspase activation and can be prevented by caspase inhibition (Chinchar et al. 2003).

In addition to apoptotic death, SGIV, the most phylogenetically distant member of the genus *Ranavirus*, triggers different forms of programmed cell death depending upon the cell type. In cultured grouper cells, a natural host for SGIV, infection triggers a nonapoptotic form of programmed cell death designated parapoptosis. Parapoptosis is characterized by the appearance of cytoplasmic vacuoles, distended endoplasmic reticulum, and the absence of DNA fragmentation, apoptotic bodies, and caspase activation. In contrast, in cells cultured from a non-host fish, e.g., fathead minnow (FHM), SGIV induces the typical form of apoptosis characterized by

caspase activation and DNA fragmentation. Furthermore, disruption of mitochondrial transmembrane potential and externalization of phosphatidylserine (PS) are not detected in grouper cells but are seen in FHM cells after SGIV infection (Huang et al. 2011a). Similar results are obtained in GIV-infected grouper kidney cells. However, whether all ranaviruses modulate the survival of host and non-host cells differently requires further study (Pham et al. 2012). In host fish, mitogen-activated protein kinase (MAPK) cascades are involved in SGIV-induced parapoptosis, including extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun N-terminal kinase (JNK) signaling (Huang et al. 2011a, b). Moreover, transcription of grouper immune genes, such as interferon regulatory factor 1 (IRF1), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α) were regulated by JNK, while only TNF-α was regulated by p38 MAPK. It is proposed that the JNK pathway is important for SGIV replication and modulates the inflammatory responses during virus infection (Huang et al. 2011b). Interestingly, activation of similar genes was seen following infection of FHM cells with FV3 (Cheng et al. 2014).

4.2 Host Shut-Off and the Selective Expression of Viral Gene Products

Despite the rapid inhibition of host macromolecular synthesis, viral DNA replication, transcription, and translation remain unaffected and abundant levels of infectious viral particles are produced within 24 h or less (Willis et al. 1985). The maintenance of viral protein synthesis in the face of the marked inhibition of cellular translation is likely the result of several factors. In the first place, a viral homolog of eukaryotic translational initiation factor 2α (vIF2α) acts as a pseudosubstrate, binds PKR, and prevents the phosphorylation and subsequent inactivation of cellular eIF2α (Jancovich and Jacobs 2011). In addition, abundant levels of highly efficient viral messages outcompete host transcripts for access to the cellular translational machinery (Chinchar and Yu 1990a, b). In contrast, it is not clear how ranaviruses selectively inhibit host transcription. Perhaps ranaviruses target host POL-II as infection progresses, and rely upon vPOL-II to synthesize viral transcripts in the cytoplasm. If this model is correct, it would be instructive to determine whether early genes continue to be synthesized at later times using vPOL-II to catalyze their synthesis. As with transcription and translation, host DNA synthesis is also blocked. However, inhibition of host DNA synthesis is thought to be a consequence of the earlier blocks to cellular protein and RNA synthesis. Furthermore, a virus-encoded endonuclease, perhaps part of a restriction-modification system, is thought to degrade host DNA. Not only will this negatively impact host transcription, but the resulting nucleotides may also be re-utilized for the synthesis of viral DNA (Feighny et al. 1981; Kaur et al. 1995).

5 Interplay Between Host and Virus Determines the Outcome of Infection at the Organismal Level

Virulence is a function of several factors but the two that are likely most important involve those that enhance virus replication and those that block host immune defenses. Large DNA viruses such as herpesviruses and poxviruses encode numerous genes that fulfill these functions and preliminary findings among ranaviruses suggest that they are no different (Eaton et al. 2007). In the case of viral genes that enhance replication, prime examples are viral homologs of the large and small subunits of ribonucleotide reductase (RR). Both Vaccinia virus (VACV) and herpes simplex virus 1 (HSV1) encode RR genes. VACV RR2 subunits form functional complexes with host RR1 and provide sufficient dNTPs for viral replication (Gammon et al. 2010). Likewise HSV and pseudorabies virus-encoded RR convert ribonucleoside diphosphates into the corresponding deoxyribonucleotides and play key roles in viral DNA synthesis by maintaining dNTP pool sizes (Conner et al. 1994a, b; Daikoku et al. 1991). Consistent with this key role in viral biogenesis, mutants defective in RR expression are avirulent *in vivo* (de Wind et al. 1993). However, in addition to their role in dNTP synthesis, HSV-1 and HSV-2 RR1 subunits also protect cells from apoptosis (Chabaud et al. 2007; Langelier et al. 2002). In contrast to the α- and β-herpesviruses, the RR1 subunit of murine cytomegalovirus is catalytically inactive and does not play a role in increasing dNTP pool sizes. Rather it has evolved a new function in which it inhibits RIP1, a cellular adaptor protein, and blocks signaling pathways involved in innate immunity and inflammation (Lembo and Brune 2009). Other viral genes that contribute to enhanced replication encode viral homologs of dUTPase and thymidine kinase (Eaton et al. 2007). It is likely that the RR1, RR2, dUTPase, and thymidine kinase homologs encoded by FV3 and other ranaviruses play similar critical roles in viral replication.

In addition to genes that enhance viral replication, viruses also encode genes that inhibit or counteract host immune responses. Poxviruses contain over two dozen genes whose sole function is to block one or more aspects of innate or acquired immunity (Seet et al. 2003), and it has been estimated the perhaps half of the genes encoded by human cytomegalovirus may be involved in immune evasion (Eberhardt et al. 2013). For example, poxviruses encode decoy receptors for IFNα/β, IFN-γ, IL-1β, IL-18, various chemokines, and complement that bind their cognate targets and downregulate their effector functions (Johnston et al. 2005; Johnston and McFadden 2003, 2004; Seet et al. 2003). In addition, virus-encoded miRNAs may also play critical roles in blocking host responses or controlling viral replication (Babu et al. 2014; Hook et al. 2014; Pavelin et al. 2013).

Sequence analysis of the genome of FV3 and other vertebrate iridoviruses has identified several putative genes that may play roles in blunting host immunity. These putative immune evasion proteins include the aforementioned viral homolog of eIF-2α (vIF-2α), a viral homolog of RNAse III, a virus-encoded CARD (caspase activation and recruitment domain)-containing protein, a viral homolog of steroid

dehydrogenase (β-HSD), a viral Bcl-2 homolog, and one or more TNFR homologs (Huang et al. 2013b; Lin et al. 2008; Tan et al. 2004). In addition to these putative immune effectors, there are other viral genes that are unique to specific viruses or genera and may represent proteins that act only within specific host species, tissues, or cells. For example, there are a dozen open reading frames that are found only among ranaviruses that might encode unique polypeptides that enhance viral replication or impair immunity within specific ectothermic hosts (Eaton et al. 2007). However, the challenge of identifying virulence proteins by similarity searches is considerable. Because the level of similarity between viral and cellular homologs, even in mammalian systems, may be low, identification of a viral protein as a potential inhibitor of a specific function is far from certain unless key sequence motifs are conserved. For a given viral protein, conclusive evidence for a specific role in virulence will require using the genetic and biochemical approaches discussed below.

6   Antiviral Immunity

Because host antiviral immunity is discussed in Grayfer et al. (2015), only a brief summary will be presented here. The host immune response to ranavirus infection has been productively examined using a model pairing FV3, the best characterized ranavirus at the molecular level, with *Xenopus laevis*, the amphibian with the most fully characterized immune system (Robert 2010). Robert and colleagues have shown that infection of immunocompetent adult frogs is usually limited to the kidney and resolves with a few weeks with minimal mortality (Gantress et al. 2003). In contrast, tadpoles, which are naturally defective in MHC I expression, and immunocompromised adult animals, are susceptible to infection and display considerable morbidity and mortality (Tweedell and Granoff 1968; Gantress et al. 2003; Robert et al. 2005). In immunodeficient or immunocompromised *Xenopus*, infection begins in the kidney, but becomes systemic and spreads to multiple organs including the liver, gastrointestinal tract, and skin (Gantress et al. 2003). Consistent with observations that fish could be protected from ranavirus- and megalocytivirus-induced disease by vaccination with inactivated virions or a DNA vaccine (Caipang et al. 2006a, b; Ou-yang et al. 2012), antibody responses were shown to play a protective role in FV3 infections (Maniero et al. 2006). Likewise, cell-mediated immunity was shown to play an important role in protection from FV3-induced disease (Morales and Robert 2007). Recently macrophages were shown to be susceptible to FV3 infection (Morales et al. 2010). Although macrophages likely play a critical role in immunity, their infection may have two untoward effects: eliminating their ability to process and present viral antigens and providing a source of persistently infected cells that may facilitate the maintenance of virus in a population.

Although their roles in survival have yet to be determined, it is likely that multiple cellular genes that play various roles in antiviral immunity and virus replication are induced following ranavirus infection. Consistent with this suggestion, Cheng et al. (2014) recently examined the response of FHM cells to infection with

either wild type FV3 or a knock out mutant lacking the ranavirus vIF-2α and observed the induction of multiple immune-response genes at the transcriptional level including IFN, IL-8, GILT, IRF-3. Likewise, vaccination of groupers with inactivated SGIV induced the expression of numerous immune-related genes including Mx1, ISG15, IL-8, IL-1β, and MHC I/II indicating that the immune response is conserved among different fish species and similar to that seen in mammals (Ou-yang et al. 2012).

Recently, Grayfer et al. (2014) reported the cloning and sequencing of interferon from *Xenopus laevis* (*Xl*-IFN). They showed that recombinant *Xl*-IFN protected A6 amphibian cells from FV3 infection and transiently protected tadpoles. As expected, FV3-infected adults synthesized *Xl*-IFN sooner and in higher amounts than tadpoles. Surprisingly, despite the more robust response of adults, viral burdens were greater in adults than tadpoles, although, as reported above, their long-term survival was higher. Moreover, although *Xl*-IFN markedly impaired virus replication, it did not prevent death. Control, vector-treated tadpoles survived for an average of 26 days and experienced 90 % mortality by day 32, whereas r*Xl*-IFN-treated animals displayed a mean time to death of 36 days, with 90 % mortality seen by day 40. These results suggest that FV3 is more pathogenic to tadpoles than formerly thought and even low viral titers could cause extensive damage to internal organs and ultimately lead to death. In support of a protective role for IFN, transfection of a vector expressing turtle IFN-γ into cultured STA cells was shown to result in an approximately 90 % reduction in viral gene expression and a tenfold reduction in the yield of Softshell turtle iridovirus (Fu et al. 2014). Based on the results shown above, host immune responses seen after ranavirus infection are broadly similar to those seen in fish following infection with other viruses (Verrier et al. 2011).

7 Determination of Viral Gene Function

With the broad outlines of ranavirus replication known, the current challenge lies in elucidating the function of specific viral genes as this may provide targets for effective chemotherapy or aid in vaccine development. Determination of ranavirus gene function has accelerated markedly in the last 10 years as a variety of powerful molecular approaches have been brought to bear. These contemporary approaches, as well as more classical methodologies, are discussed below.

7.1 Biochemical and Genetic Approaches

The earliest attempts at identifying ranavirus gene function relied upon a combination of genetic and biochemical approaches. In the latter, various inhibitors were employed to block specific aspects of viral replication. For example, cycloheximide (CHX) blocked global protein synthesis and limited viral transcription to IE

mRNAs, whereas flurophenylalanine (FPA), phosphonoacetic acid (PAA), and cytosine arabinoside (araC) permitted the expression of only IE and DE viral gene products (Goorha and Granoff 1979; Willis et al. 1985). It is thought that FPA is incorporated into one or more viral proteins required for late gene expression (e.g., the viral homologs of the two largest subunits of RNA polymerase II) and inhibits function by altering protein conformation. PAA and araC block viral DNA synthesis, an event that is required for full late gene expression, by specifically (e.g., PAA) or nonspecifically (e.g., araC) inhibiting viral DNA polymerase activity. The requirement for ongoing DNA replication for full late gene expression may reflect either the need for an increased number of, or conformationally altered, DNA templates.

The role of host RNA polymerase II in the transcription of early, but not late, viral mRNA was demonstrated using α-amanitin, an inhibitor of host RNA polymerase II (Goorha 1981). When α-amanitin was added before or at the time of infection, it blocked all viral gene expression. However, when added at late times, it had no effect on viral functions suggesting that host RNA polymerase II was only required at the beginning phase of infection (Goorha 1981). The critical role of DNA methylation in viral replication was explored using the methylation inhibitor, 5′-azacytidine (azaC). In the presence of azaC, viral RNA and protein synthesis are not compromised, and viral DNA synthesis was only modestly affected (Goorha et al. 1984). However, in the presence of azaC, newly synthesized viral DNA lacked methylated cytosine residues and experienced single-stranded breaks. As a result, viral yields were reduced by more than a 100-fold.

Although the above studies successfully elucidated the roles of several virus-encoded proteins, because the inhibitors targeted a limited number of viral gene products (e.g., viral DNA polymerase, viral DNA methyltransferase), they were able to identify the roles of only a few of the approximately 100 putative FV3 ORFs. Attempts at identifying viral genes and their functions using traditional genetic approaches focused on the generation and characterization of drug-resistant (PAA[R] and azaC[R]) and temperature-sensitive (*ts*) mutants. Characterization of an azaC-resistant mutant linked a 26 kDa protein to DMTase activity (Essani et al. 1987); studies using the PAA-resistant mutant confirmed that the drug target was the viral DNA polymerase (Chinchar and Granoff 1984). Temperature-sensitive mutants identified (via complementation analysis) 19 genes essential for viral replication (Chinchar and Granoff 1986; Naegele and Granoff 1971; Purifoy et al. 1973). These included mutants defective in RNA synthesis (five complementation groups) and viral DNA synthesis (two complementation groups). Analysis of the two DNA-deficient complementation groups supported the concept of two stages of DNA synthesis, i.e., the synthesis of unit size genomes within the nucleus, and the formation of large concatemers in the cytoplasm (Goorha and Dixit 1984; Goorha et al. 1981). The presence of at least five complementation groups displaying defects in late viral gene expression likely reflect mutations targeting the large and small subunits of viral RNA polymerase as well as other gene products required for late mRNA synthesis, e.g., transcription elongation factor SII.

The majority of *ts* mutants (12 complementation groups) synthesized both early and late viral proteins and viral DNA, but failed to form infectious particles at the nonpermissive temperature (Chinchar and Granoff 1986). This finding indicated that ranavirus particle assembly was not simply a concentration dependent event in which the MCP was the major, if not only, component. Electron microscopy showed that with some mutants outwardly mature but noninfectious virions were formed, whereas with others virion formation was aborted (Sample 2010). Given recent studies indicating that multiple proteins make up mature iridovirus virions (see above), it is likely that these defects reflect mutations in either critical structural proteins (e.g., MCP, 53R, anchor, finger, and zip proteins), putative scaffold proteins required for virus assembly, or in one or more virion-associated proteins required for the initiation of replication (e.g., the putative transactivator of IE transcription).

Attempts to map the genomic positions of *ts* mutants have been challenging. Although the relative positions of a number of *ts* mutants have been determined based on recombination frequencies (Chinchar and Granoff 1986), physical assignment of specific mutations to defined restriction fragments, as was done with herpesviruses and poxviruses, was unsuccessful. The inability of plasmids bearing ranavirus DNA to rescue *ts* mutants is thought to be due to the degradation of input plasmid DNA by a virus-encoded endonuclease that targets unmethylated plasmid DNA. Fortunately, with complete sequencing of ATV, FV3 and other ranavirus genomes, alternative approaches for determining gene function have become available.

7.2 Knock Down Strategies: Antisense Morpholino Oligonucleotides and RNA Interference

Determination of the complete nucleotide sequence of FV3 and other ranavirus genomes has opened up the possibility of directly ascertaining gene function by targeting specific viral genes. Both knock down (KD, described here) and knock out (KO, described in the following section) have been used successfully. In the former, FV3 gene function was inhibited (i.e., knocked down) using either antisense morpholino oligonucleotides (asMOs) or small, interfering RNAs (siRNAs) (Sample et al. 2007; Whitley et al. 2010, 2011). asMOs are oligonucleotides (~25 nucleotides in length) that are complementary to regions upstream of, or directly surrounding, the initiating AUG codon. They downregulate gene expression by blocking ribosomal movement ("scanning") and inhibiting protein synthesis (Hudziak et al. 2000; Summerton and Weller 1997; Summerton 2007). siRNAs are small (~22 nucleotide) double-stranded molecules. Following their incorporation into an RNA-induced silencing complex, the strand complementary to the target message binds the target message leading to either RNA degradation or translational inhibition (Hannon 2002). In contrast to asMOs, siRNAs may bind within either coding or non-coding regions. Although algorithms exist to predict which sequences make effective siRNAs, they are not definitive and experimental validation of potential siRNAs must be carried out to ensure successful inhibition.

Antisense morpholino oligonucleotides have been used successfully to target several FV3 genes, including those encoding the MCP, the 18K immediate early protein, vPOL IIα, and 53R, a putative myristoylated membrane protein, as well as a SGIV-encoded histone-binding protein (Sample et al. 2007; Tran et al. 2011; Whitley et al. 2010, 2011). KD was verified by the absence or marked reduction of the target protein following SDS-PAGE or immunoblotting, and its effect on virus replication was monitored by transmission electron microscopy and determination of viral yields. KD of MCP and 53R resulted in marked reductions in the abundance of the target proteins, but had little to no effect on non-targeted proteins. Inhibiting the synthesis of MCP and 53R resulted in ~90 % reductions in virus yield and the appearance, respectively, of atypical elements (MCP) and granular particles (53R) within viral assembly sites. It is likely that a marked reduction in critical structural elements resulted in the accumulation of aberrant structures. In contrast to the above, KD of vPOL IIα resulted in a global reduction in late protein synthesis and the absence of all structural elements within viral assembly sites. KD of the 18K IE protein only affected the target protein and had no adverse impact on virus yield. These results indicate that whereas MCP, 53R, and vPOL IIα are essential proteins required for replication *in vitro*, 18K is nonessential for replication in cultured FHM cells (Sample et al 2007; Whitley et al. 2010).

Attempts to extend these studies to other viral gene products were stymied by the inability to detect the loss of the targeted protein by either SDS-PAGE or immunoblotting. Nevertheless, we observed FV3 yields ranging from 8 % (ORF 41R) to 43 % (ORF 95R) of control following exposure to asMOs targeting ORF 2L (putative membrane protein), ORF 9L (NTPase/DEAD/H helicase), ORF 41R (an unknown protein, mol wt 129 kDa), ORF 32R (Neurofilament triplet H1 protein), ORF 38R (RRα), ORF 57R (Ser/Thr kinase), ORF 80L (Ribonuclease III-like protein), ORF 91R (46 kDa, immediate early protein), and ORF 95R (DNA repair protein, RAD2) (Whitley et al. 2011; VGC, unpublished observations). Partial yield reductions may reflect the fact that the targeted viral protein supplements an existing host function (e.g., ribonucleotide reductase) and may not be absolutely required for replication in cultured cells. Alternatively, a partial reduction may be due to incomplete KD. Moreover, the inability to confirm KD by SDS-PAGE may be due to either co-migration of the target protein with a more abundant protein of the same size (as was the case with 53R, Whitley et al. 2010) or its presence at only low levels in infected cells (Whitley et al. 2010). Although immunoblotting may be useful in identifying co-migrating or low abundance proteins, antibodies for detecting specific viral proteins are not readily available. Taken together KD studies have the potential for identifying three classes of viral proteins: (1) "essential" proteins that are absolutely required for virion production, e.g., the MCP and vPOL-IIα; (2) "efficiency" proteins that enhance virus replication in certain environments, e.g., viral homologs of ribonucleotide reductase, but that are not absolutely required for replication; and (3) "immune evasion" proteins that target innate and acquired components of the host antiviral immune response, e.g., viral homologs of eukaryotic translational initiation factor 2α (vIF-2α) or β-hydroxysteroid dehydrogenase (β-HSD).

In addition to KD studies using asMOs, a limited number of studies have been conducted using siRNAs to silence viral gene expression (Dang et al. 2008; Kim et al. 2010; Whitley 2011; Xie et al. 2005). siRNA-mediated KD of FV3 MCP, vPOL-IIα, and the virus-encoded DNA methyltransferase markedly reduced levels of the cognate messages leading to 90 % or greater reductions in virus yields, and showed little to no evidence of virion formation by transmission electron microscopy (Whitley et al. 2011; Xie et al. 2005). However, in contrast to KD mediated by asMOs, which could be detected following infection at multiplicities of infection (MOI) of 10 PFU/cell or higher, siRNA-mediated KD was only seen when MOIs of 0.01–0.1 were used. At higher inputs (i.e., MOI of 1–10), virus yields were not reduced by treatment with siRNAs. Although the reason for this inability is unclear, it is possible that ranaviruses, as do some other viruses, encode a gene product that blocks RNA interference (RNAi) perhaps by binding dsRNA and preventing its ability to form RNA silencing complexes.

Collectively, studies using siRNA and asMOs to inhibit the expression of specific viral genes have been extremely useful for elucidating viral gene function and determining whether a given gene is "essential" for replication *in vitro*. However, for viral genes that impact humoral and cell-mediated aspects of host immunity, siRNA and asMO approaches may not be suitable because the function of the targeted gene may only be required *in vivo*. For this reason, studies of putative virus-encoded immune evasion genes may be better suited for the knock out studies discussed below.

7.3 Knock Out Mutants

Until recently, ranavirus research has been limited by an inability to achieve recombination between an introduced plasmid and an infecting viral genome. This difficulty prevented the physical mapping of *ts* mutants by marker rescue and the generation of recombinant viruses. Recently, methodologies utilizing efficient selection techniques capable of isolating rare recombinants have been developed. For example, recombinant SGIV, expressing enhanced green fluorescent protein (EGFP) fused to the envelope protein VP55, was constructed and used to evaluate the dynamics of viral replication (Huang et al. 2011c). Likewise, ranaviruses bearing drug resistance genes have been introduced into ATV, Rana grylio virus (RGV), and FV3 (see below). Thus, despite initial difficulties in generating recombinant ranaviruses, techniques based on the selection of fluorescent or drug-resistant viruses have emerged which allow their efficient isolation.

The first recombinant ranavirus was generated in BIV using homologous recombination to introduce the neomycin resistance gene under the control of the ICP18 promoter and the adult globin gene from *Bufo marinus* under the control of the promoter for the viral MCP into the vIF-2α locus (Pallister et al. 2007). While not focused on characterizing the function of the vIF-2α gene, the technology developed in this study facilitated the development of protocols for generating recombinant ranaviruses and confirmed the nonessential nature of the vIF-2α gene product.

Building on this study, knock-out (KO) and knock-in (KI) mutants have been generated in ATV, FV3, and RGV (Chen et al. 2011; He et al. 2012b; Huang et al. 2011c; Jancovich and Jacobs 2011). The first step in the generation of KO ranaviruses involves constructing a plasmid that contains a selectable marker flanked by sequences up- and downstream from the targeted gene. Cells are infected with wild type virus and subsequently transfected with the recombination construct. Homologous recombination within the flanking regions surrounding the selectable marker results in replacement of the targeted gene with a gene encoding the selectable marker. Recombinant viruses are isolated by their ability to replicate in the presence of the specific inhibitor (e.g., neomycin or puromycin) or by identifying fluorescent plaques, in those cases in which the green fluorescent protein (GFP) gene was introduced. Since viral and cellular growth are sensitive to neomycin and puromycin treatment, genes that confer resistance to these antibiotics have been used to select successfully KO ranaviruses (Jancovich and Jacobs 2011; Chen et al. 2011). In addition, GFP has been used as a selectable marker (Huang et al., 2011c). Once isolated, characterization of the KO mutant allows gene function to be determined by changes in phenotype. For example, replication of vIF-2α KO mutant *in vitro* was not significantly altered indicating that vIF-2α is a nonessential gene (Chen et al. 2011; Jancovich and Jacobs 2011; Pallister et al. 2007). However, when frogs or salamanders were infected with the vIF-2α KO mutant, a reduction in host mortality was observed suggesting that vIF-2α played an important role *in vivo*. In addition to the vIF2α homologue, the viral 18K immediate early gene has also been targeted (Chen et al. 2011). Deletion of the FV3 18K gene had little impact on viral replication *in vitro* but resulted in lower mortality in infected tadpoles, again suggesting that this gene was contributing to viral virulence. Preliminary characterization of two additional FV3 KO mutants targeting vCARD and βHSD suggested that both were nonessential for replication *in vitro* (J Robert and G Chen, University of Rochester, and VGC, unpublished observations). Collectively, these studies show the power of KO mutants in ascertaining the role of "nonessential" viral genes.

7.4 Conditionally Lethal Mutants

Because loss of an essential gene eliminates a virus's ability to replicate, KO strategies can only be directed against nonessential genes. There are two potential solutions to this impasse: (1) the construction of complementing cell lines that expresses the deleted gene product in *trans* and thus allow virus replication, and (2) the generation of conditionally lethal (*cl*) mutants that fully express the targeted gene in the presence of the appropriate inducer (e.g., IPTG or tetracycline), but show little to no expression of the target gene in the absence of the inducer. Conditional lethal mutants have been constructed in African swine fever virus (ASFV, family *Asfarviridae*) and vaccinia virus (VACV, family *Poxviridae*; Garcia-Escudero et al. 1998; Nichols et al. 2008), two virus families that along with members of the *Iridoviridae*, *Ascoviridae*, and *Phycodnaviridae* families comprise a

group of phylogenetically related viruses termed, Nuclear Cytoplasmic Large DNA Viruses (Colson et al. 2012; Koonin and Yutin 2010).

Recently, Zhang and her co-workers have used homologous recombination to construct *cl* mutants targeting the 53R and 2L genes of the ranavirus, RGV (He et al. 2012b, 2013, 2014). Briefly, the *lacI* gene (encoding the lac repressor protein) under the control of the promoter for the ranavirus ICP18 immediate-early gene was introduced into the RGV TK gene by homologous recombination and a recombinant virus designated RGV-lacI was generated. Subsequently, the 53R or 2L genes were placed under the control of a hybrid promoter (p50-lacO) in which the *lacO* sequence (an "operator" sequence which when bound by the *lacI* repressor protein silences downstream transcription) is located immediately downstream of a TATA-like box within the promoter for RGV gene ORF50. A plasmid containing this construct was introduced into RGV-lacI via homologous recombination to generate the *cl* mutants, i53R-RGV-lacIO and i2L-RGV-lacIO. In the absence of the inducer, IPTG, the lac repressor binds the lacO sequence and inhibits transcription of the downstream gene. In contrast, when the inducer is present, IPTG binds the repressor and relieves the transcriptional block allowing full expression of the viral gene product. In the case of the 53R and 2L *cl* mutants, expression of the targeted transcripts and proteins were markedly reduced, but not abolished, in the absence of IPTG. Thus, at 72 h after infection, virus yields were reduced more than 90 % following a 75 % reduction in the level of 53R transcripts. Similar results were seen with the 2L mutant. Taken together these results indicate that both 53R and 2L are essential for replication *in vitro* and that, as with ASFV and VACV, *cl* mutants can be used to probe the function of essential viral genes. One potential drawback to their widespread use is that repression of the targeted gene is often not complete. However, as shown with both mutants, unless low level expression of the targeted gene is sufficient for full replication, the repression achieved (in the case of 53R, 75 % at the transcriptional level) is sufficient to ascertain the essential nature of the gene product.

7.5   *Ectopic Expression of Recombinant Viral Proteins*

In addition to KD studies and studies using *ts*, *cl*, and KO mutants, another profitable way in which to explore viral gene function is by ectopically expressing recombinant viral proteins and monitoring their activity. Studies using this approach have been performed to determine the locations and functions of a number of ALRV proteins (e.g., vIF-2α, β-HSD, dUTPase, ERV1, 50L, 2L, and 53R) and are summarized immediately below and in Table 2. The *in vitro* expression of recombinant viral proteins is especially useful in determining the role of catalytic proteins that impact cellular or immune functions, but less so with viral structural proteins or those that must interact with additional viral proteins for proper functioning.

vIF-2α. vIF-2α, a viral homolog of the alpha subunit of eukaryotic translational initiation factor 2, is thought to play a critical role in maintaining viral protein

Table 2 Assessment of Ranavirus gene function using recombinant proteins

Gene (virus and ORF)[a]	Phenotype	Reference
vIF-2α (RCV)	Maintains viral translation by blocking eIF-2α phosphorylation; found among most, but not all, ranaviruses	Rothenburg et al. (2011)
ICP46 (SGIV ORF 162L)	Promotes GP cell growth and contributes to SGIV replication as a structural protein of the nucleocapsid	Xia et al. (2010)
ICP18 (SGIV ORF 086R)	Promotes GP cell growth and contributes to SGIV replication as a viral non-envelope protein	Xia et al. (2009)
LITAF (SGIV ORF 136R)	Plays crucial roles in cell death by inducing apoptosis	Huang et al. (2008)
LITAF (FV3 ORF 75L)	The C-terminal half of ORF 75L is markedly similar to cellular LITAF; 75L and LITAF associate in virus-infected cells	Eaton et al. (2013)
TNFR (SGIV ORF 096R)	Contributes to viral replication by modulating the host apoptotic response	Huang et al. (2013b)
dUTPase (SGIV ORF 049R)	Contains a nuclear export signal	Gong et al. (2010)
dUTPase (RGV ORF 67R)	Regulates levels of dUTP, contributes to synthesis of dTTP and virus replication	Zhao et al. (2007)
H3 binding protein (SGIV ORF 158L)	Facilitates viral replication, and functions as a histone H3 chaperone protein to control cellular gene expression and viral replication	Tran et al. (2011b)
Viral Insulin-like growth factor [IGF] (SGIV ORF 062R)	Stimulates cell growth and virus replication by promoting G1/S transition; over-expression leads to increased apoptosis in non-host cells	Yan et al. (2013)
Viral Semaphorin (SGIV ORF 155R)	Regulates host cytoskeletal structure and, immune responses; facilitates viral replication.	Yan et al. (2014)
VP088 (SGIV ORF 088)	Plays a role in viral entry as a viral envelope protein	Zhou et al. (2011)
VP019 (SGIV ORF 019)	Viral envelope protein	Huang et al. (2013a)
VP18 (SGIV ORF 018R)	A putative Ser/Thr kinase; plays critical roles in virion assembly and expression of viral late genes	Wang et al. (2008a)
ORF 38R (SGIV)	Viral protein with an RGD motif; may play a role in entry	Wan et al. (2010b)
ORF 20R (TFV)	Viral protein with an RGD motif; may play a role in entry; similar to SGIV ORF 38R	Wang et al. (2008b)
ORF 97R (FV3)	The ORF 97R product localizes to the ER and induces invagination of the ER and outer nuclear membrane into the nucleus	Ring et al. (2013)
β-HSD (RGV ORF 52L)	Plays a key role in host steroid synthesis; overexpression in EPC cells suppresses CPE	Sun et al. (2006)
ERV1/ALR (RGV ORF88R)	A sulfhydryl oxidase; by analogy to its ASFV homolog, ERV1/ALR is thought to play a key role in virion assembly	Ke et al. (2009)

<div align="right">(continued)</div>

Table 2 (continued)

Gene (virus and ORF)[a]	Phenotype	Reference
53R (RGV ORF 53R)	Putative myristoylated membrane protein; plays a key role in virion formation	Kim et al. (2010), Zhao et al. (2008)
50L (RGV ORF 50L)	Virion-associated protein, plays roles in virus assembly and viral gene expression	Lei et al. (2012a)
2L (RGV ORF2L)	Putative membrane protein plays an essential role in virus replication	He et al. (2014)

[a]Viral gene products are identified either based on their putative function (e.g., dUTPase) or by reference to their FV3 homolog (e.g., ICP 46). In addition, the virus in which the gene product is found and the specific ORF are indicated. If the function is not known or homology to FV3 is not present, the gene is simply identified by its ORF designation

synthesis in the face of the global translational block triggered by dsRNA-activated PKR. vIF-2α is hypothesized to act as a pseudosubstrate and, similar to VACV K3L, to bind PKR thereby preventing the phosphorylation and inactivation eIF-2α (Beattie et al. 1991; Langland and Jacobs 2002). To demonstrate a role for vIF-2α in maintaining viral protein synthesis in infected cells, Rothenburg et al. (2011) utilized a heterologous yeast system and showed that ectopic expression of vIF-2α from the ranavirus, Rana catesbeiana virus-Z (RCV-Z), blocked the growth inhibiting effects of human and zebrafish PKR. RCV-Z vIF-2α was shown to act as an inhibitor of both human and zebrafish PKR and block the PKR-mediated phosphorylation of eIF-2α, whereas VACV K3L displayed host specificity and only blocked the activity of human PKR. Moreover, experiments with vIF-2α deletion constructs showed that the N-terminal and helical domains were sufficient for PKR inhibition whereas the C-terminal domain was dispensable. Because vIF-2α does not substitute for eIF-2α but instead inhibits PKR function, the authors suggested renaming it RIPR, Ranavirus Inhibitor of Protein kinase R. Although this study strengthens the view that vIF-2α/RIPR is critical for maintaining viral protein synthesis in the face of a shut-off of host translation, it should be noted that both FV3 and soft-shell turtle iridovirus (STIV) encode truncated versions of vIF-2α that are missing the N-terminal three-fourths of the full length product (Huang et al. 2009; Tan et al. 2004). The ability of both FV3 and STIV to turn off host translation and maintain high levels of viral protein synthesis indicate that although vIF-2α is important for the maintenance of viral translation in the face of host shut-off, other proteins must also play a part in this process and perhaps, like the E3L/K3L system of VACV, provide a level of redundancy (Langland et al. 2006; Langland and Jacobs 2002).

βHSD. 3β-Hydroxysteroid dehydrogenase plays a key role in cellular steroid synthesis and the corresponding VACV homolog is thought to play a critical role in blunting the host antiviral immune response (Reading et al. 2003). A 1,068 bp/355 amino acid β-HSD homolog of RGV was cloned and shown to be an immediate-early gene product (Sun et al. 2006). Confocal microscopy revealed that an ectopically expressed βHSD-EGFP fusion protein co-localized exclusively with the mitochondrial marker

pDsRed2-Mito in EPC cells. Moreover, overexpression of βHSD-EGFP suppressed RGV-induced cytopathic effect (CPE) in EPC cells. Given the putative role of βHSD in steroid biosynthesis, it is likely that the role of this protein in a productive *in vivo* infection is to impair the host immune response. Whether βHSD plays another role *in vitro* which allows it to suppress CPE remains to be determined. As seen with VACV mutants that lack βHSD (Reading et al. 2003), we hypothesize that mutants targeting the βHSD gene will fail to inhibit the host immune response as fully as *wt* virus and display an attenuated phenotype *in vivo*.

dUTPase (*dUTP pyrophosphatase*): dUTPase is a ubiquitous enzyme responsible for regulating dUTP concentrations (whose incorporation into DNA would be deleterious for virus replication) and raising levels of dTTP via the salvage pathway (Kato et al. 2014; Oliveros et al. 1999). dUTPase catalyzes the conversion of dUTP to dUMP. RGV-encoded dUTPase is a 164 amino acid protein that was characterized as an early gene product by RT-PCR and Western blot analysis. An ectopically expressed dUTPase-EGFP fusion protein was found within the cytoplasm, and immunofluorescence confirmed the cytoplasmic location of dUTPase in productively infected cells (Zhao et al. 2007). Overexpression of dUTPase had no detectable effect on RGV replication suggesting that it did not negatively or positively impact replication *in vitro*. Thus, at least in EPC cells, dUTPase overexpression does not enhance virus replication, perhaps because the level of expression provided by *wt* virus is sufficient for full virus replication. In addition, studies of the SGIV dUTPase homolog identified a nuclear export signal that was crucial for the translocation of SGIV dUTPase from the nucleus to the cytoplasm (Gong et al. 2010). Lastly, although BLAST analysis suggests that RGV dUTPase functions in the control of dUTP/dTTP levels, dUTPase genes found within herpesviruses have evolved novel functions including the dysregulation of immune functions (Davison and Stow 2005; Glaser et al. 2006).

ERV1: The yeast protein ERV1 (Essential for Replication and Viability) and its mammalian homolog ALR (Augmenter of Liver Regeneration) are sulfhydryl oxidases that play critical roles in protein folding (Thorpe et al. 2002). Consistent with that role, ASFV contains an ERV1/ALR homolog, 9GL that is found within viral factories and plays a critical role in virion maturation (Lewis et al. 2000). A 9GL deletion mutant displayed a 2-log drop in viral yield, and, of the virions that formed, 90–99 % contained acentric nucleoid structures. Like ASFV, the RGV ERV1 homolog (88R) is a late protein (Ke et al. 2009). It contains the highly conserved ERV1 motif Cys-X-X-Cys and was detected in both the nucleus and cytoplasm. However, unlike the ASFV ERV1/ALR KO mutant, knock down of 88R expression by RNAi did not result in a drop in virus yield, suggesting either that ERV1 plays another role in virus replication, knock down was incomplete, or host cells retained sufficient sulfhydryl oxidase capacity to compensate for any defect in the viral enzyme.

50L: RGV 50L encodes a protein 499 amino acids in length with a predicted mol wt of 55.5 kDa. Full-length homologs ranging in size from 499 (STIV)—541 (EHNV) amino acids are present in a variety of ranaviruses, whereas a shorter homolog of

249 amino acids encompassing only the C-terminal half of the protein is found in FV3. RGV 50L was cloned, expressed in *E. coli*, and used to prepare anti-50L antibody in mice. In addition, 50L was cloned into the eukaryotic expression vector pcDNA3.1, to yield 50L-pcDNA3.1 (Lei et al. 2012a). Immunofluorescent staining detected 50L within the cytoplasm, viral assembly sites, and nucleus. Moreover, the presence of 50L within the nucleus was dependent upon a nuclear localization signal within the central region of the protein. Infection of cells transfected with 50L-pcDNA3.1 displayed higher levels of 53R mRNA suggesting that 50L expression may affect the expression of RGV genes.

LITAF: Both SGIV and FV3 encode proteins with homology to a cellular protein designated LITAF, LPS-induced TNFα factor. LPS is a potent stimulator of monocytes and macrophages and triggers the secretion of TNFα and other pro-inflammatory cytokines. LITAF was identified as a novel transcriptional factor that modulated TNFα expression and played a role in the regulation of inflammatory cytokines (Tang et al. 2006). SGIV ORF136 encodes an early viral gene product that is a homolog of LITAF and which, following transfection of grouper cells with a vector expressing ORF136, is predominantly associated with mitochondria. Overexpression of SGIV LITAF *in vitro* induces apoptosis, as shown by increased apoptotic bodies, depolarization of mitochondrial membrane potential, and activation of caspase-3, suggesting that SGIV LITAF might play crucial roles in SGIV-induced cell death (Huang et al. 2008). Similar to SGIV ORF136, FV3 ORF 75L encodes a protein that shows high sequence similarity to a conserved domain found in the C-terminal half of cellular LITAF (Eaton et al. 2013). Following co-transfection or viral infection, cellular LITAF and FV3 75L were shown to co-localize to late endosomes/lysosomes within both baby green monkey cells (BGMK) and A6 amphibian cells. Interestingly, reexamination of SGIV ORF 136 confirmed localization to mitochondria within BGMK cells, but showed that, as with FV3 75L, SGIV ORF 136 localized to endosomes/lysosomes in amphibian A6 cells. To date, the exact function and temporal class of the viral LITAF homologs remain uncertain. It has been suggested that viral LITAF acts in a dominant-negative fashion to block the function of cellular LITAF (Eaton et al. 2013). If cellular LITAF does have an antiviral function, RNAi or asMO knock down of cellular LITAF levels should generate higher titers of FV3 *in vitro* and suggest a role for its viral homolog. Alternatively *in vivo* infection with a LITAF knock out mutant should result in an attenuated infection and lower viral titers.

RGD motif-containing proteins: Proteins containing the RGD motif are present among all iridovirid genera and include SGIV VP38 (Wan et al. 2010), Tiger frog virus ORF 20R (Wang et al. 2008b), and yellow croaker iridovirus (YCIV, genus *Megalocytivirus*) 037L (Ao and Chen 2006). Although differing markedly in size, all three genes contain an RGD motif that has been reported to play an important role in virus attachment and entry. In all three studies, recombinant protein was used to generate specific antiserum that was used to identify the protein within viral assembly sites and the viral envelope. Collectively, these studies indicate that RGD-containing proteins are found within viral assembly sites and envelopes and may play

a role in subsequent viral entry. However, suggestive of other roles, a YCIV-encoded thioredoxin-037L fusion protein was shown to induce cell rounding, detachment, and aggregation following its transfection into BF-2 cell monolayers. Additional ranavirus ORFs whose functions have been explored using recombinant proteins are described briefly below and summarized in Table 2.

Additional Recombinant SGIV- and ISKNV-encoded Proteins. Vectors expressing the SGIV immediate-early (IE) genes, ORF162L and ORF086R, encoding putative homologs of FV3 ICP46 and ICP18, respectively, were constructed. Both gene products are distributed predominantly within the cytoplasm, and their overexpression promoted the growth of grouper embryonic cells and contributed to SGIV replication. SGIV ORF096 (VP96) encodes a putative homolog of TNFR, which contains two extracellular cysteine-rich domains (CRDs) but lacks the C-terminal transmembrane domain. Overexpression of ORF096 *in vitro* enhances cellular proliferation and improves cell survival suggesting that SGIV might utilize a viral homolog of TNFR to modulate the host apoptotic response for effective replication (Huang et al. 2013b). As with RGV, SGIV encodes a dUTPase homolog (ORF049R) with a leucine-rich nuclear export signal (NES) at its C-terminus. SGIV dUTPase is a cytoplasmic protein, and its NES is crucial for the translocation of dUTPase from the nucleus to the cytoplasm (Gong et al. 2010). SGIV ORF158L is observed in nuclei and virus assembly sites and its knock-down results in a significant decrease in virus yield in grouper embryonic (GP) cells. Further, analysis suggests that ORF158L may function as a histone H3 chaperon, enabling it to control host cellular gene expression and facilitate viral replication (Tran et al. 2011). ORF 158L is not found in all ranaviruses, but homologs displaying approximately 33 % similarity to the SGIV product are found in EHNV, ADRV, and CMTV. SGIV ORF062R encodes a novel insulin-like growth factor that stimulates the growth of grouper GP cells and perhaps SGIV replication by promoting G1/S phase transition. In addition, overexpression of ORF062R slightly increased apoptosis in SGIV-infected non-host FHM cells (Yan et al. 2013). SGIV ORF155R encodes a semaphorin homolog, which could promote viral replication *in vitro* and attenuate the cellular immune response. Ectopically expressed ORF155R was shown to alter the cytoskeletal structure of fish cells. This alteration was characterized by a circumferential ring of microtubules near the nucleus and a disrupted microfilament organization (Yan et al. 2014). An abundant viral protein, ORF018R, has been identified which may play critical roles in serine/threonine phosphorylation and virion assembly (Wang et al. 2008a). Two late genes, SGIV ORF088 and ORF019, encode viral envelope proteins. Furthermore, rVP88 was shown to bind a 94 kDa host cell membrane protein, suggesting that VP88 might function as an attachment protein and play a role in viral entry (Huang et al. 2013a; Zhou et al. 2011). Finally, similar approaches have been applied to ISKNV (genus *Megalocytivirus*) and used to identify putative functions among a viral TRAF protein (He et al. 2012a), a viral protein that mediates formation of a mock basement membrane and provides attachment sites for lymphatic endothelial cells (Xu et al. 2010), a viral ankyrin repeat protein that may inhibit TNFα-induced NF-κB signaling (Guo et al. 2011a), and a viral-encoded vascular endothelial growth factor

(Wang et al. 2008c). Collectively, the generation and expression of recombinant viral proteins provides a powerful methodology for determining the cellular location and putative function of virus-encoded proteins.

8 Final Thoughts

Using contemporary molecular techniques, researchers are slowly elucidating the specific steps by which ranaviruses replicate *in vitro* and trigger disease *in vivo*. This work has been facilitated by pioneering studies performed with herpesviruses, poxviruses, and ASFV that have provided insights into which viral genes play important roles in virus replication and disease progression. Optimistically, identi-fication of ranavirus genes that contribute to enhanced virus replication and the evasion of host immune responses will allow us to construct vaccines that are able to effectively protect endangered amphibians, fish, and reptiles. Success in this area is critical given the ongoing decline of amphibians in many parts of the world and the growing reliance upon mariculture and aquaculture as a source of protein for human consumption. In addition, understanding how viral genes interact and modu-late immunological responses will broaden our understanding of immunity among "lower vertebrates" and shed light on the origins of the immune system.

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