Ranaviruses: History and Future Directions

Ranaviruses: Emerging Pathogens of Cold-blooded Vertebrates

OnLine Course 2016

VG Chinchar, U. Mississippi Medical Ctr.

Jackson, MS

vchinchar@umc.edu

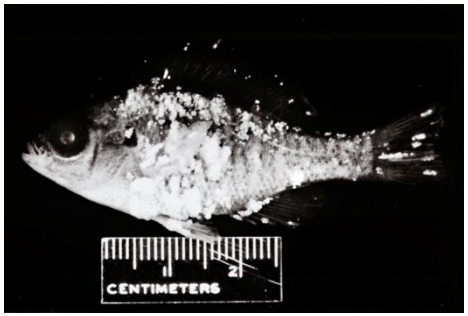
Outline

- Past: 100+ years of iridoviruses
- Present:
  - Molecular Virology: Elucidation of FV3 life cycle and gene function
  - Ecology: Understanding the role of ranaviruses in die-offs and extinctions
- Future: Molecular, Genetic, Immunological, and Ecological studies.

Iridovirus/Ranavirus Timeline: 100+ years of Iridoviruses

1900 — 1954 — 1965 — 1986 — 2016

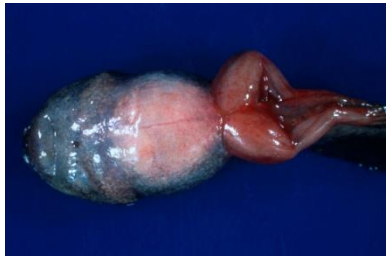
Lymphocystis disease identified



First Insect Iridovirus Identified

Xeros (1954)

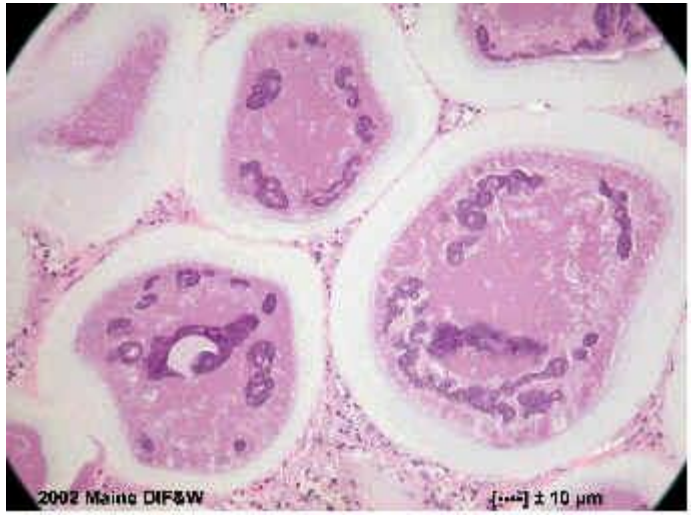
FV3 identified



Granoff et al., 1965 (NY Acad. Sci.)

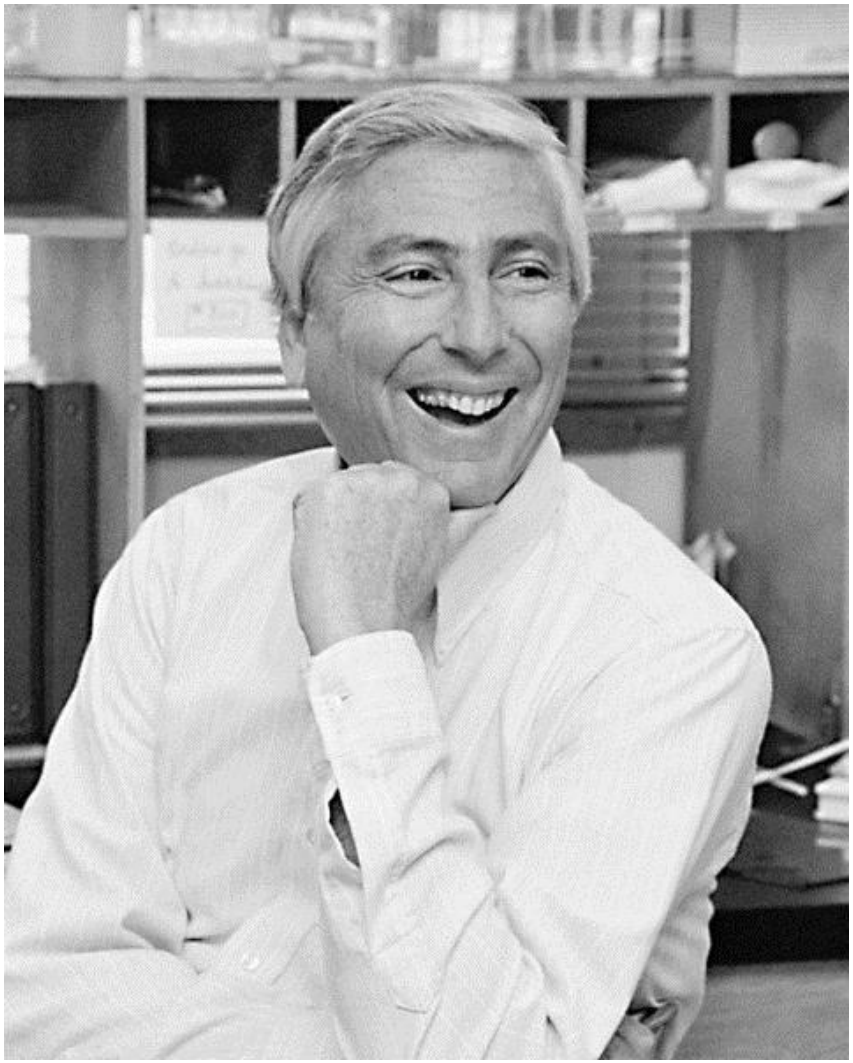
1890s: Lymphocystis Disease - the first identified iridovirus disease

1914: Weissenberg postulates LD to be a viral disease
1924: Transmission via transplanted skin
1945: Transmission following ultrafiltration
1962: TEM showed that “tumors” contain icosahedral virions



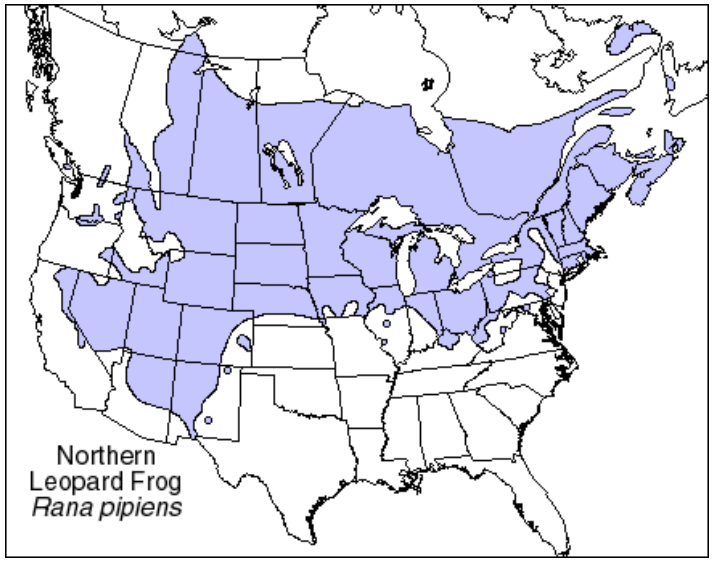
1954 – Invertebrate iridoviruses

In a search for crane fly (*Tipula spp*.) larvae infected with polyhedrosis virus, Claude Rivers applied St. Ives fluid to pasture land in Shropshire, UK. As the larvae wriggled to the surface to escape the irritating phenolic solution, Rivers was amazed to see larvae with brilliant patches of **iridescent blue color!**



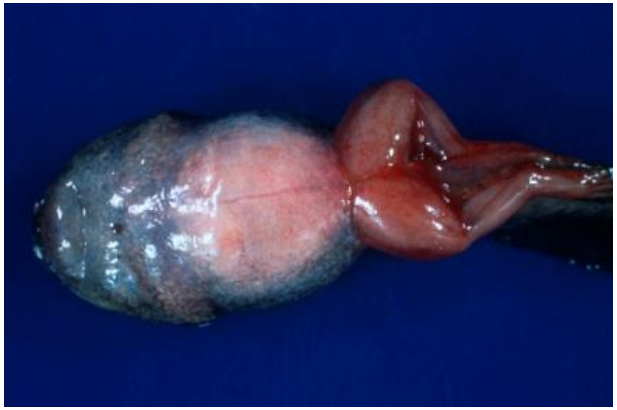
Allan Granoff (1923 - 2012): Chair Division of Virology, SJCRH (1962 – 1988); Deputy Director – Research (1988), Interim Director (1992).

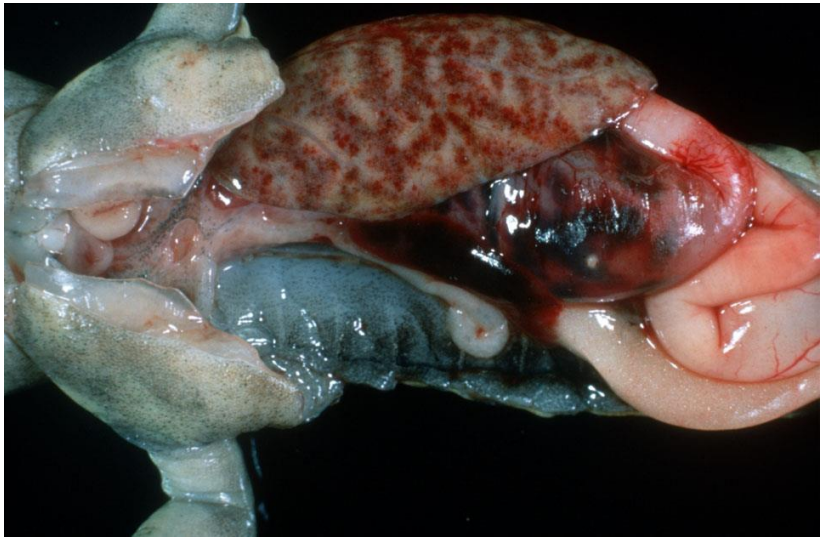
1965 – Granoff isolates Frog virus -1, -2, and -3



FV-1 and FV-2 were isolated from “healthy” frogs.
FV-3 from a tumor-bearing frog.

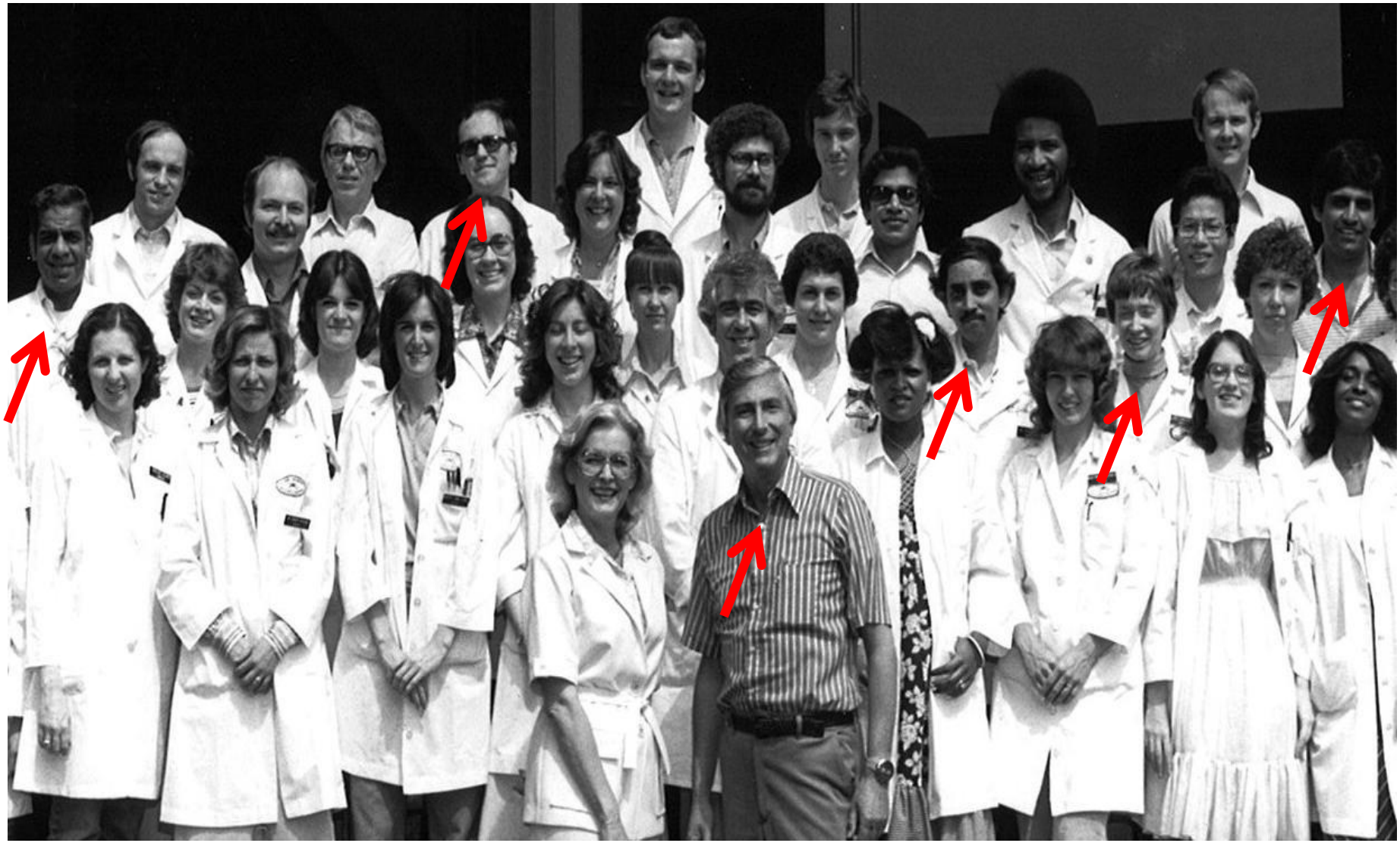
Ranaviruses target frogs, salamanders, and turtles and trigger systemic infection





Box turtle with ranavirus by: Scott Farnsworth

St. Jude Childrens' Research Hospital, Division of Virology, circa 1980 – Six virologists responsible for many of the early FV3 studies



Allan Granoff, Rakesh Goorha, Dawn Willis, Raj Raghow, Gopal Murti, Greg Chinchar

Other early ranavirus workers

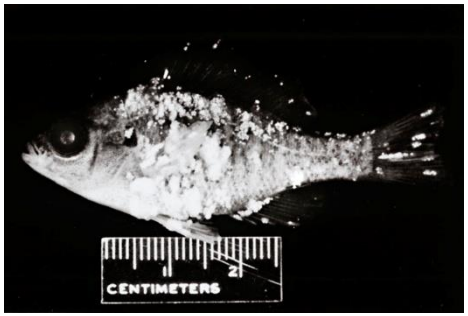
- Molecular Studies:
  - Aubertin, Drillien, Kirn (FR)
  - McAuslan (USA)
  - Elliot and Kelly (UK)

- Identification and Virus Characterization
  - Karzon, Clark
  - Wolf (tadpole edema virus)

Iridovirus/Ranavirus Timeline: 100+ years of Iridoviruses

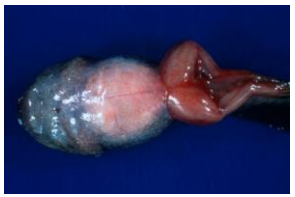
1900 — 1955 — 1965 — mid-1980's — 2016

Lymphocystis disease identified



First Insect Iridovirus Identified

FV3 identified



FV3 Life Cycle

1. **Identification and characterization of ranaviruses and other iridoviruses infecting fish, reptiles, and amphibians.**
2. **Elucidation of viral gene function and anti-viral response.**

A Ranavirus Renaissance

- Langdon and Humphrey (1987) **Redfin perch** [AU – EHNV]
- Ahne et al. (1989) **Sheatfish** [Germany – ESV]
- Kanchanakhan (1989) ***R. tigrina*** [SE Asia]
- Pozet et al. (1992) ***Ictalurus melas*** [France – ECV]
- Speare and Smith (1992) **ornate burrowing frog** [Australia – BIV]
- Bloch and Larsen (1993) Turbot [Scandinavia]
- Chua et al. (1994) and Qin et al. (2001) brown spotted grouper [Singapore/Taiwan/PRC – SGIV/GIV]
- Cunningham et al. (1996) *R. tempora* [UK]
- Plumb et al., (1996) largemouth bass [USA/SC – LMBV]
- Mao et al., (1997) doctor fish [SE Asia –DFV]
- Jancovich et al. (1999) *Ambystoma tigrinum* [USA/AZ – ATV]
- Chen et al. (1999) soft shell turtle [China – STIV]
- Allender et al. (2006) box turtles [USA]
- Cheng et al., (2014) North American Bohle-like virus
- Mavian et al. (2012) Common midwife toad virus (Spain)
- Wang et al., (2014) Chinese giant salamander ranavirus

An explosion of Irido/Ranavirus-related publications (Pubmed)

- 1975 – 1985: 25 publications
- 1986 – 1995: 40 publications
- 1996 – 2005: 125 publications
- 2006 – 2015: 387 publications
  - 2006 & 2007: 47
  - 2008 &2009: 49
  - 2010 & 2011: 87
  - 2012 & 2013: 95
  - 2014 & 2015: 109

3rd International Ranavirus Symposium – Gainesville, FL - 2015

What has happened in the past 10 years?

- **Genomes** of more than 20 different iridoviruses have been sequenced and used to construct concatenated phylogenetic trees.
- **Knock down** (asMOs and siRNA) and **Knock out** (deletion & conditionally lethal mutants) studies have proven invaluable in elucidating viral gene function.
  - Viral replicative, efficiency, and immune evasion genes have been identified and characterized

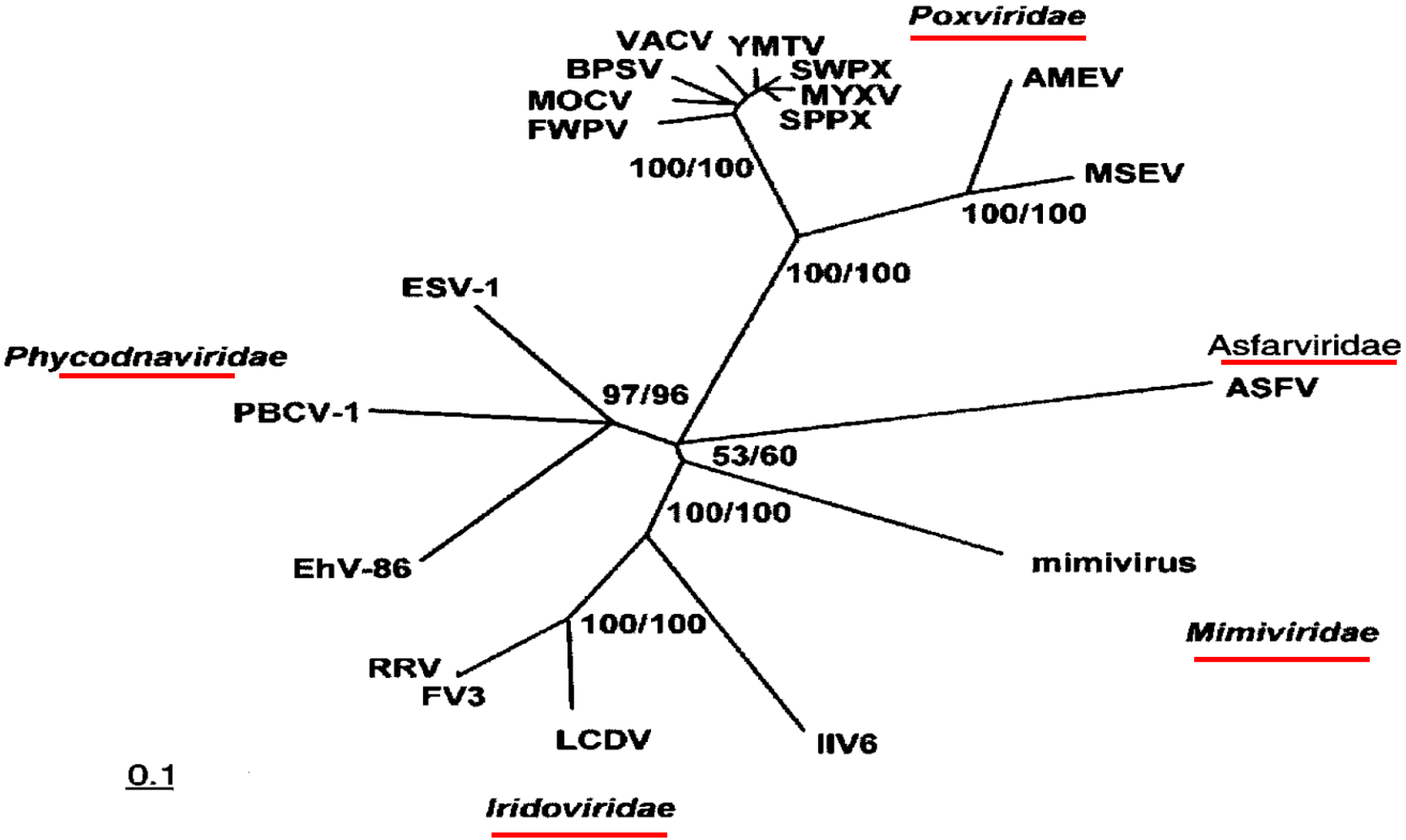
…last 10 years

- **Ectopic expression** of recombinant viral proteins has facilitated determination of function.
- Polyclonal and monoclonal **antibodies** have permitted subcellular localization of viral proteins.
- The roles of innate and acquired **immunity** in resolving ranavirus infection have been revealed.
- **Field studies** have deepened our understanding of ranavirus ecology and its impact on susceptible populations.

Taxonomy

Viral Taxonomy

- *Order Megavirales (a.k.a. NCLDVs)
  - Family *Ascoviridae*
  - Family *Poxviridae*
  - **Family *Iridoviridae***
  - Family *Asfarviridae*
  - Family *Mimiviridae*
  - *Family *Marseilleviridae*
  - Family *Phycodnaviridae*
    - Colson P et al., ***Intervirology*** 55: 321 – 332 (2012)



Allen et al., (2006) Virology J 3:15.

Nuclear Cytoplasmic Large DNA-containing Viruses (NCLDV) = **Megavirales**

Family: *Iridoviridae*

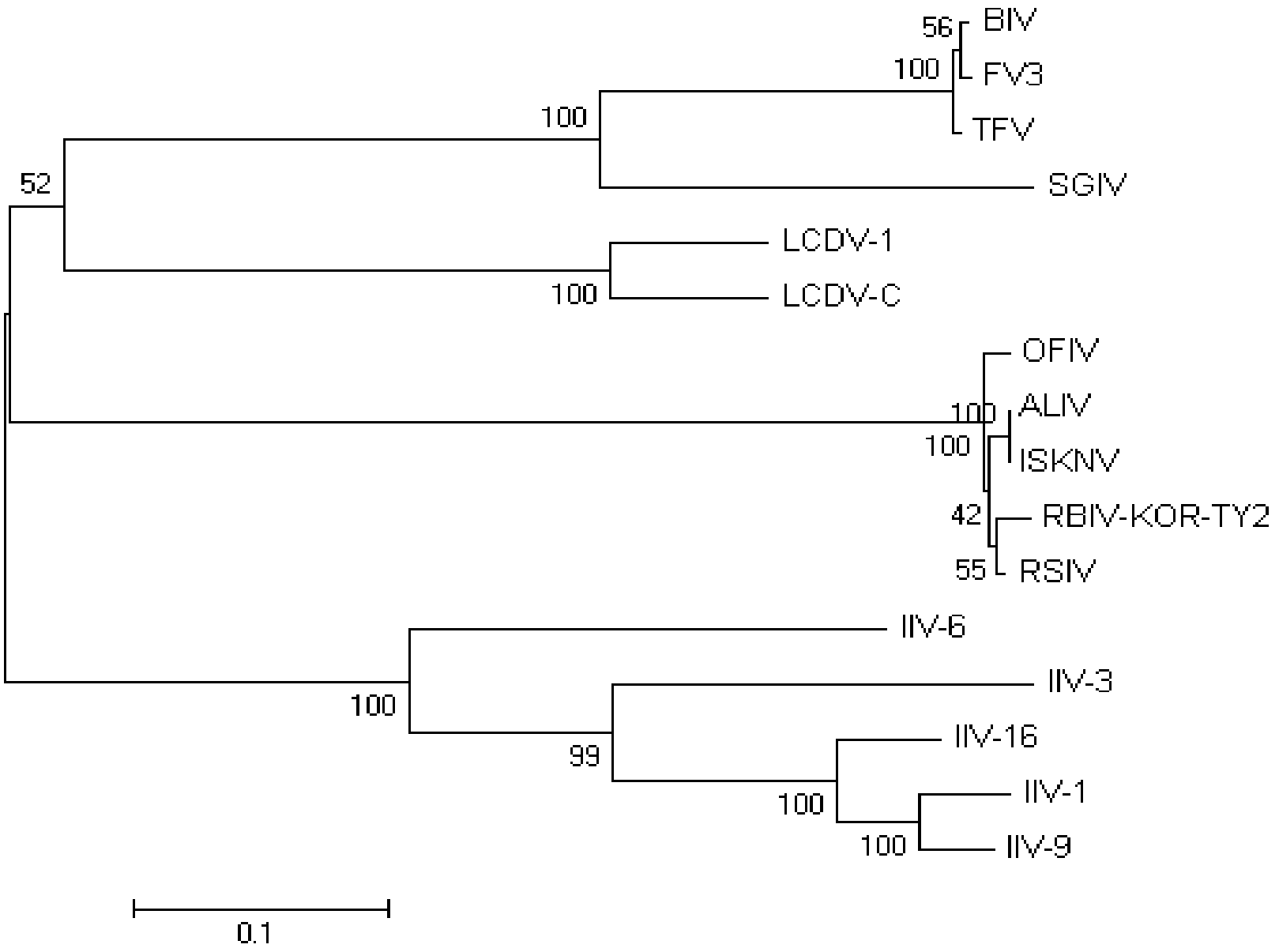
- ***Subfamily*: *Inveriridovirinae***
  - ***Iridovirus*: IIV6 (Invertebrate iridovirus 6)**
  - ***Chloriridovirus:* IIV3**
- ***Subfamily: Chordiridovirinae***
  - ***Lymphocystivirus: LCDV-1, LCDV-C***
  - ***Megalocytivirus: ISKNV, RSIV, TRBIV***
  - ***Erythrovirus: Erythrocytic necrosis virus (ENV)***
  - ***Ranavirus: FV3***

*** Tentative taxanomic designation**

Vertebrate Iridoviruses

- **Subfamily*: Chordiridovirinae***
  - ***Lymphocystivirus***…wart-like disease in freshwater and marine fish, disfigurement, but low mortality
  - ***Megalocytivirus***…life-threatening systemic infections in >52 species of marine and freshwater fish in SE Asia…and elsewhere
  - ****Erythrovirus**:* Erythrocytic necrosis virus (ENV)
  - ***Ranavirus***…systemic disease in fish, reptiles, and amphibians accompanied by variable mortality.
    - **FV3, ATV, BIV, EHNV, ECV, SCRV**, *SGIV*

Phylogenetic Tree: MCP



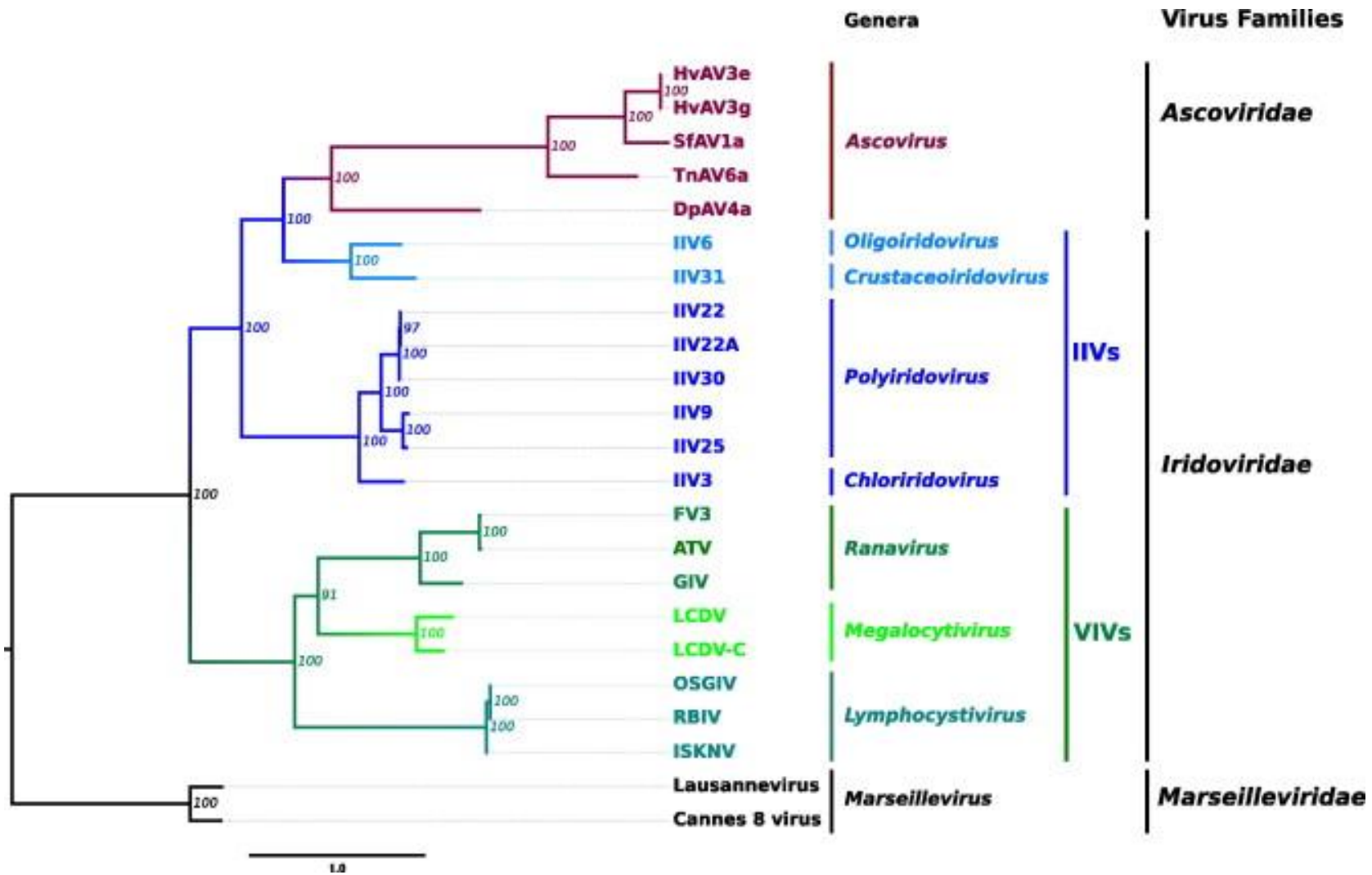


Fig. 4. Core protein tree. Numbers in italics at nodes indicate bootstrap values (%) retrieved from 1000 replicates. Branch lengths were proportional to genetic distances. Color codes are the same as those used in Fig. 1. The taxonomic levels from the genera t...

Piegu B et al., Evolutionary relationships of iridoviruses and divergence of ascoviruses from invertebrate iridoviruses in the superfamily Megavirales. Mol. Phylogen. Evol. 84: 44 – 532, 2015

Taxonomic Questions

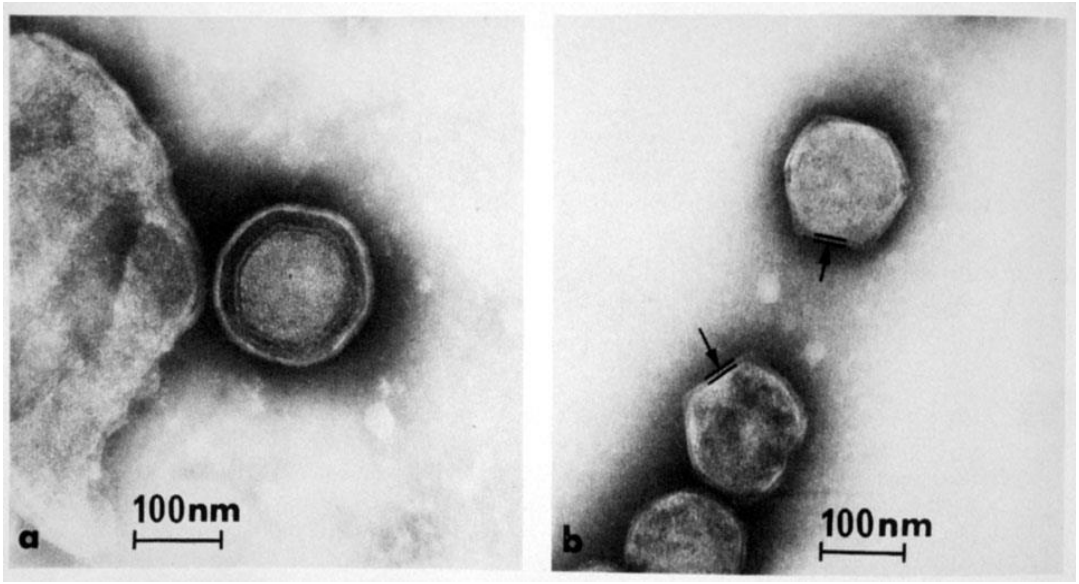
- Should “Iridovirids” be placed within the proposed Order *Megavirales*?
- How many genera of Iridovirids are there? 5, 6, 8, or more?
- How many viral species are within the genus *Ranavirus*? What is the definition of a species?
  - Are differences in hosts, size, GC content sufficient to define a new species/genus?
  - Can species be defined based on sequence data alone? And is so, where are the break points?
    - A VIRUS SPECIES IS A POLYTHETIC CLASS THAT CONSTITUTES A REPLICATING LINEAGE AND OCCUPIES A PARTICULAR ECOLOGICAL NICHE

Why is taxonomy important?

- Provides a framework for identifying and understanding pathogenic and ecologically-important viruses.
- May have commercial/trade implications
  - EHNV-infected fish cannot be shipped to EHNV-free regions of the world. If ECV/ESV = EHNV, can EHNV-infected fish be shipped to Europe?
  - ISKNV was originally detected in SE Asia, but now ISKNV-like viruses are found in Australia and North America. Does this represent introduction or natural wide-spread prevalence?

Morphology and Life Cycle

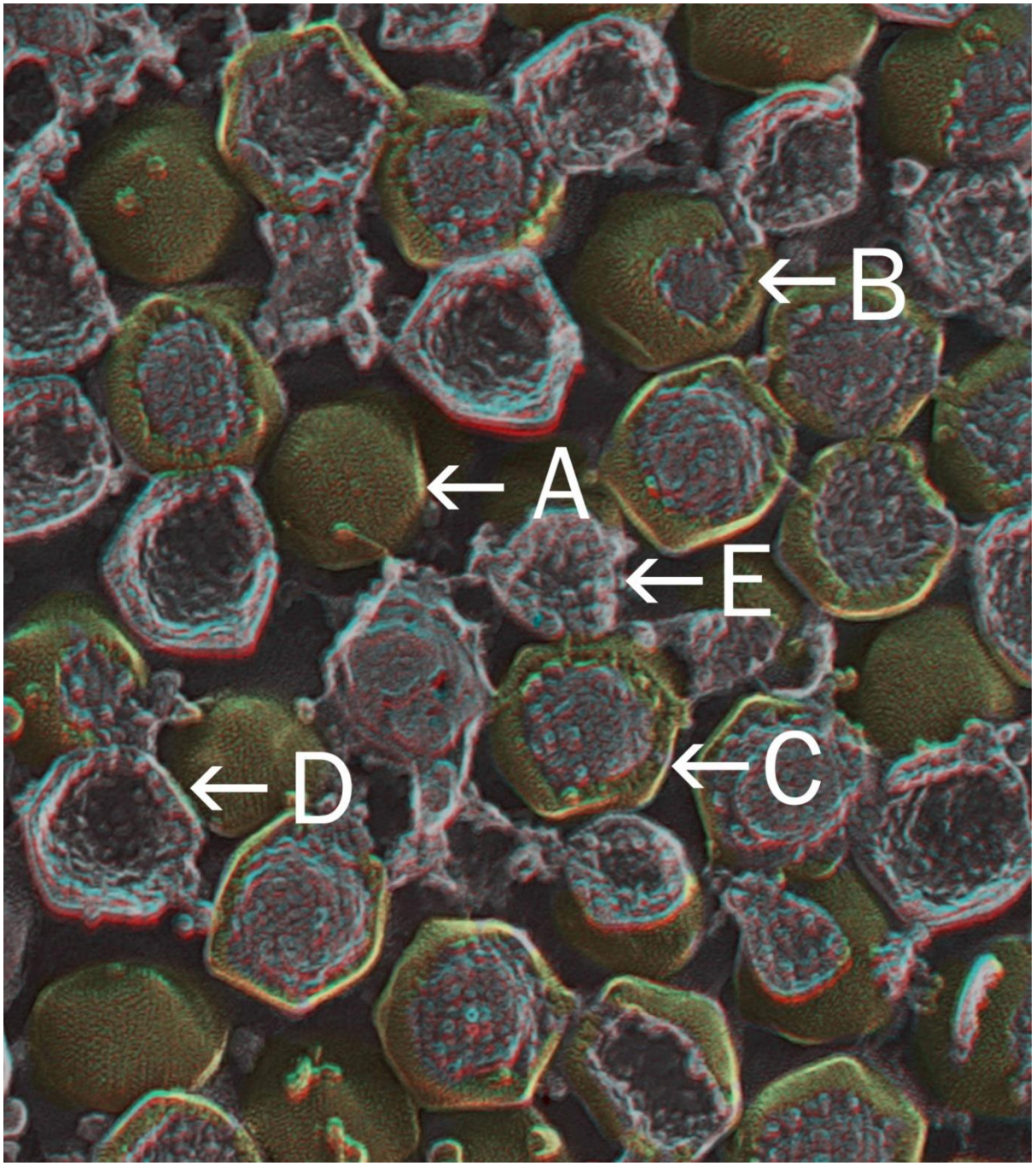
All vertebrate iridoviruses are about the same size (150 nm); some invertebrate iridoviruses are a bit larger.



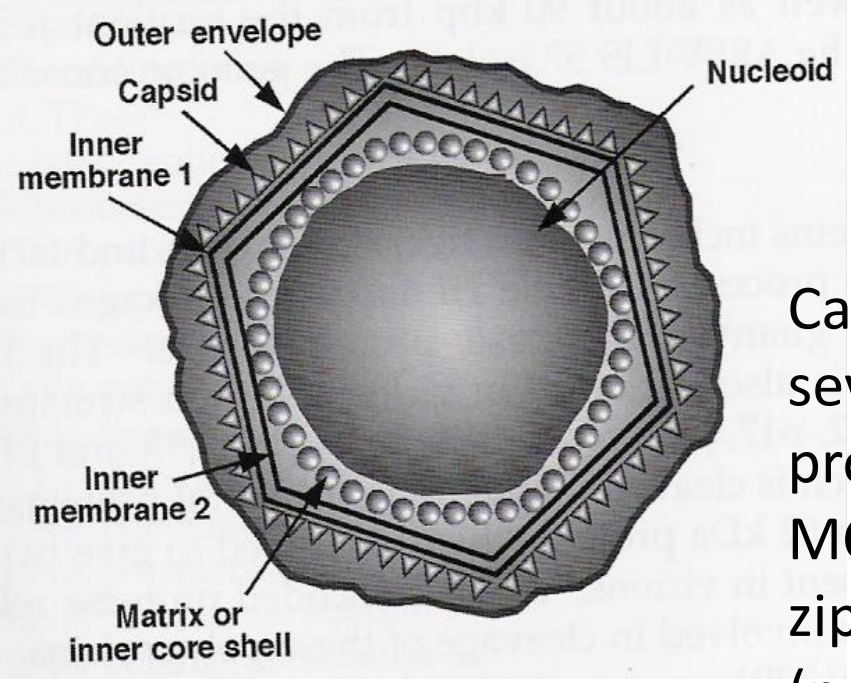
Enveloped Virus

Non-enveloped FV3

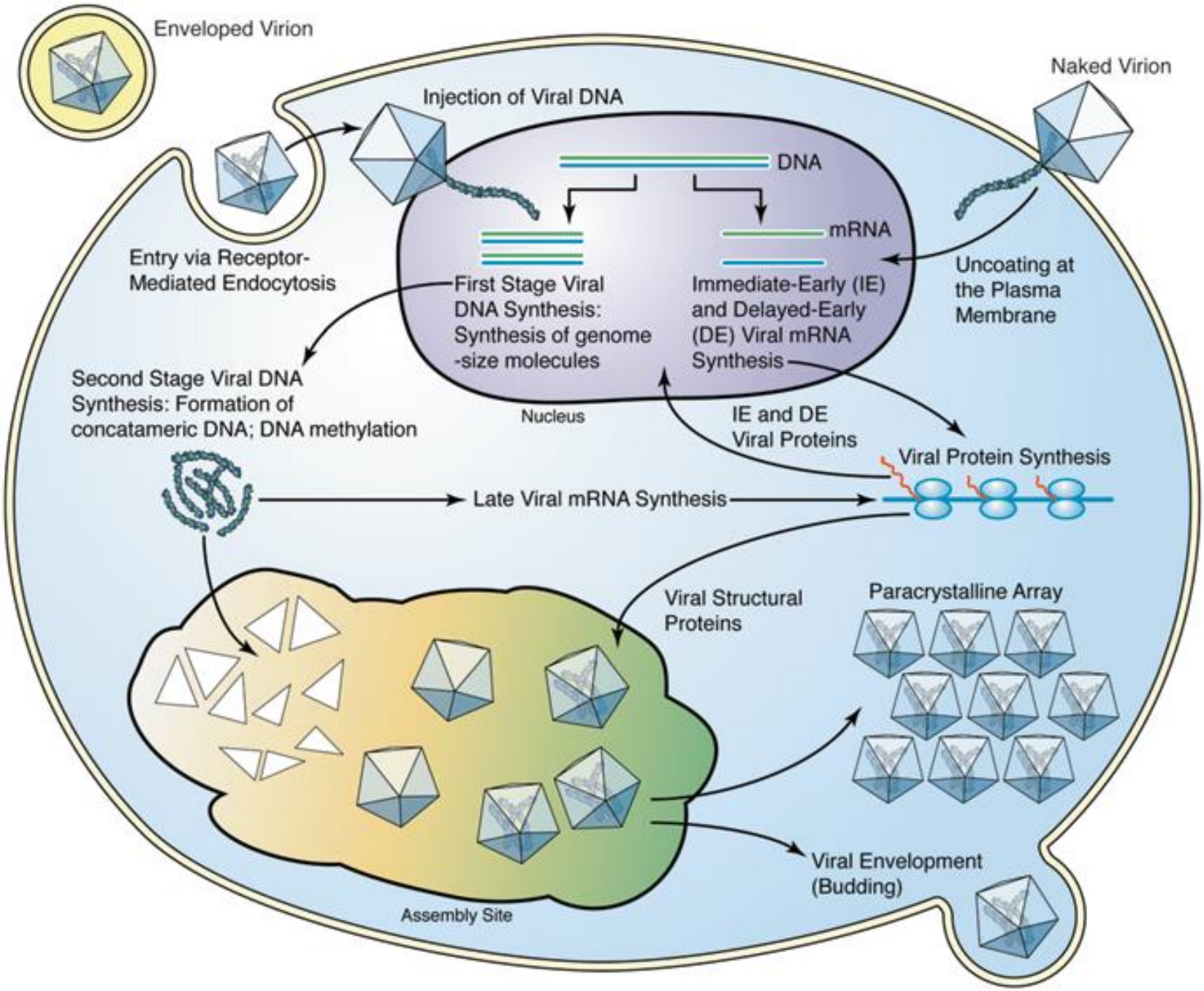
Freeze-Fracture analysis of FV3 virions detects 10 nm knob-like particles in association with the internal lipid membrane.



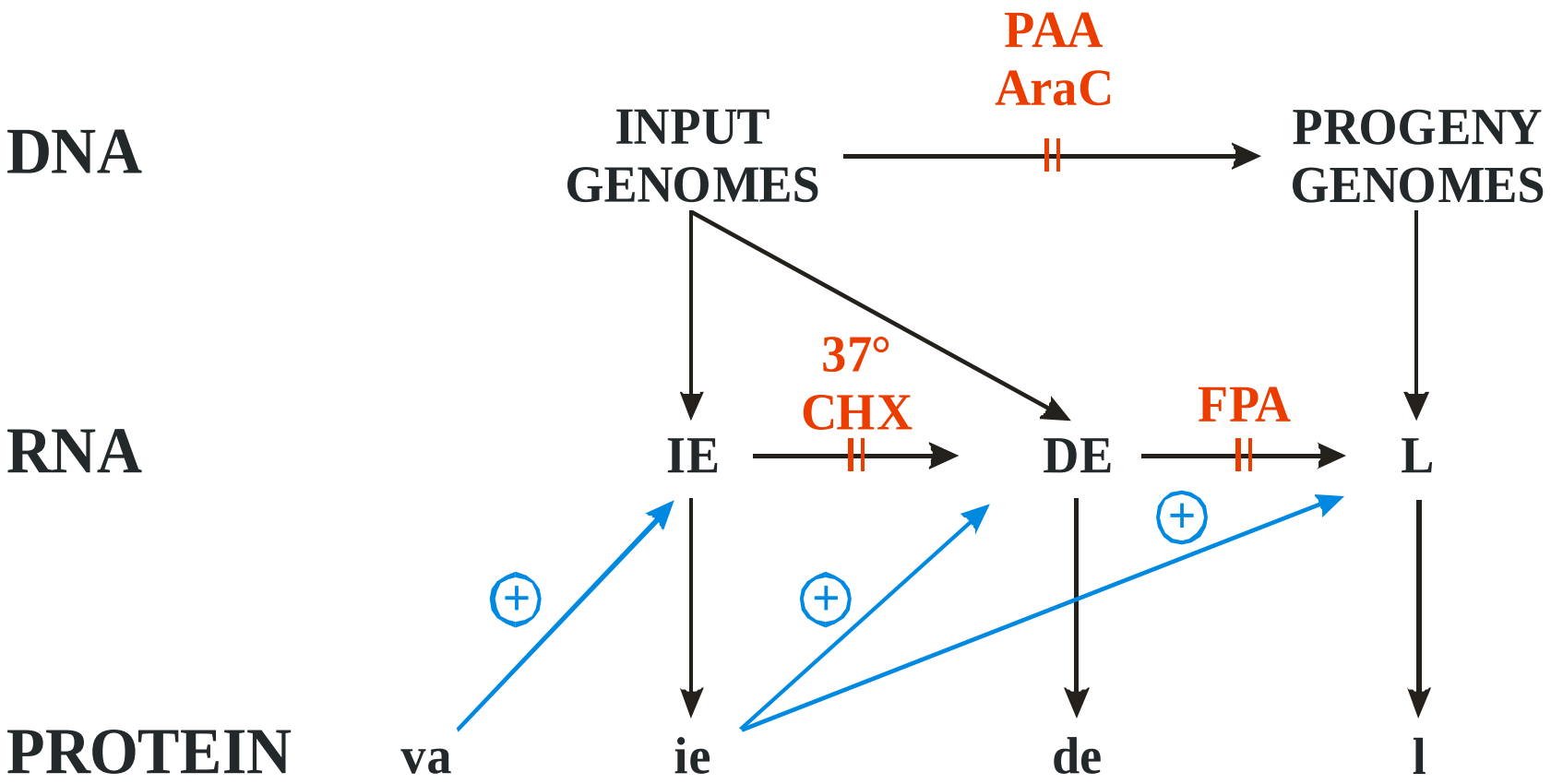
Ranavirus Virion Morphology: Envelope, Capsid, Inner Membrane; Core



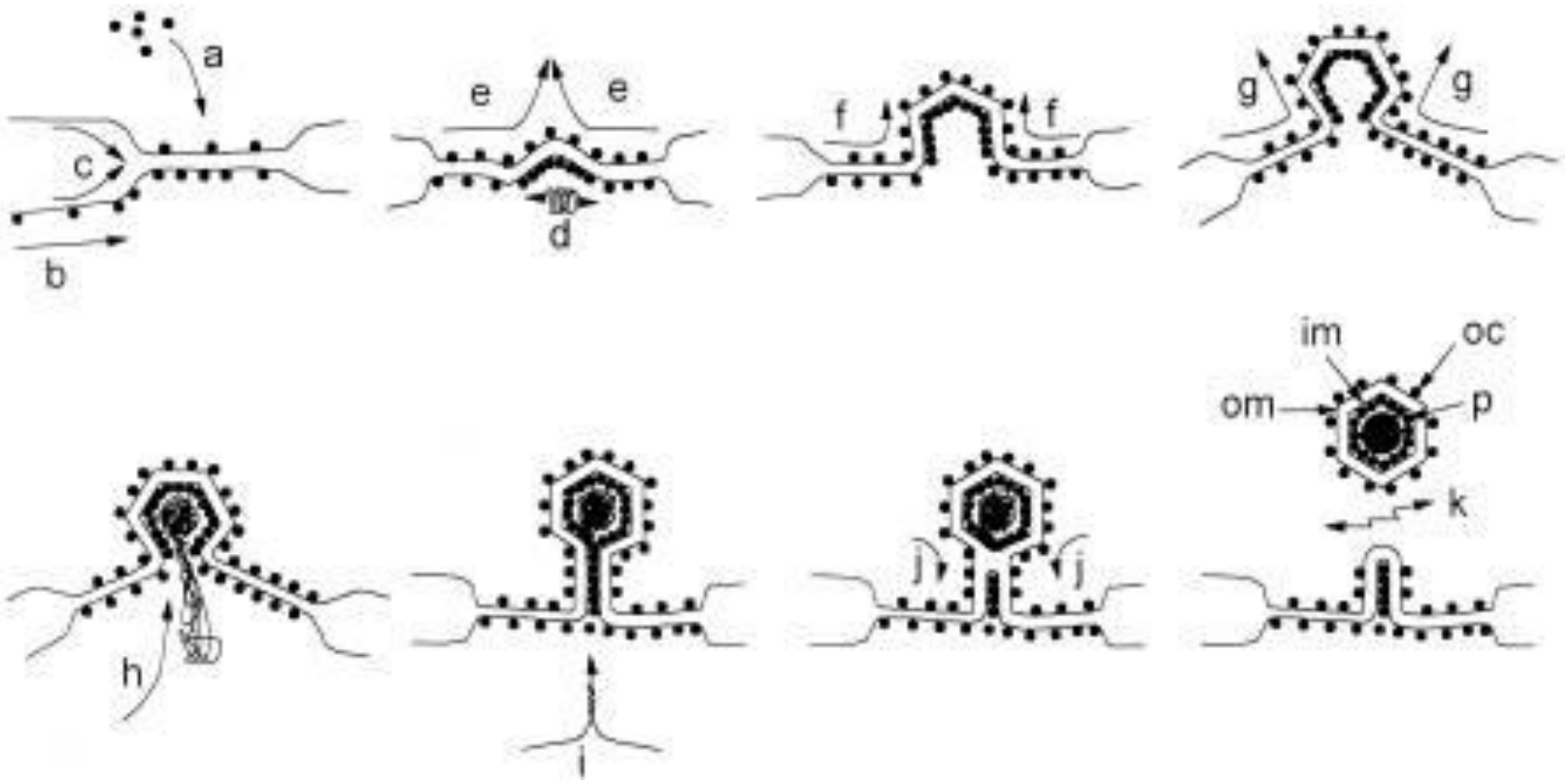
Capsid composed of several structural proteins … MCP>>ORF53, zipper, hinge….and (perhaps) other non-structural catalytic proteins.


Enveloped Virion
Naked Virion
Injection of Viral DNA
DNA
mRNA
Entry via Receptor-Mediated Endocytosis
Uncoating at the Plasma Membrane
First Stage Viral DNA Synthesis: Synthesis of genome -size molecules
Immediate-Early (IE) and Delayed-Early (DE) Viral mRNA Synthesis
Second Stage Viral DNA Synthesis: Formation of concatameric DNA; DNA methylation
Nucleus
IE and DE Viral Proteins
Viral Protein Synthesis
Late Viral mRNA Synthesis
Viral Structural Proteins
Paracrystalline Array
Viral Envelopment (Budding)
Assembly Site

Regulatory events in FV3 replication



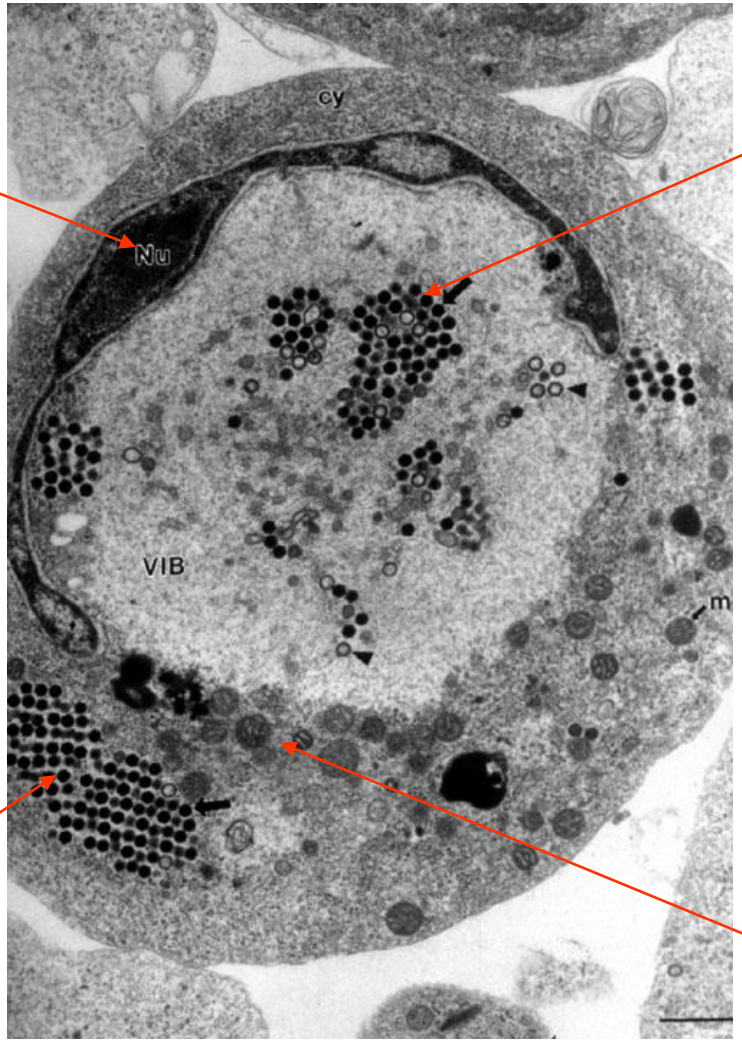
FV3 genomic DNA is NOT infectious. A virion-associated protein (va) and host RNA polymerse II are needed to synthesize immediate early (IE) viral transcripts. At least one IE gene product is required for subsequent DE and L viral transcription. Early viral gene products include the viral DNA polymerase and the two largest subunits of the viral transcriptase, the latter catalyze the synthesis of L viral mRNAs. L gene expression is also dependent upon ongoing viral DNA synthesis.



Possible scheme of FV3 virion formation based on the ASFV model: "a" may represent MCP and/or p53 proteins. They are thought to bind bits of cellular membrane and in the process comprise the capsid wall. Progressive addition of MCP/p53 leads to folding of the planar sheet into an icosahedron that is filled by a headful mechanism. Reference: Rouiller et al., ***J Virology*** 72: 2372 – 2387 (1998).

Nucleus showing chromatin condensation

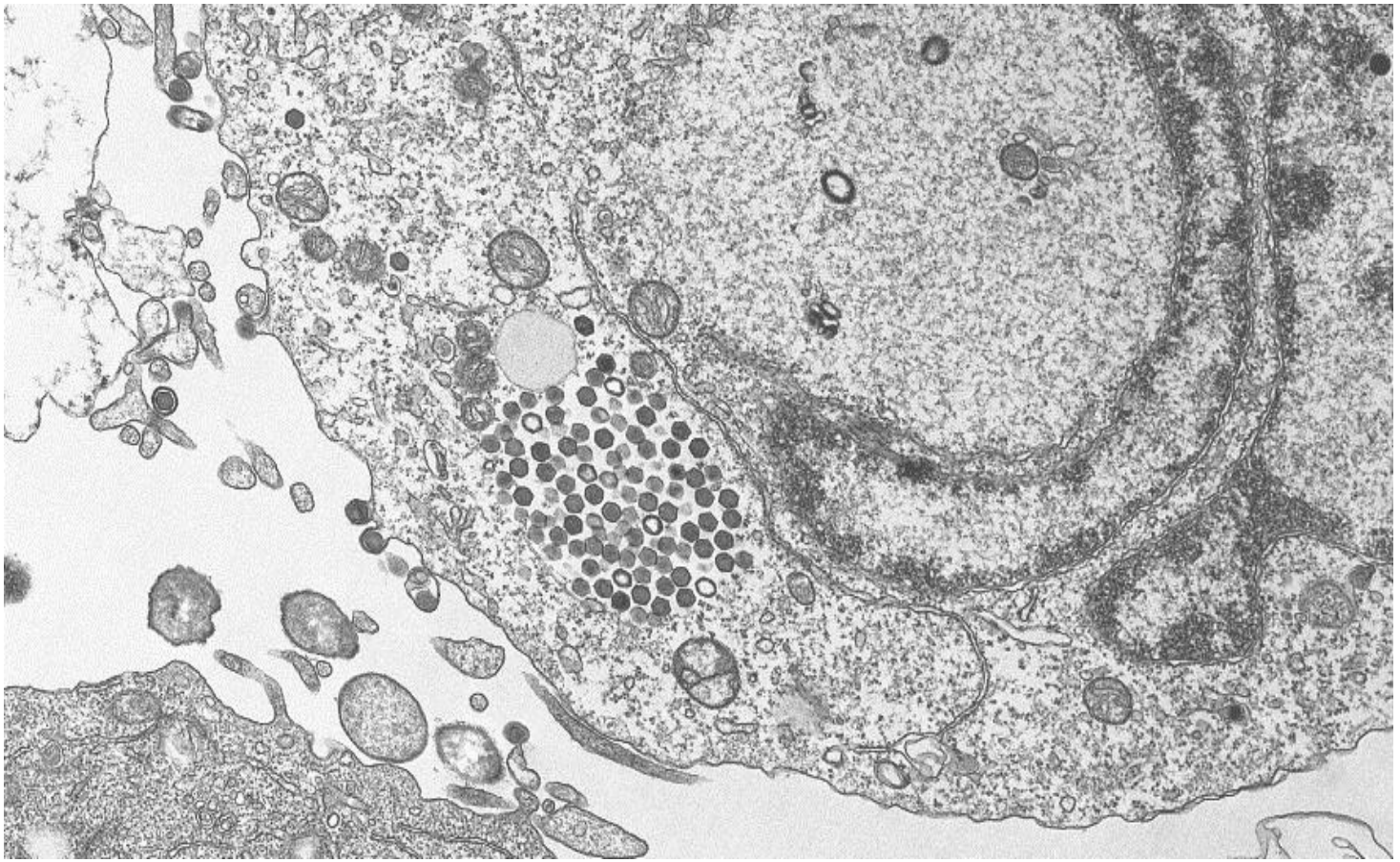
Virions within viral assembly sites



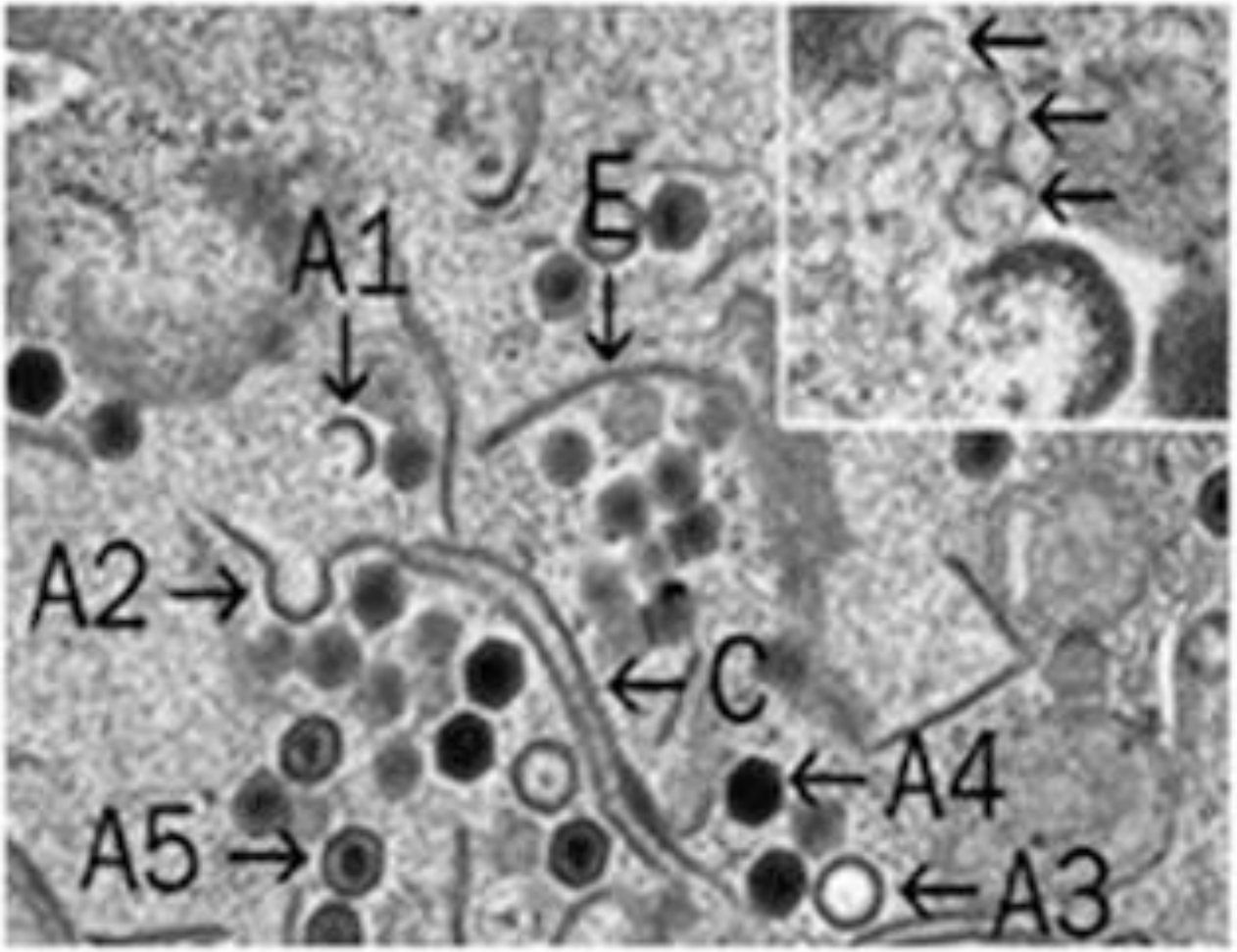
Virions within para-crystalline array

Mitochondria

Alex Hyatt (AAHL)



Low magnification image of an FV3-infected FHM cell. A large, centrally-located viral assembly site, a paracrystaline array of virus particles, and a few virions budding from the lower are shown.

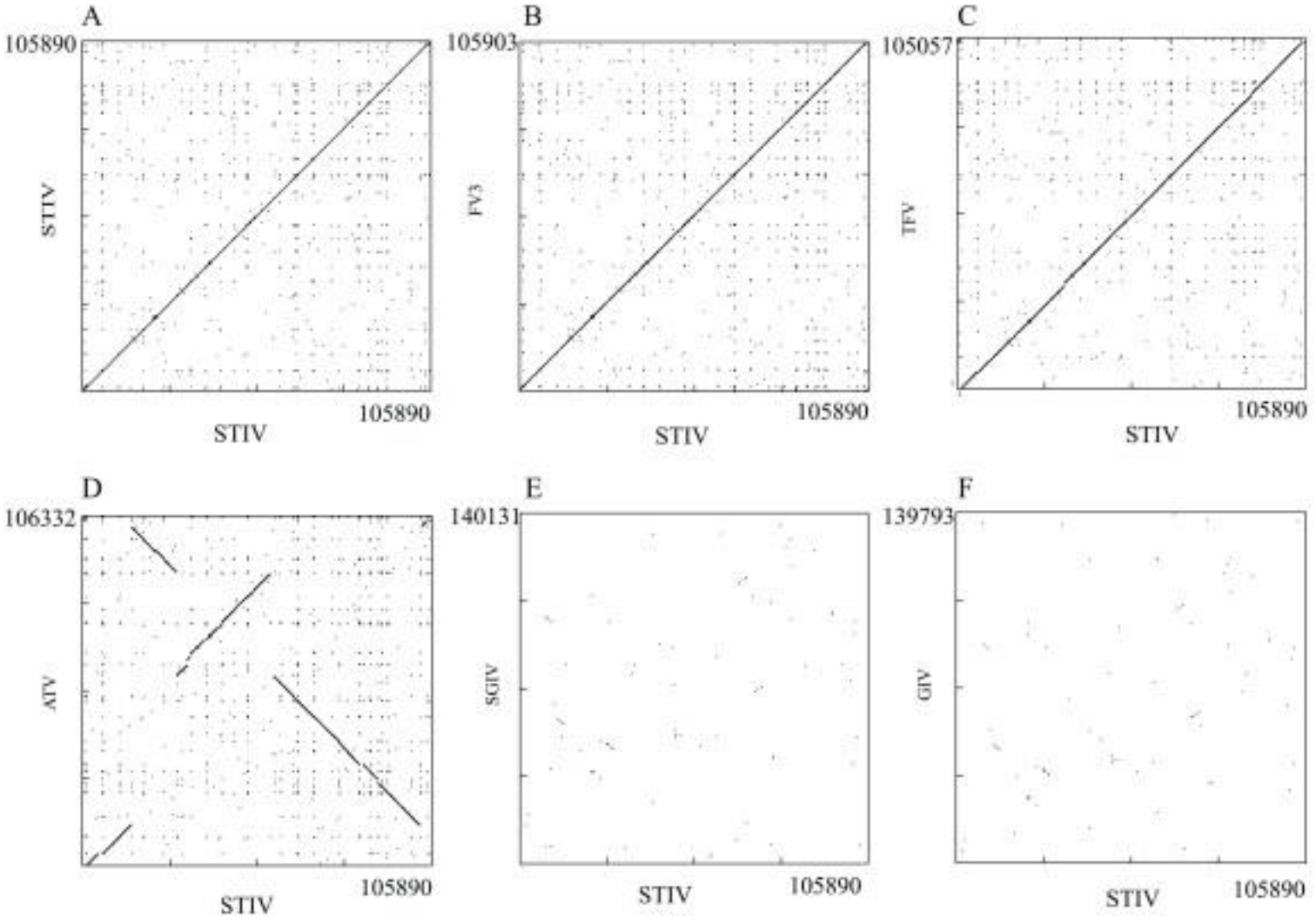


Putative stages in assembly of FV3 virions: Arrows in Inset, host-derived scaffold membranes above an assembly intermediate; A1 and A2, assembly intermediates; A3, empty capsid; A4 and A5, full virions; E and C, aberrant forms often seen late during infection cycle.

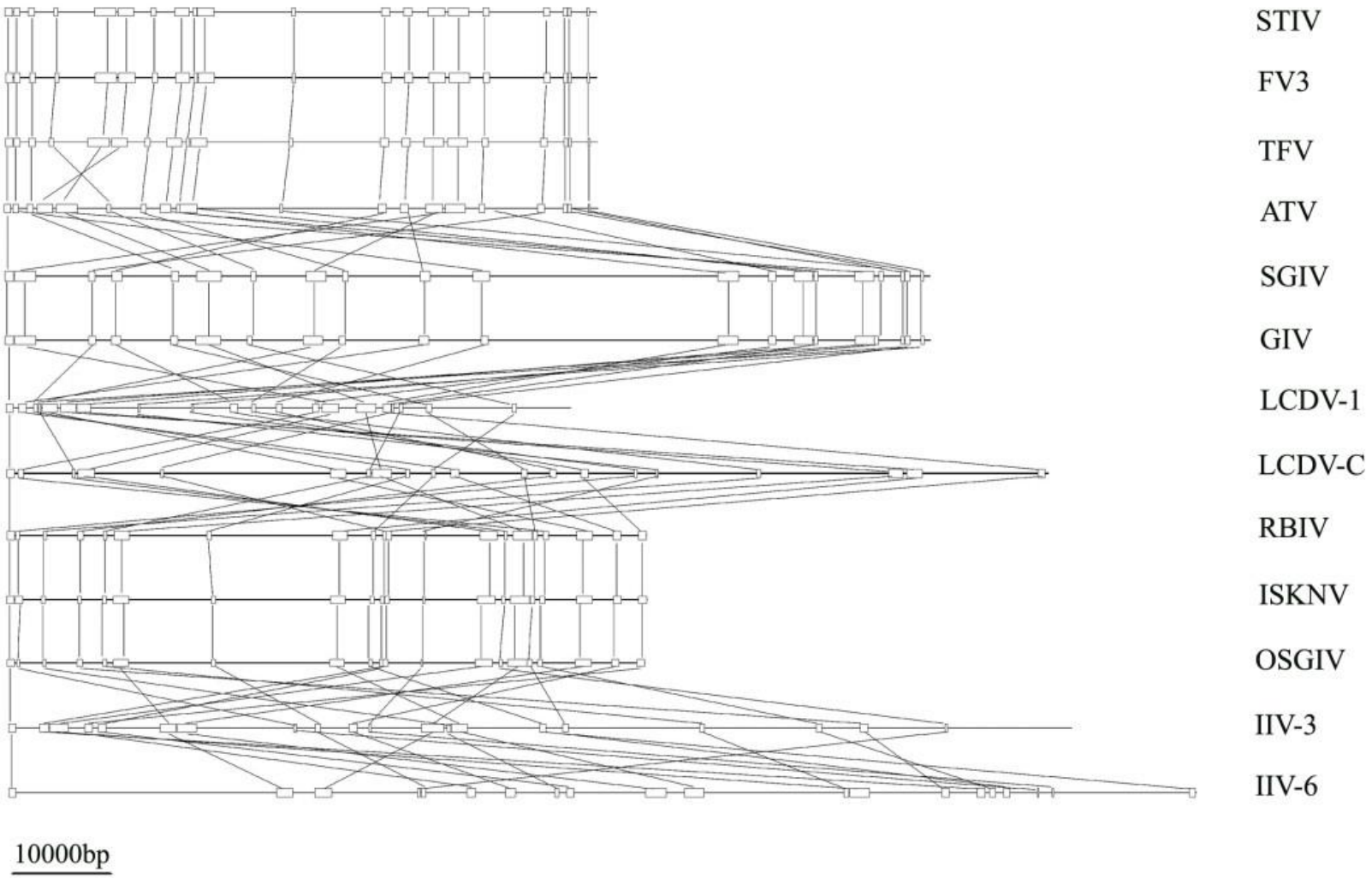
Genomes and Genes

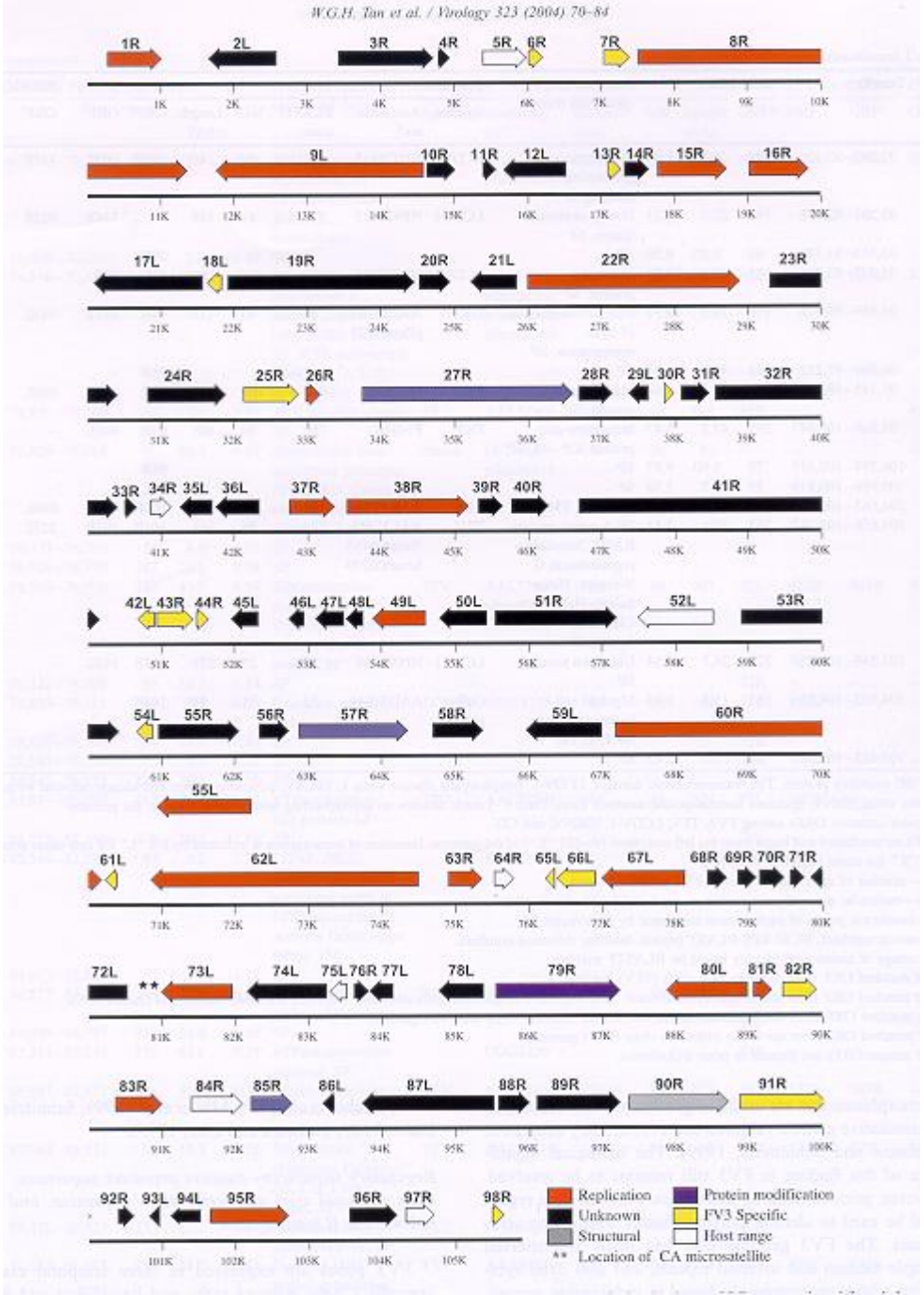
Iridovirid Genomic Sequences

Genus	Species[a]	Size (bp)	No. ORFs[b]	% 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	TFV	105,057	105	55	AF389451
	ATV	106,332	92	54	AY150217
	FV3	105,903	97	55	AY548484
	RGV	105,791	106	55	JQ654586
	CMTV	106,878	104	55	JQ231222
	STIV	105,890	103	55	EU627010
	EHNV	127,011	100	54	FJ433873
	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



Dot-Plot Analyses: Displays differences in the orientation of viral genes within a genome.


STIV
FV3
TFV
ATV
SGIV
GIV
LCDV-1
LCDV-C
RBIV
ISKNV
OSGIV
IIV-3
IIV-6
10000bp



FV3 ORFs:

19 RED (Replication)

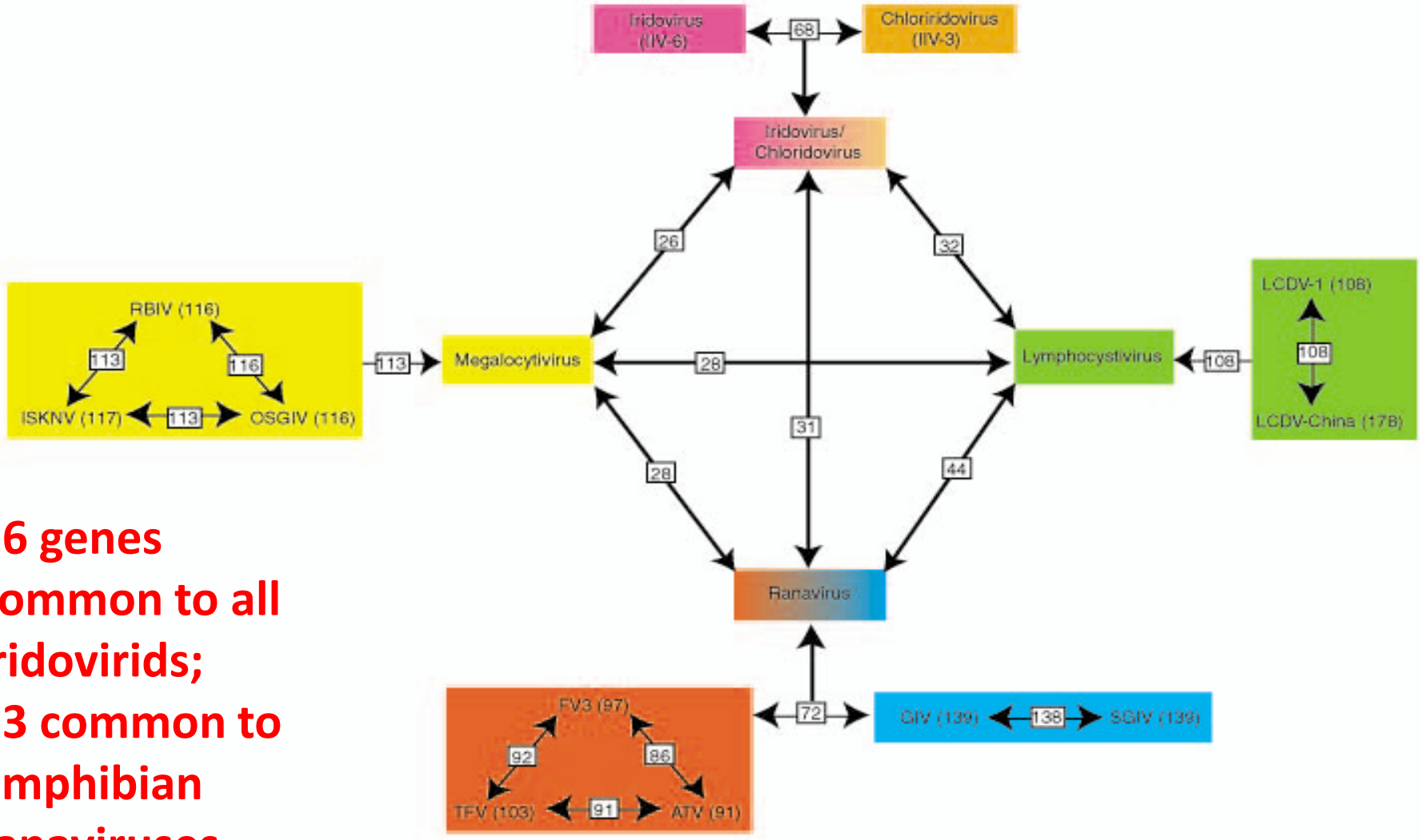
5 Blue/Gray (structural)

51 Black (UNKNOWN)

15 Yellow (FV3-specific)

~100 ORFs (non-overlapping)

Tan et al., 2004



26 genes common to all iridovirids; 13 common to amphibian ranaviruses

Eaton et al., 2007 Virology J 4:11 and Jancovich et al., 2010.

FV3 Genes

- **Replicative Genes**
  - DNA/RNA Pol
  - Major capsid protein
  - DNA repair
  - Myristylated membrane protein
- **Immune evasion, HR, Virulence/Efficiency**
  - **vIF-2α**
  - **Steroid synthesis (β-HSD)**
  - **vCARD**
  - **TNF receptor**
  - **Bak-like, IAP-like**
  - **dTTP synthesis: RR, TK, dUTPase**
  - **13 amphibian RV-specific genes; 27 RV-specific genes**

How does one determine viral gene function?

- Temperature sensitive (*ts*) mutants
- Knock down studies using asMOs and siRNAs
- Knock out studies using homologous recombination to generate deletion mutants or conditionally-lethal mutants
- Ectopic expression of viral proteins

FV3 *ts* mutants

- Naegele and Granoff, Virology 44: 286 – 295, 1971
- Purifoy et al., Virology 54: 525 – 535, 1973
- Chinchar and Granoff, J. Virology 58: 192 – 202, 1986.
- 28 mutants placed into 19 complementation groups and 3/4 classes
  - Class I: 12 CG, 16 mutants – E^+ L^+ DNA^+ AS^+ $Virions^+$ [Assembly/Infectivity]
  - Class II: 4 CG, 5 mutants – E+ L- DNA+ AS+/-
  - Class III: 1 CG, 1 mutant – E+ L- DNA+ AS-

  } Late viral RNA synthesis

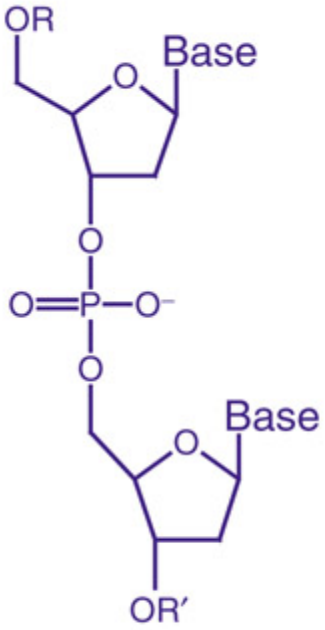
  - Class IV: 2 CG, 5 mutants – E+ L- DNA- AS- [DNA synthesis]

Knock Down:
Antisense Morpholino Oligonucleotides (asMOs) and siRNAs

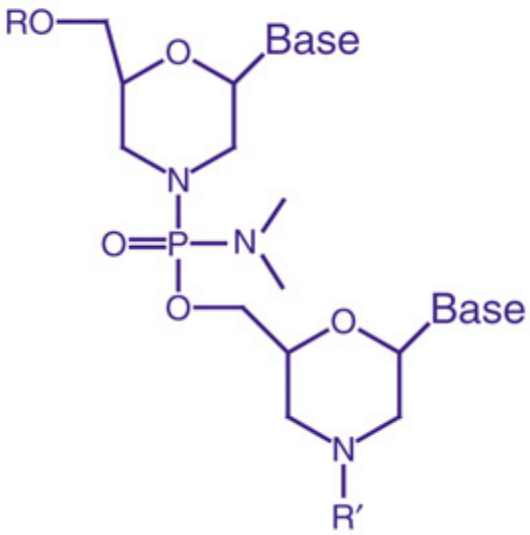
asMOs: single-stranded 25-mers that bind within the 5' NTR, or in the immediate vicinity of the AUG initiation codon, and block protein synthesis by inhibiting ribosomal movement.

siRNAs: double-stranded molecules 21 – 22 nts in length with 2 nt overhangs. The complementary strand binds mRNA at various points within coding or non-coding regions. Subsequently, DICER triggers mRNA degradation. Alternatively, siRNA bindings leads to a translational block.

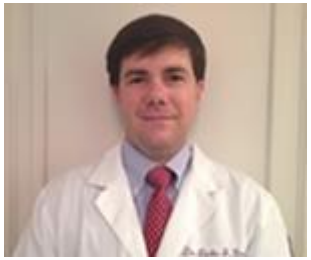
Structure of asMOs

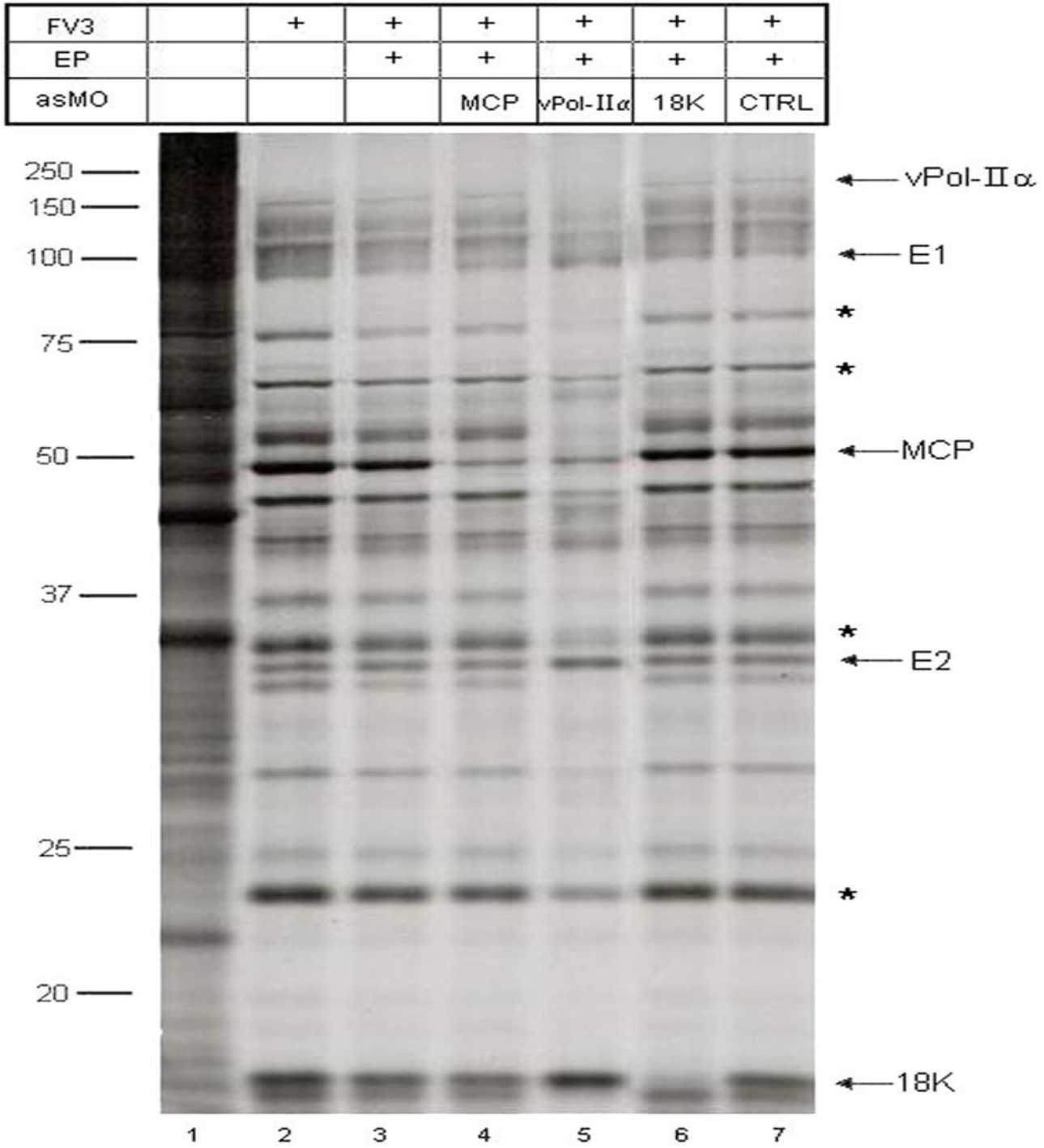


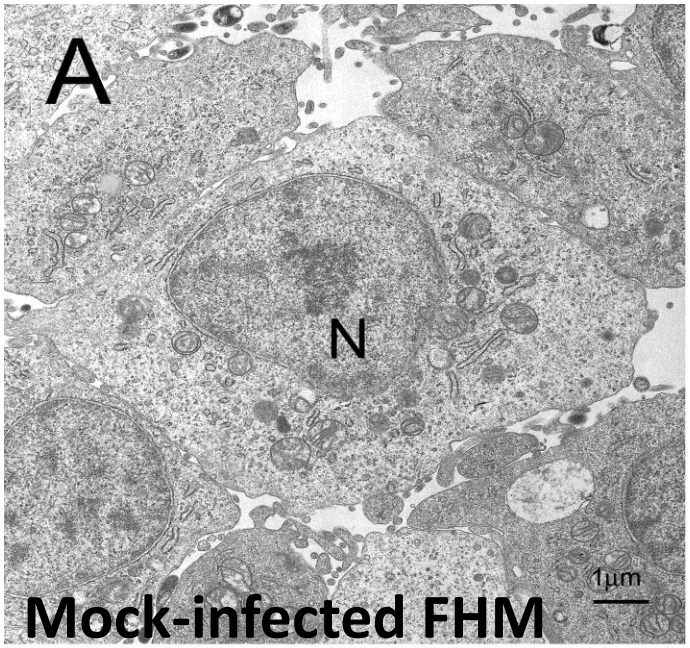
Phosphodiester
DNA

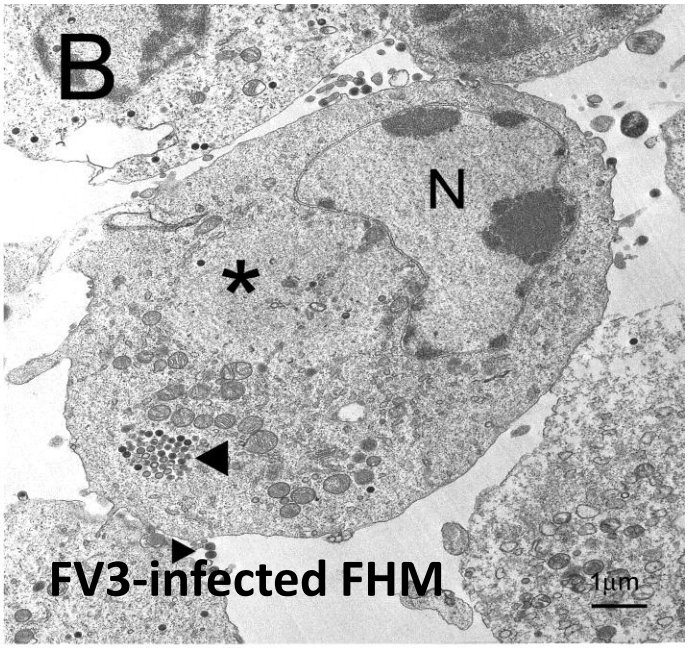


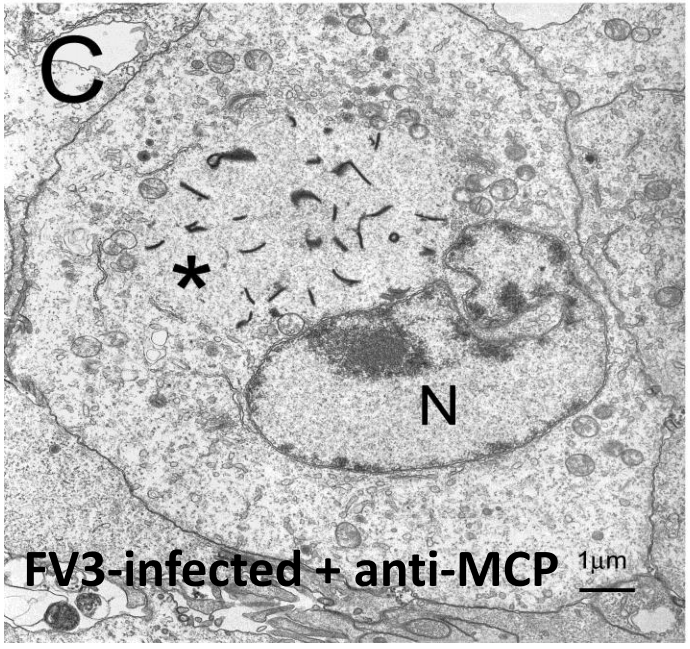
Morpholino

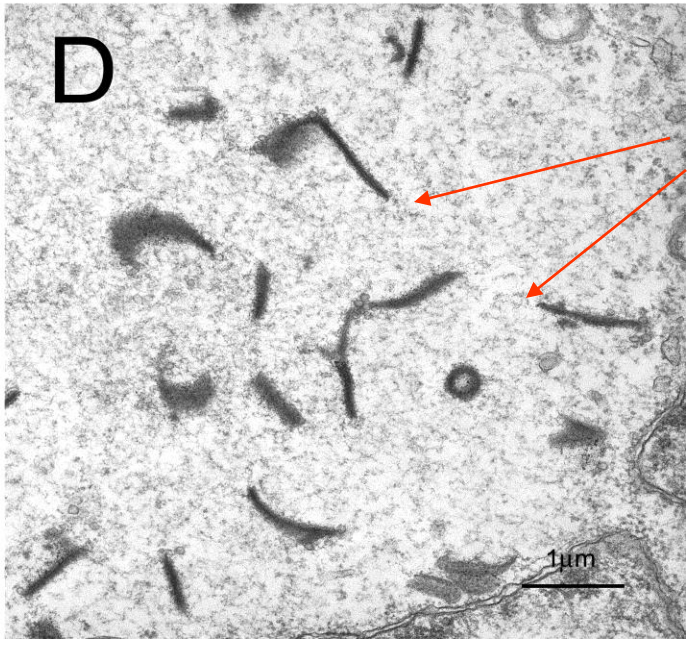



FV3 + + + + + +
EP + + + + +
asMO MCP vPol-IIα 18K CTRL
250
150
100
75
50
37
25
20
vPol-IIα
E1
*
*
MCP
*
E2
*
18K
1 2 3 4 5 6 7

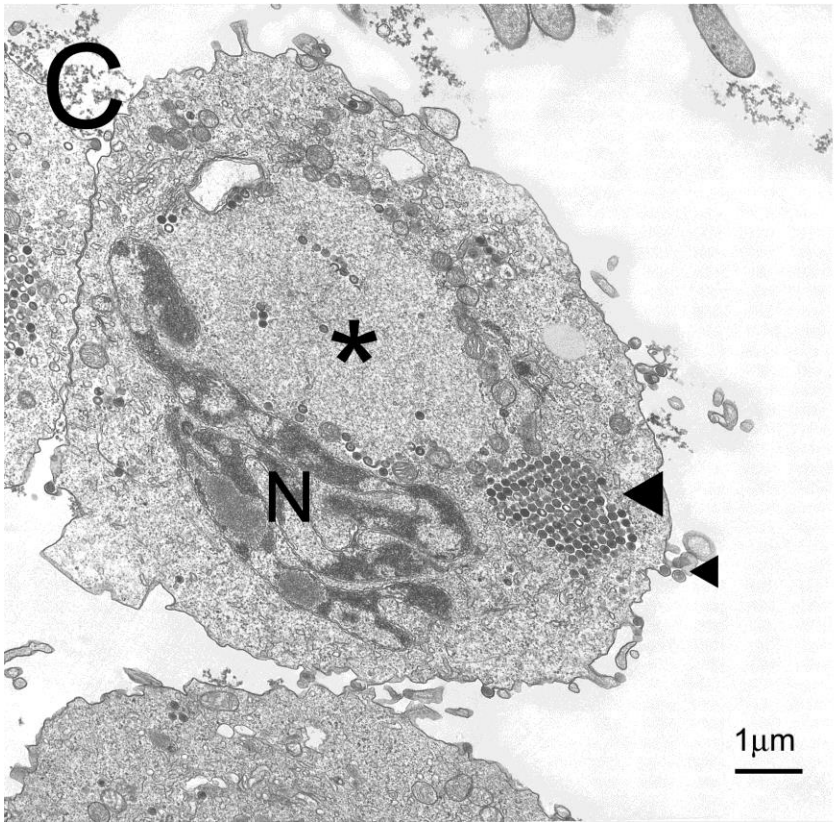

A
N
1µm
Mock-infected FHM


B
N
*
FV3-infected FHM
1µm

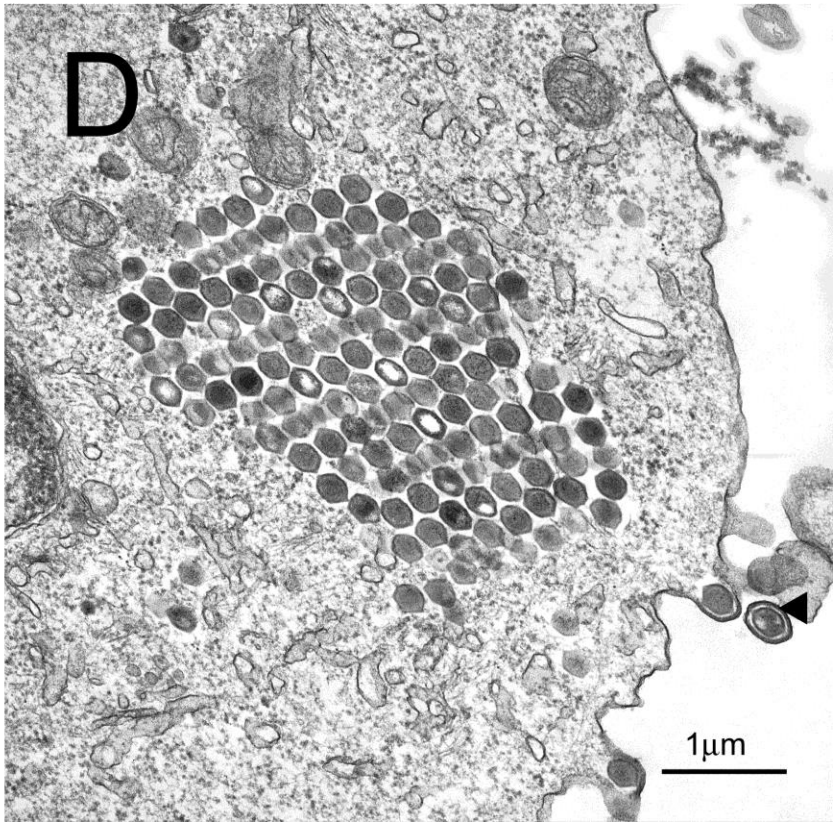

C
*
N
FV3-infected + anti-MCP
1µm


D
AE
1µm

Knock down of 18K synthesis does not affect virion formation or viral infectivity



FV3 + anti-18K MO

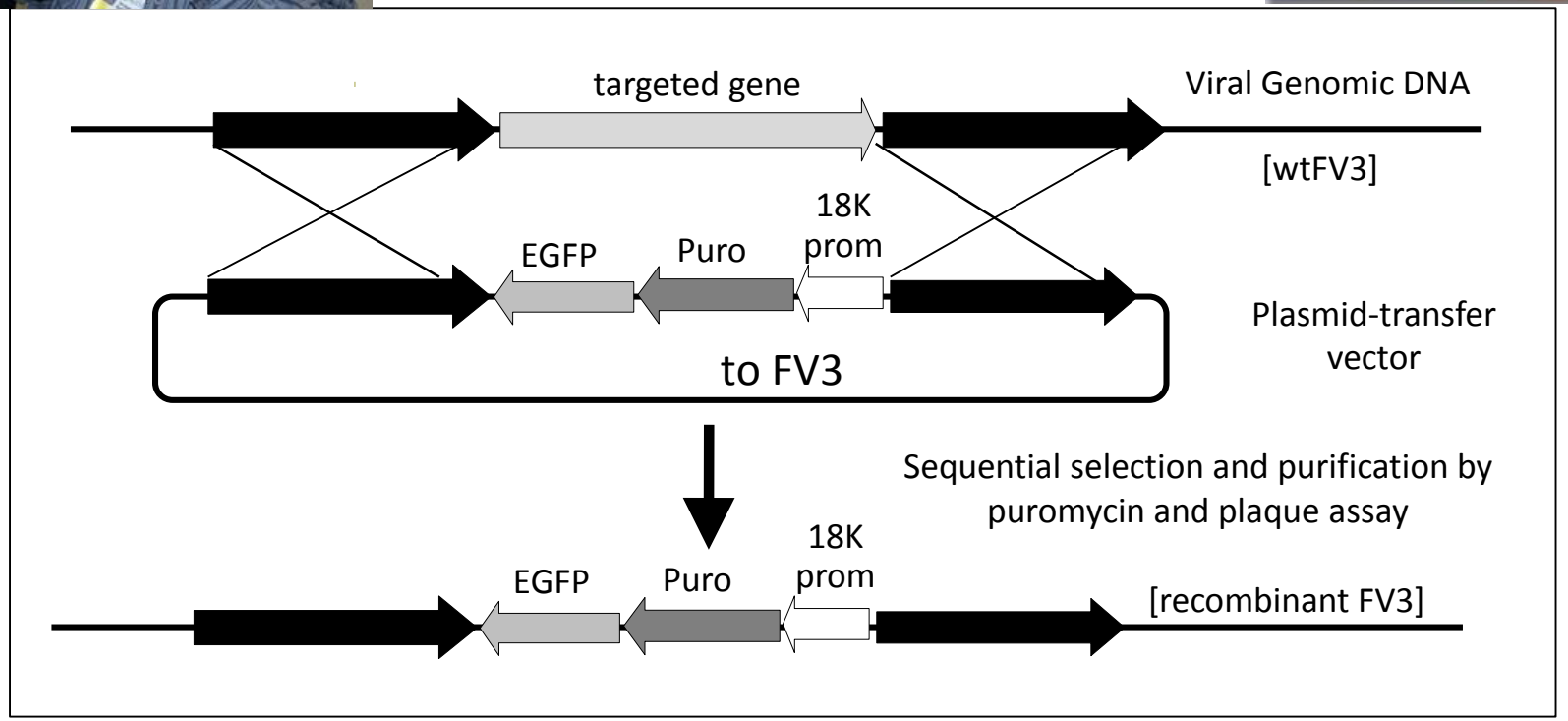


FV3 + anti-18K MO

Knock Down Studies: Summary

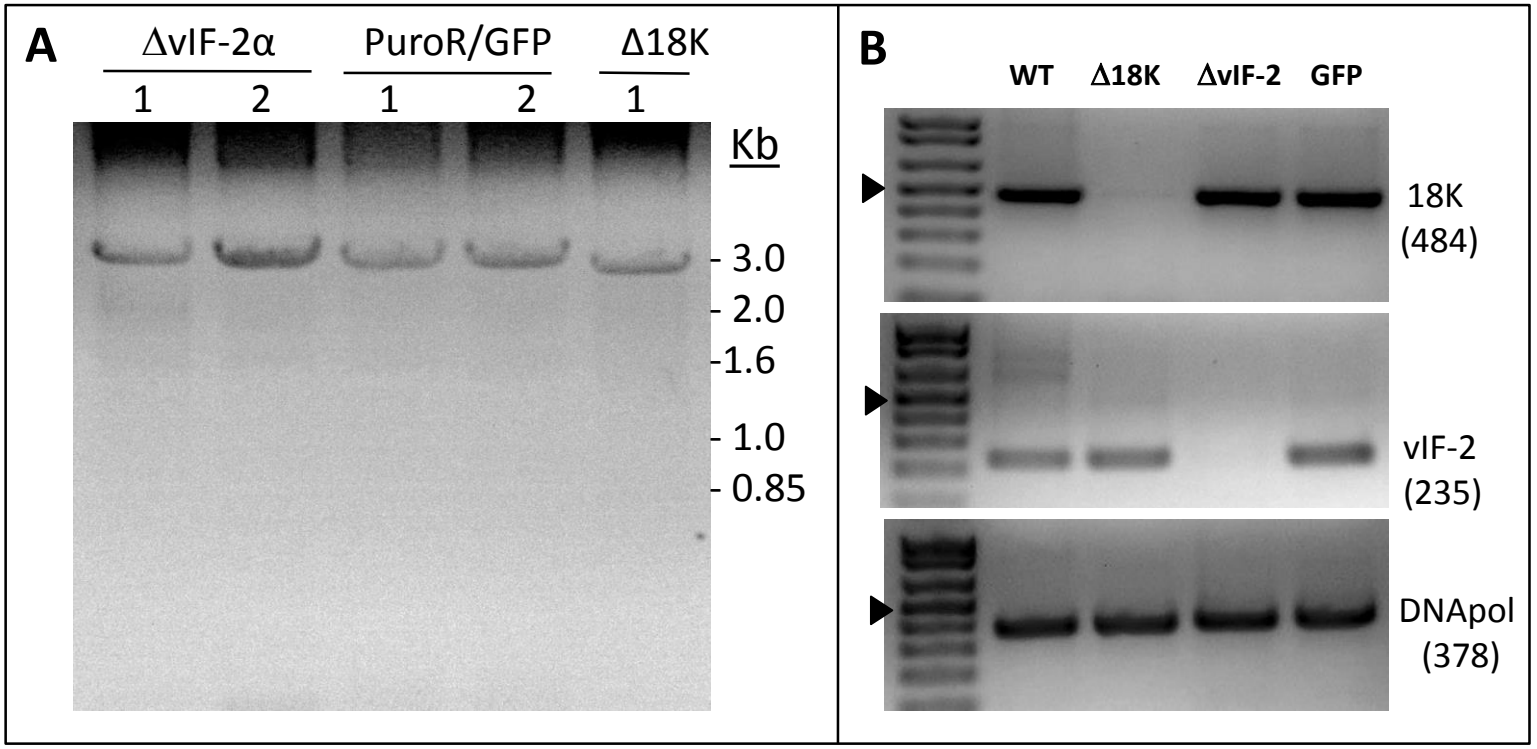
- KD of MCP, vPOL-IIα, 53R, 46K and 32R (asMOs) and MCP, vPOL-IIα and DMTase (siRNa) resulted in a marked drop in viral replication.
- KD distinguishes essential from non-essential genes.
- KD studies are limited by
    - inability to detect some viral proteins by SDS-PAGE,
    - sequence of target mRNA,
    - inability to function *in vivo*.

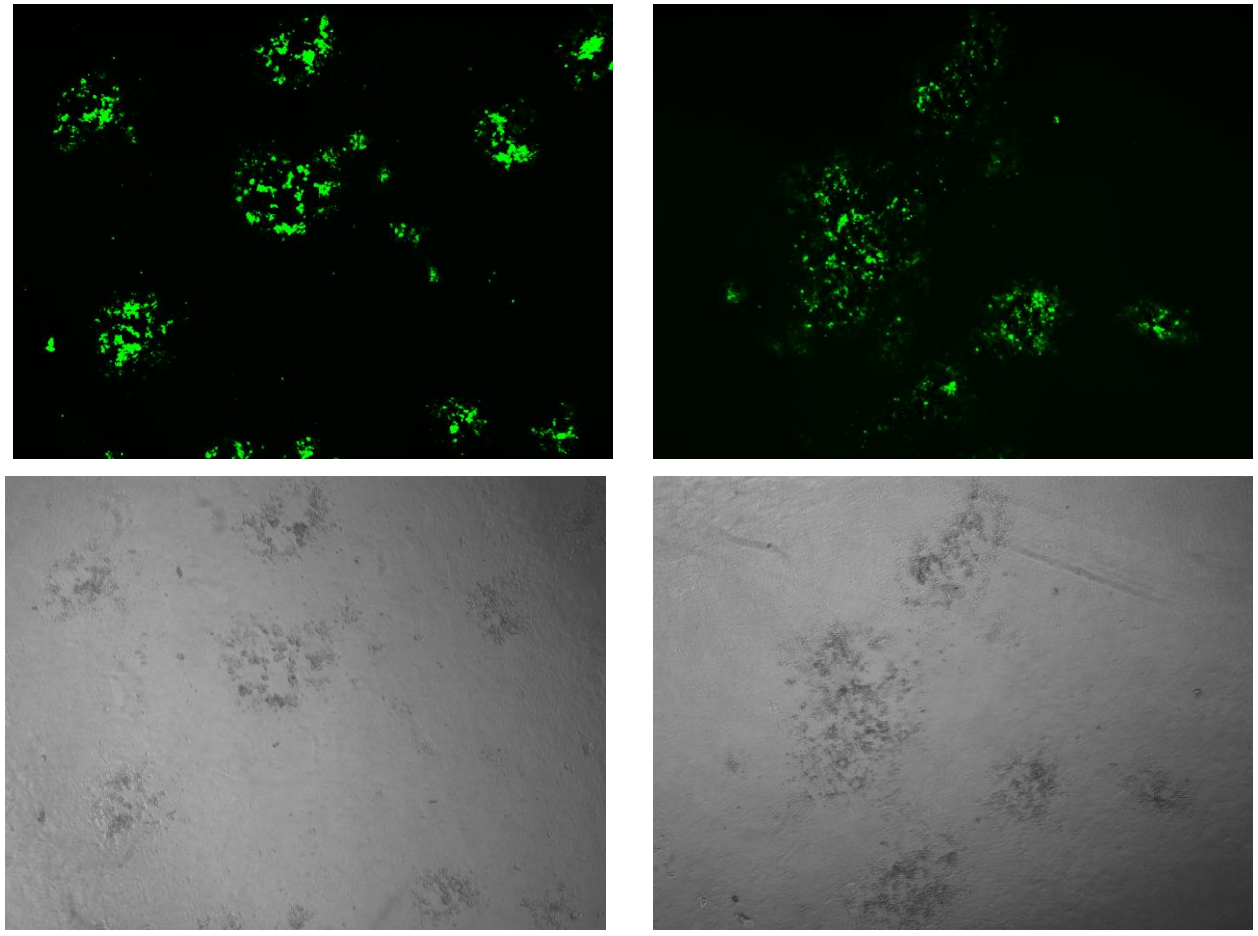
Knock Out



Chen and Robert (U. Rochester Medical Center);
Jancovich and Jacobs (Arizona State University)

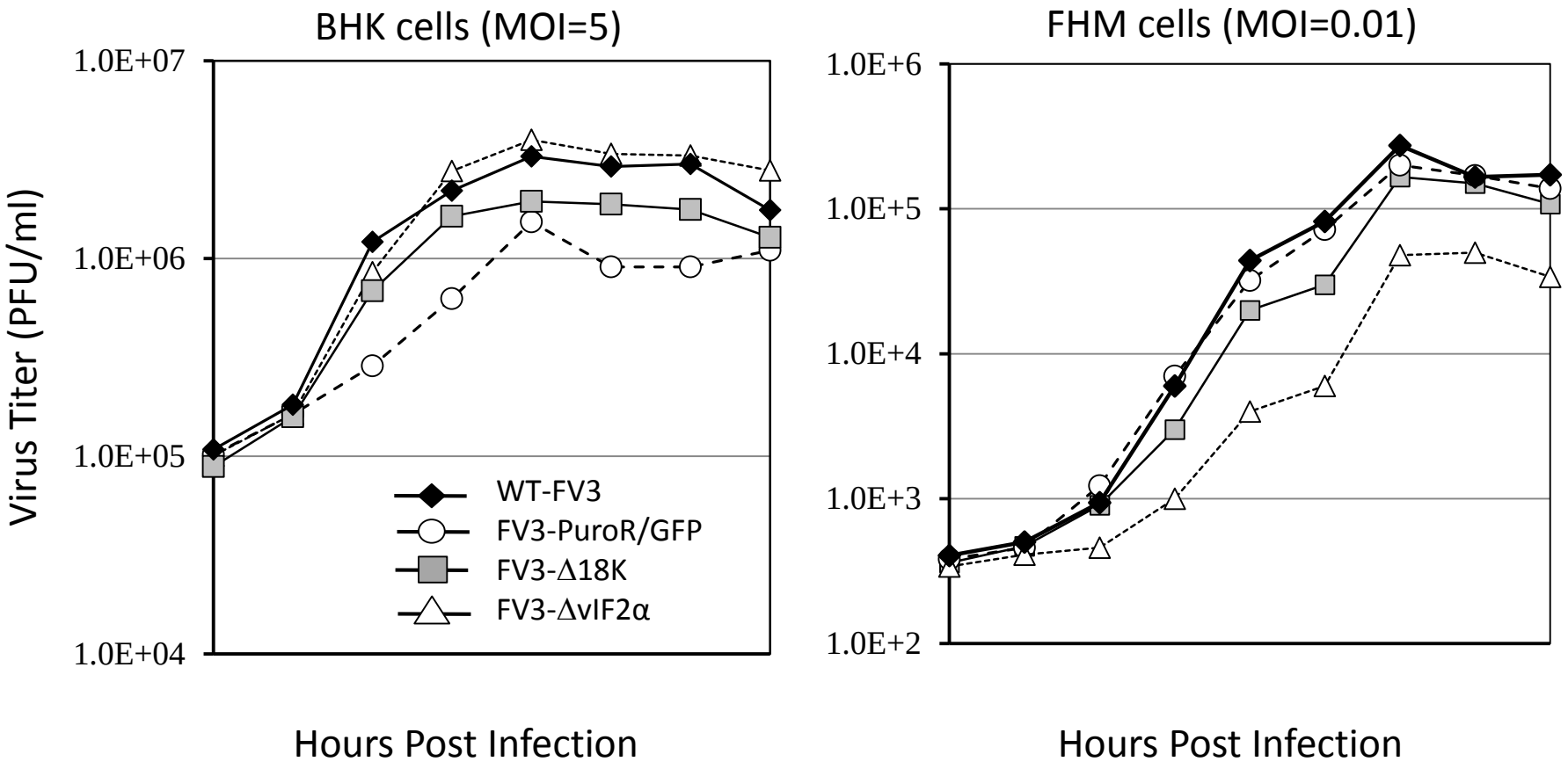
Confirmation of PuroR/GFP insertion and 18K and vIF-2 deletion

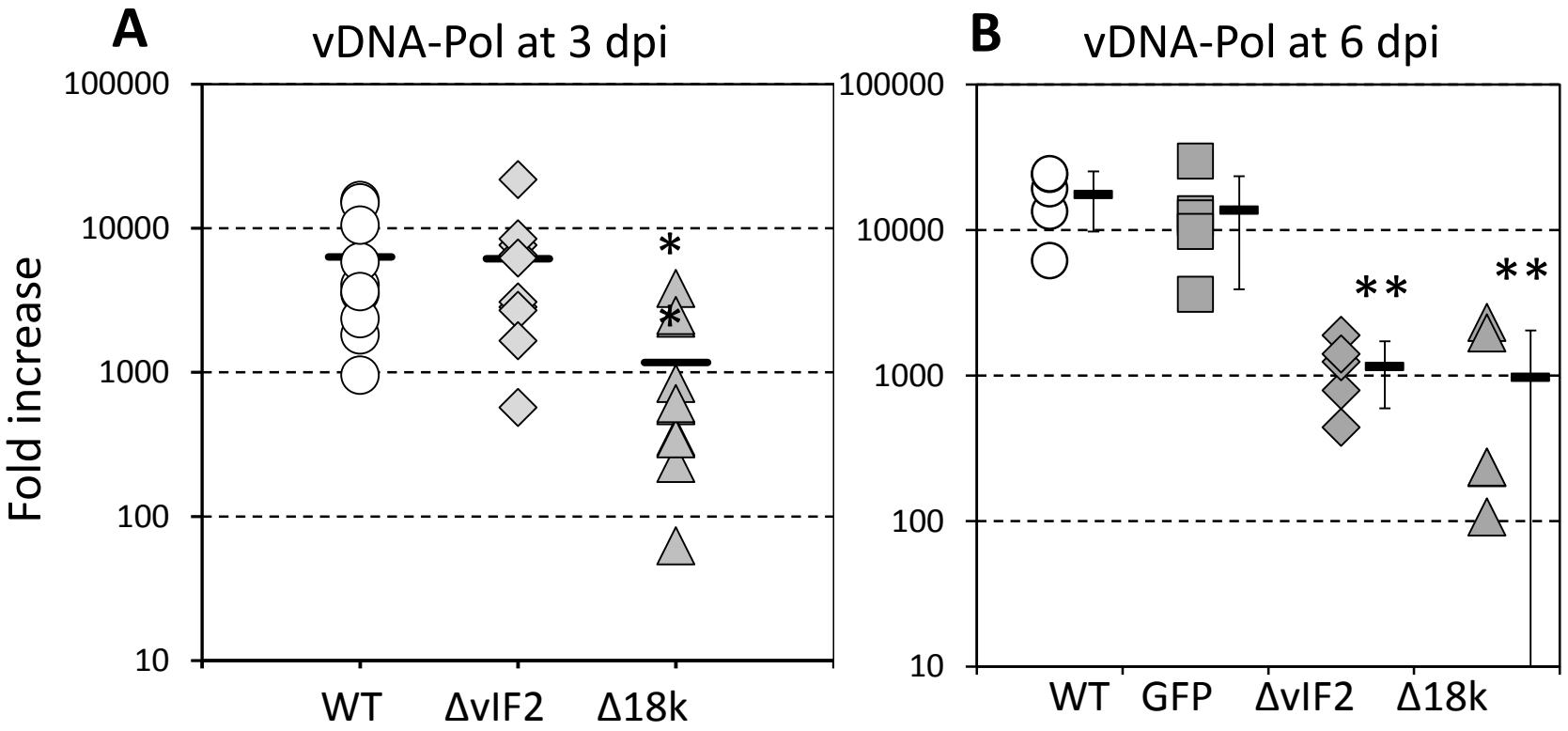




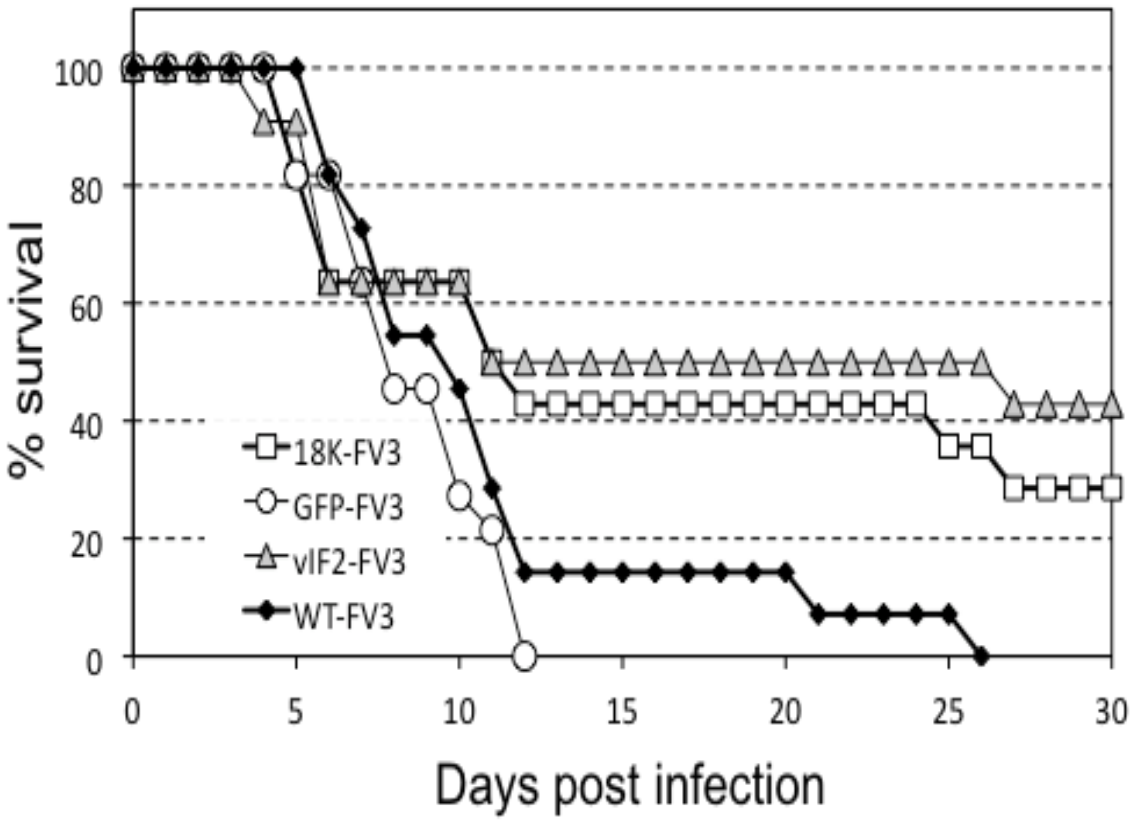
FV3-ΔvIF-2α

FV3-Δ18K


BHK cells (MOI=5)
1.0E+07
1.0E+06
1.0E+05
1.0E+04
Virus Titer (PFU/ml)
WT-FV3
FV3-PuroR/GFP
FV3-Δ18K
FV3-ΔvIF2α
Hours Post Infection
FHM cells (MOI=0.01)
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
Hours Post Infection


A
vDNA-Pol at 3 dpi
B
vDNA-Pol at 6 dpi
Fold increase
100000
10000
1000
100
10
*
*
**
**
WT
ΔvIF2
Δ18k
WT
GFP
ΔvIF2
Δ18k

P<0.01

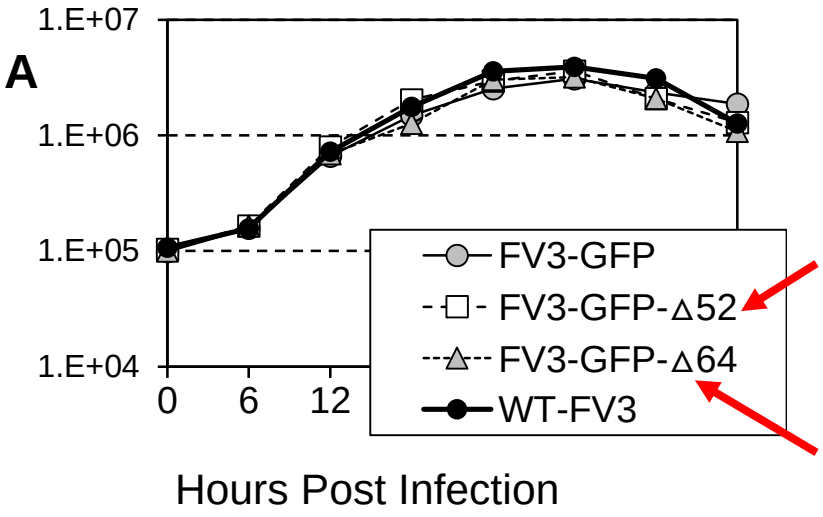


Challenge of *Xenopus* tadpoles with control and KO mutants

Single-Step Growth Curve (MOI=5

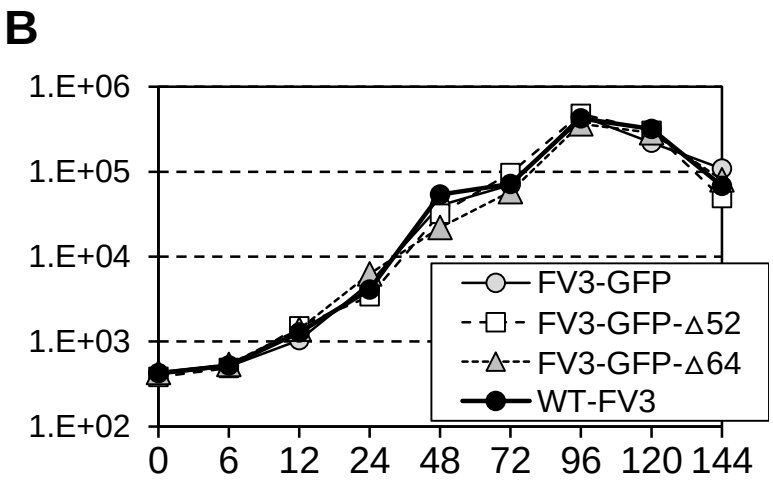
VacV **HSD** is thought to upregulate steroid synthesis, depress antiviral responses and enhance virus replication.

vCARD may short-circuit CARD dependent signaling, trigger apoptosis and/or depress innate immunity, and enhance virus replication.



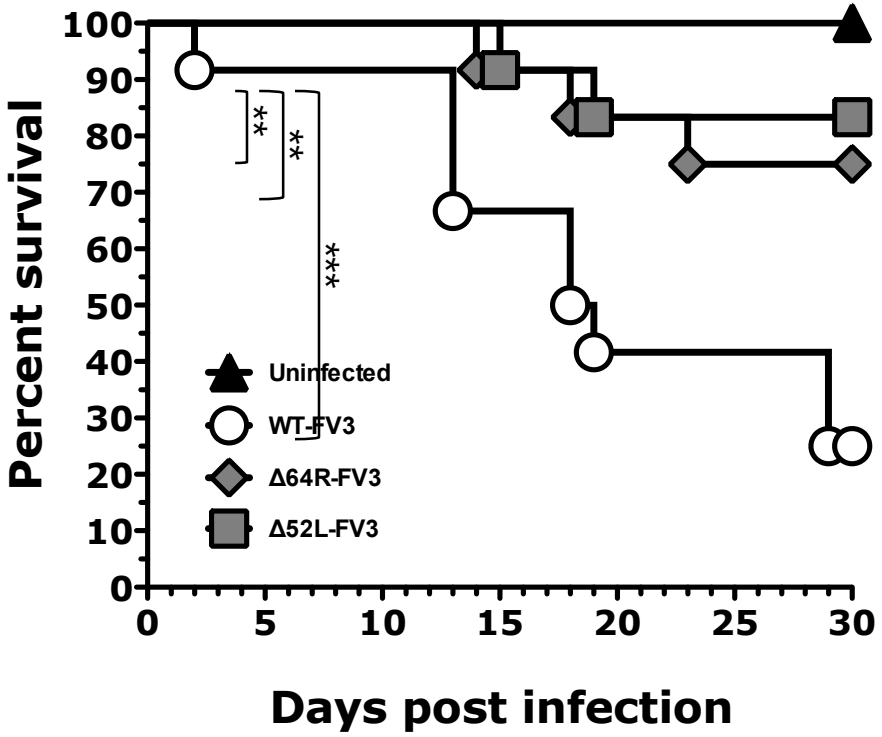
Beta hydroxysterioid dehydrogenase (HSD)

vCARD (caspase activation and recruitment domain)



Mutiple-Step Growth Curve (MOI=0.01)

vCARD and HSD KO mutants are also markedly attenuated *in vivo*.

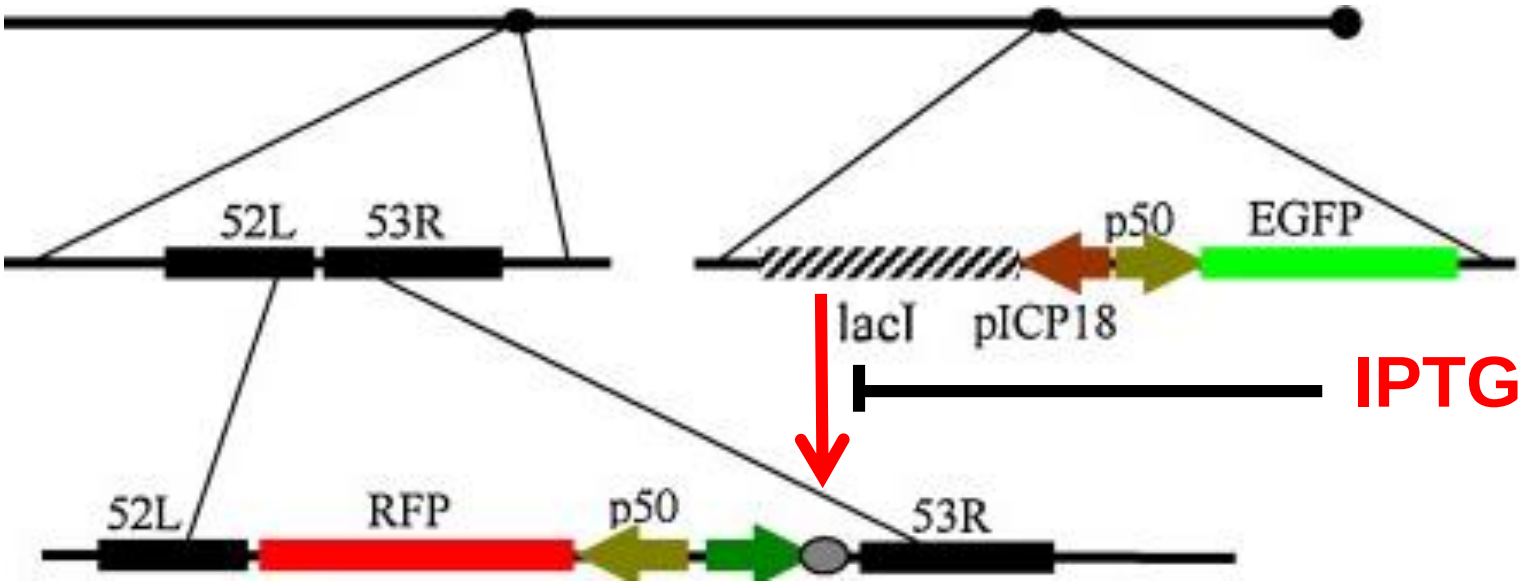


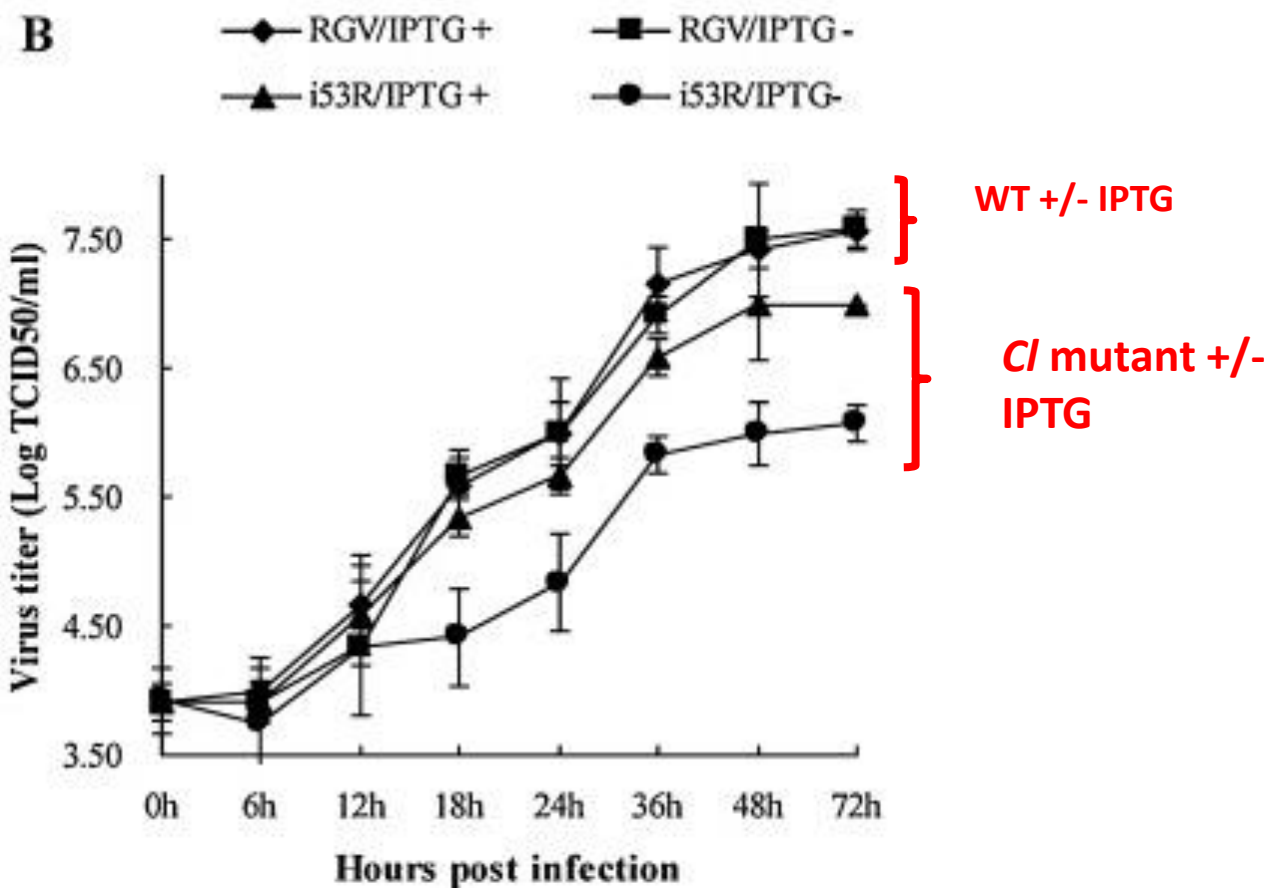
FV3 KO mutants

- 4 KO mutants have been generated
  - FV3Δ-18K, -vIF-2α, -βHSD, -vCARD
- KOs can be used both ***in vitro* and *in vivo,*** and should be constant in their phenotype.
- Identify **non-essential genes** that may play key roles in virulence, host range, and immune evasion.

Conditionally-lethal mutants

- He LB, Gao XC, Ke F, Zhang QY (2013) A conditional lethal mutation in *Rana grylio* virus ORF 53R resulted in a marked reduction in virion formation. **Virus Research** 177: 194 – 200.


52L
53R
p50
EGFP
lacI
pICP18
IPTG
52L
RFP
p50
53R

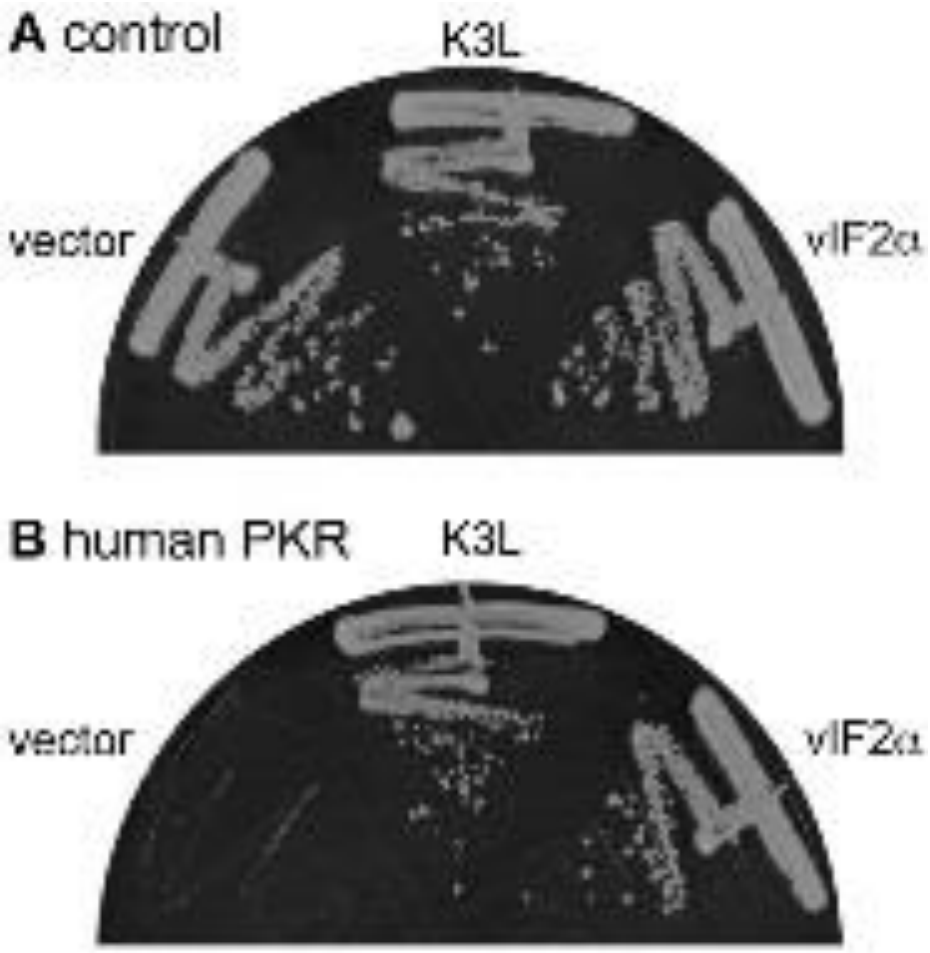

B
RGV/IPTG+
RGV/IPTG-
i53R/IPTG+
i53R/IPTG-
Virus titer (Log TCID50/ml)
7.50
6.50
5.50
4.50
3.50
0h
6h
12h
18h
24h
36h
48h
72h
Hours post infection
WT +/- IPTG
CI mutant +/- IPTG

Conditionally-lethal Mutants:

- **PRO:**
  - Target both essential and non-essential genes
  - Mutants can be propagated in *vitro* in the presence of the inducer.
  - The effects of knock down can be studied both *in vitro* and *in vivo* in its absence.
- **Con:**
  - In the absence of the inducer, expression may be leaky making assignment of function difficult.

Ectopic Expression

- Transfection of a vector that drives the expression of an isolated ranavirus gene provides another way to ascertain viral gene function.
  - Xia et al., Identification and characterization of SGIV ORF162L, an immediate early gene involved in cell growth control and virus replication. Virus Res. 39 (2010).
  - Rothenburg S et al., Characterization of a ranavirus inhibitor of the antiviral protein kinase PKR BMC Microbiol. 1:56 (2011)



Rothenburg et al., 2011. When PKR is ectopically expressed in yeast, cell growth is blocked. Cell growth can be restored by ectopic expression of either VacV K3L or ranavirus vIF-2α.

Ectopic Expression

- PRO
  - Facile method to ascertain gene function based on changes in phenotype
  - Provides a way to generate recombinant protein for use in developing antigen-specific antibodies
- CON
  - Over-expression of the expressed gene may generate phenotypes that do not reflect the authentic function of the gene product.

Ecological/Population/Immunological Studies

- RVs and species declines (Gray, Storfer, et al.)
- Viral transmission and persistence (Brunner, Jensen, Picco)
- Host susceptibility and pathology (Hoverman, Green, Miller)
- Pesticides and RV infections (Kirby)
- Host shifts among RVs (Jancovich)
- Host-Pathogen co-evolution (Lesbarreres, Storfer)
- Host anti-viral immunity (Robert) and viral anti-host immunity (Robert, Chinchar)
- Identify viruses from various hosts and geographic regions (Balseiro, Schock, Duffus, Mazzoni, Une, Kanchankhan, Waltzek, Marschang, Allender, Ariel)

Ranaviruses: Past and Present

Old view

- RVs are relatively harmless viruses that provide insight into a poorly-characterized virus family.
- They are useful molecular models for the study of DNA methylation and its effect on transcription, host-shutoff, etc.

New realization

- RVs are responsible for localized die-offs among ecologically and commercially important ectothermic animals .
- The "die-off trigger" is not known, but likely involves interplay between intrinsic viral functions and extrinsic factors (e.g., host immunity, stress, etc.) .
- FV3 and *Xenopus laevis* are excellent models with which to explore the correlates of anti-viral immunity in lower vertebrates.

The Future

- Identify viral gene function and understand their role in replication and virulence
  - Immune evasion, host range; virion assembly
- Identify host and reservoir species and ascertain their roles in initiating infections and maintaining virus in the environment
  - Is FV3 really a “frog virus” ? Is LMBV an isolate of DFV ?
- Understand what host, viral, and environmental factors trigger disease/persistence/recrudescence
  - FV1 and FV2 were isolated from “healthy” frogs; what makes LMBV pathogenic?
- Determine if susceptible species can be protected by vaccination with KO mutants?
- Does the genus *Ranavirus* consist of 6+ unique species, or are there fewer species but multiple isolates displaying various host preferences and degrees of pathology?
  - A Regulatory/Taxonomic issue?

References

- “Lesser known large dsDNA viruses,” James Van Etten (Ed.), Springer, 2009.
- “Ranaviruses: Lethal pathogens of ectothermic vertebrates,” MJ Gray and VG Chinchar (Eds.), Springer Open, 2015.