Bio-engineering Vaccinia viruses for increased oncolytic potential

Adrian Pelin

Supervisor

Dr. John C. Bell, Ph. D

This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the Doctorate in Philosophy degree in Biochemistry

Department of Biochemistry, Microbiology & Immunology

Faculty of Medicine

University of Ottawa

Abstract

Vaccinia virus has a large and still incompletely understood genome although several strains of this virus are already in clinical development. For the most part, clinical candidates have been attenuated from their wild type vaccine strains through deletion of metabolic genes like the viral thymidine kinase gene. In the present work, we thoroughly examined the genetic elements of vaccinia which could be modulated to improve tailor the virus as a cancer therapeutic. Using a variety of cancer cell lines and primary tumor explants, we performed a fitness assay that directly compares multiple wild-type Vaccinia strains to identify the genetic elements that together create an optimal “oncolytic engine”. Using a transposon insertion strategy and deep sequencing of viral populations we systematically examined Vaccinia genes that do or do not play a role in the therapeutic activity of the virus. Our studies allowed us to identify a variety of genes in the vaccinia genome that when deleted, augment the oncolytic activity of a newly engineered Vaccinia virus. In the context of this thesis, I define enhanced oncolytic activity as superior therapeutic activity, increased immunogenicity and an improved safety profile, all aspects which we used to compare this novel virus to Vaccinia viruses currently in the clinic.

Acknowledgments

It was an honour to have Dr. John Bell as my PhD supervisor and mentor for the last few years. John, you have always made time to discuss and evaluate new results and ideas. During our discussions and meetings, you have always given me constructive criticism and helped me grow as a scientist. I cannot thank you enough for giving me the freedom to explore and learn, for encouraging me to pursue independent ideas, for sending me to amazing conferences and for supporting all my scholarship applications and academic endeavours.

I would like to thank my TAC members Dr. Guy Ungerechts, Dr. Jean-Simon Diallo and Dr. Christopher Boddy. Your critical evaluation of my work, challenging questions and ideas have not only added value but helped me better understand my research. I would also like to thank Dr. Carolina Ilkow, Dr. Tommy Alain, Dr. Harold Atkins and Dr. Rebecca Auer for always giving me your input.

My research would not have been possible without past and present members of the Bell, Diallo, Ilkow, Auer and Ungerechts labs. Fabrice, Dom and MC, you were some of the first people I have interacted with and I really appreciate all the helpful discussions and techniques you taught me. Mohsen, Mike P., Mike H., Matt T. and Serge, you have taught me and helped me out with a lot of cloning, Westerns and other techniques, I appreciate it. Leonard, Katherine and Fanny, I really appreciated all your help with flow cytometry and discussions related to immunology. A very special thanks goes to Julia Petryk, you have spent hundreds of hours (I did the math) helping me perform animal experiment which have been critical to all of my research. I cannot ignore the fact that I was not always prepared yet you were always adapting and more than willing to help, thank you for always being there. Jessie, being my student for over a year you have helped me produce and reproduce a lot of my results and have done a lot of valuable

work that is now part of this thesis, thank you for that. Ragunath, nothing interfered with you making me understand anti-viral signaling. Thanks for all the work you have helped with and for all the feedback. It was a pleasure working with you all, for the smaller and bigger things you helped out with, big thanks go to our hard-working technicians: Anna K., Leah C., Annie, Andrew, Naveen, John C., Catia and Christiano as well as the rest of the lab members: Jiahu, Stephen B., Vicki, Mohammed, Nikolas, Larissa, Giuseppe, Mathieu, Brian K., Ashley, Briana, Justin C., Marie-Eve, Abera, Taylor, Hayley, Joanna, Anna J. and Harsimrat.

Thanks to my parents and parents in law for always asking about my work and for all the support. Finally, I am very grateful to my wife, Elena Scut, who has not only been immensely supportive and encouraging, especially during challenging times, but has also helped me out with countless flow experiments. I couldn't have gotten this far without you.

Table of Contents

Abstract II

Acknowledgments III

Table of Contents V

List of Abbreviations IX

List of Figures XI

List of Tables XIII

Chapter 1 – Introduction 1

- Preface 1
- The Problem 1
- The Solution: Oncolytic viruses as Cancer Therapeutics 2
  - Anti-viral response in Cancer 2
  - Cancer proliferation as an OV target 4
  - Tumors generate an immunosuppressive microenvironment 5
  - Oncolytic viruses can reverse cancer-induced immune-suppression 7
- Vaccinia as a therapeutic agent 9
  - Life cycle and biology 11
  - Virulence factors 14
  - Oncolytic Vaccinia 19
  - Targeting Vaccinia to the tumor 19
  - Enhancing Vaccinia immunogenicity 21
  - Combination of Vaccinia with other therapies 23
- Shortcomings and thesis rationale 24
- Objectives 25

Chapter 2 – Deletion of apoptosis inhibitor F1L in vaccinia virus increases safety and oncolysis for cancer therapy 30

- Preface 30
- Abstract 31
- Introduction 32
- Results 35

F1L KO increases immunogenic apoptosis 35
Removal of F1L increases VACV safety 40
Cop-ΔTK/F1L is more efficacious in glioblastoma tumors 43
Discussion 46
Materials and Methods 49
Cell Lines 49
Construction of plasmids 49
Generation of recombinant Vaccinia 50
In vitro virus yield 51
Viral pathogenicity 51
Mice and tumor models. 51
In vivo imaging 52
Immunohistochemistry 52
Statistical analysis 52
Funding 53
Author contributions 53
Chapter 3 – Viral genome editing reveals non-essential genes in the development of a safe and efficient oncolytic platform 54
Preface 54
Abstract 56
Introduction 57
Results 59
Identifying which strain replicates best in cancer cells 59
Genome-wide analysis reveals non-essential genes for oncolysis 68
Naturally occurring deletions of non-essential genes 71
CopMD5p3p is more tumor selective 84
CopMD5p3p is more immune stimulating 92
CopMD5p3p synergizes with cancer immunotherapy 97
Expression of antigens, antibodies and other transgenes 98
Discussion 104
Materials and Methods 109
Cell Lines 109
Viruses 109

Sequencing and assembly of virus genomes 110
Fitness Assay 110
Transposon Library Generation 111
Transcriptomic and RPF analysis 112
Cell Viability Assay 113
Flow cytometry 113
Mice and tumor models 114
Bioluminescent *in vivo* imaging 114
Virus biodistribution 115
General discussion, future directions and concluding remarks 116
Choosing a Vaccinia strain as the backbone of a therapeutic virus 116
Targeting Vaccinia to the tumor 117
CopMD5p3p is a tumor selective therapeutic capable of transgene expression 119
Thesis Shortcomings and Pitfalls 120
Concluding statement 121
References 122
Contribution of Collaborators 135
Appendices 136
A1. Supplemental information for Chapter 2 136
Supplemental Figure 136
A2. Supplemental information for Chapter 3 138
Supplemental Tables 138
Supplemental Figure 140
A3. Enhanced susceptibility of cancer cells to oncolytic rhabdo-virotherapy by expression of Nodamura virus protein B2 as a suppressor of RNA interference 142
Preface 142
Abstract 143
Introduction 144
Results 146
Discussion 161
Materials and methods 166
Acknowledgements 173
Funding 173

Availability of data and materials 174
Authors' contributions 174
References 175
Rights and Permissions 180
Chapter 2 180
Appendix A3 180
Curriculum Vitae 181

List of Abbreviations

APC	Antigen presenting cell
ARG1	Arginase 1
ATCC	American type culture collection
CEF	Chicken embryonic fibroblasts
cGAS	Cyclic GMP-AMP synthase
CSF	Colony-stimulating factor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CVA	Chorioallantois Vaccinia virus Ankara
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
ED50	50% Effective Dose
EEV	Extracellular enveloped virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor (gene)
eGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
FLT3LG	Fms-related tyrosine kinase 3 ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor (protein, cytokine)
HRE	Human Renal Epithelial cells
HSV	Herpes simplex virus
ICIs	Immune checkpoint inhibitors
ICS	Intracellular cytokine staining
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducibile nitric oxide synthases
IP	Intraperitoneal (route of administration)
IRF	Interferon regulatory Factor
ISGs	Interferon response genes
IT	Intratumoral (route of administration)
ITR	Inverted terminal repeats
IV	Intravenous (route of administration)
JAK	Janus kinase
MDSCs	Myeloid-derived suppressor cells
MOI	Multiplicity of infection
MV	Mature Virion
MVA	Modified Vaccinia Ankara
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing

OCT	Optimal cutting temperature
OV	Oncolytic virus
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PD1	Programmed cell death protein 1
PDL1	Programmed death-ligand 1
PFU	Plaque-forming unit
PKR	Protein kinase R
RACK1	Receptor for activated C kinase 1
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I like receptors
RPMI	Roswell park memorial institute
RR	Ribonucleotide reductase
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
subQ	Subcutaneous
TAM	Tumor associated macrophage
TK	Thymidine kinase
TLR	Toll-like receptor
TPM	Transcripts per million
Treg	Regulatory T cell
UTR	Untranslated region
VacV	Vaccinia Virus
VGF	Vaccinia growth factor
VSV	Vesicular stomatitis virus
WR	Western Reserve

List of Figures

Figure 1.1: Overview of Vaccinia genome features.

Figure 1.2: Overview of Vaccinia genes known to counter anti-viral response.

Figure 1.3: Comparison of deleted genes between CopMD5p3p, MVA and NYVAC.

Figure 2.1: Deletion of F1L increases cancer cell death without affecting replication.

Figure 2.2: Vaccinia Delta F1L induces rapid cancer killing in a wide variety of tumour types.

Figure 2.3: Deletion of F1L increases the safety and tumour selectivity of Vaccinia.

Figure 2.4: Treatment with Vaccinia delta F1L extends survival in a glioblastoma model and delays tumor growth in a syngeneic mouse colon model.

Figure 3.1: Bayesian analysis of completely sequenced Poxvirus genomes.

Figure 3.2: Schematic representation of the fitness assay.

Figure 3.3: Copenhagen is the Vaccinia strain which replicates the fastest in cancer cells.

Figure 3.4: Vaccinia Copenhagen produces more EEV particles compared to other wild-type strains.

Figure 3.5: PCR strategy to screen recombinants for a double deleted virus.

Figure 3.6: CopMD5p3p kills cancer cells faster maintaining replication levels compared to parental Copenhagen.

Figure 3.7: Vaccinia CopMD5p3p produces more EEV particles compared to parental wild-type Copenhagen.

Figure 3.8: Vaccinia CopMD5p3p forms larger plaques compared to parental wild-type Copenhagen.

Figure 3.9: Vaccinia CopMD5p3p can express transgenes at levels comparable to parental virus.

Figure 3.10: Vaccinia CopMD5p3p triggers an anti-viral response in normal cells.

Figure 3.11: Vaccinia CopMD5p3p does not express virion assembly genes in normal cells.

Figure 3.12: Increased tumour specificity and safety profile of CopMD5p3p virus.

Figure 3.13: CopMD5p3p is immunogenic and synergizes with immune checkpoint inhibitors.

Figure 3.14: Vaccinia CopMD5p3p induces a wide variety of cytokine release after systemic administration.

Figure 3.15: CopMD5p3p generates a strong T cell response against an encoded antigen.

Figure 3.16: Ribsosome profiling and RNA sequencing of transgene expressing CopMD5p3p.

Figure S2.1: F1L deletion decreases neurovirulence.

Figure S3.1: Relative fitness of five Vaccinia wild-type strains after passaging three times in various cancer models.

List of Tables

Table 3.1: List of transposon mediated gene interruptions rescued in Vaccinia Copenhagen. Information includes genomic position (GenBank M35027) of transposon insertion, insert location relative to the entire CDS of the gene as well as functional category of the affected gene.

Table 3.2: List of genes and their respective function deleted in CopMD5p, CopMD3p and CopMD5p3p viruses

Table S3.1: List of poxvirus genomes and their respective GenBank Accession numbers used for phylogenetic analysis performed in Figure 3.1

Chapter 1 – Introduction

Preface

This thesis describes the work that I have done in the Bell Lab as a Ph.D. candidate. The results will be divided into two chapters; the first tests the effect of deleting a virally encoded apoptosis inhibitor on cancer therapy. The second chapter outlines the bio-selection and engineering of a Vaccinia oncolytic virus for cancer therapy.

This chapter serves as an introduction, outlining relevant literature and background to cancer biology, oncolytic viruses and using Vaccinia virus as a therapeutic.

The Problem

Malignant tumors are composed of uncontrollably proliferating cells that have undergone fundamental and permanent genetic changes [1]. These changes include avoiding anti-proliferative regulation, inducing angiogenesis and evading immune-mediated clearance. Although much has been achieved in terms of research and treatment, cancer is responsible for 75,000 Canadian deaths annually [2]. When cancer invades and forms metastatic foci [3], patients present with unique therapeutic challenges –the quality of life and overall survival are often decreased, and unfortunately, treatment can become refractory [4]. Although effective in some cases [5], current treatment options include the use of non-specific chemotherapies, which often lead to severe, sustained treatment side effects while remaining unsatisfactory in

terms of efficacy [6]. Initiation of tumorigenesis, as well tumor progression, are heterogeneous between various cancer types and between patients with the same tumor type [7]. For this reason, most standard of care therapies which focus on a single molecular target fail to achieve success. There is a clear demand for novel cancer therapeutics with increased specificity and reduced toxicity that can target the tumor on multiple fronts.

The Solution: Oncolytic viruses as Cancer Therapeutics

In the late 1800s, cancer remissions were observed in patients sick with other malignancies, including naturally occurring viruses [8]. This eventually led to research on the topic of oncolytic viruses (OVs), which are viruses engineered to selectively infect and kill cancer cells while leaving normal cells intact [9] and can be delivered systemically in patients [10]. These viruses can achieve this by exploiting multiple malignant features common in cancer cells.

Anti-viral response in Cancer

Interferons (IFNs) are signaling proteins (i.e. cytokines) expressed by both normal and immune cells. These cytokines bind to their respective receptors and trigger the Janus kinase (JAK)–Signal Transducer and Activator of Transcription (STAT) pathway. In response to IFNs, cells begin transcribing interferon-stimulated genes (ISGs) which serve to both amplify and control the signal [11]. Type I IFNs, which include paralogues IFNα and IFNβ, are the main signaling response associated with a viral infection. ISGs, produced as a result of type I IFN signaling, induce an anti-viral

and anti-proliferative state in both infected and nearby uninfected cells [12]. Since proliferation is a hallmark of cancer [1], it is unsurprising that incompatible anti-proliferative signaling such as IFN is defective in many cancers [13]. Moreover, immune cells in cancer patients show decreased ability to produce and respond to IFN signaling, suggesting tumors are inducing systemic immune slowdown. This study also revealed basal ISG levels to be significantly lower in cancer patients, suggesting susceptibility to viral infection. [14]. In melanoma patients, IFN signaling is impaired due to low levels of the IFN receptor and restoring IFN receptor levels in mice showed delayed tumor progression [15]. In some tumor types, maintenance of a proliferative state has been correlated to active Wnt signaling [16], which also directly suppresses an anti-proliferative and anti-viral state induced by IFN [17]. Similarly, active Notch signaling in tumors was shown to shape immune function in the tumor microenvironment [18] with Notch ligands implicated in regulating anti-viral response [19].

OVs take advantage of this immune deficiency in cancer, selectively targeting and destroying tumors cells [20]. These viruses are typically attenuated in some form, allowing them to replicate in cells deficient in interferon-mediated antiviral response [21]. One example, Vesicular Stomatitis Virus (VSV), was shown to replicate poorly in normal cells, but not cancer cells, are pre-treated with IFN. A screen of VSV mutants revealed a strain mutated in the 51st codon position of the M protein that preferentially replicates in cancer cells [22]. In order to ensure this mutant VSV does not revert back to wild-type, deletion of the 51st codon of the M protein was engineered into a now broadly used oncolytic VSV virus [23]. A similar screen and approach have been used in the case of Maraba virus, to generate a tumor-selective version called MG1 [24]. Both

rhabdoviruses have broad tissue tropism, given that their entry receptor LDLR is ubiquitously expressed on most cell types [25]. As a consequence of their selectivity to IFN deficient tissues, these viruses cannot replicate and are rapidly cleared from normal tissues but thrive in cancer cells.

Although promising, OVs dependent on deficient IFN signaling can fail to replicate in cancerous tissue and control tumor growth. This may be due to tumor heterogeneity both between patients and within a patient's tumor [26]. For instance, certain tumors maintain a small basal level of interferon signaling or can induce interferon production upon viral sensing [27]. This causes OVs reliant on interferon deficiency to be quickly cleared from the tumor microenvironment before any substantial replication or tumor lysis can take place.

Cancer proliferation as an OV target

Presence of interferon signaling in some tumor types has turned attention to Vaccinia virus (VacV), a dsDNA orthopoxvirus which encodes an array of genes blocking IFN signaling [28]. Since this allows the virus to replicate in cancer cells with interferon signaling, versions of VacV have been engineered as OVs and are currently tested both pre-clinically and clinically [29]. One of the most common methods to attenuate VacV includes the deletion of the viral *thymidine kinase* (TK) gene [30], an enzyme involved in nucleotide metabolism. This is thought to restrict the virus to proliferating tissue, namely cancer cells where TK levels are typically higher than those in normal tissues [31]. In fact, the first TK deletion in a virus that dates back to the 1990s when herpes simplex virus (HSV) TK knockout (TK-) was used successfully in a

glioblastoma model [32]. Interestingly, the only FDA approved OV T-Vec is a herpes dsDNA virus that has intact TK, presumably as means to stop viral infection with the use of drugs such as acyclovir which target HSV TK activity [33].

Although VacV is relatively safe, as a smallpox vaccine and OV, this virus has been shown to replicate off target. For instance, some patients receiving higher doses of VacV TK- intravenously have developed pox lesions which later resolved themselves [34]. This is unsurprising, as cancer cells are not the only proliferating cells in human patients, others including keratinocytes, hepatocytes and immune cells [35].

Tumors generate an immunosuppressive microenvironment

Aside from impaired inflammatory pathways such as IFN, another feature of the tumor microenvironment is immune-suppressing signaling. In fact, tumor signaling has been shown to recruit myeloid progenitor cells to the tumor microenvironment which contribute to carcinogenesis [36]. Upon establishment, tumor cells continuously produce secreted factors, such as M-CSF, G-CSF and VEGF, which create a gradient causing myeloid progenitor cells to leave the bone marrow and enter circulation, eventually being recruited and becoming residents of the tumor microenvironment [37]. These cytokines along with growth factors constantly secreted by tumor cells cause myeloid progenitor cells to differentiate into tumor-associated immune-suppressing cells such as tumor-associated macrophages (TAMs) [38]. Healthy myeloid cells are an important component of the innate immune system, performing functions such as phagocytosis and antigen presentation. Once recruited to the tumor, myeloid cells stop performing their normal function and contribute to immune suppression. Immune

suppression of recruited myeloid cells can happen through various mechanisms, such as the production of inducible nitric oxide synthase (iNOS), arginase-1 (ARG1), and indoleamine 2,3 deoxygenase (IDO) [39]. These enzymes have previously been shown to suppress T cell-mediated adaptive immune responses [40]. Myeloid progenitor cells recruited to the tumor can also differentiate into myeloid-derived suppressor cells (MDSCs), another immune population involved in suppression by the production of secreted anti-inflammatory cytokines, such as IL-10 and TGFβ [41]. This promotes the recruitment of other immune-suppressing cells such as regulatory T cells (T_{regs}) [42]. Importantly, MDSCs are not observed in healthy individuals but highly abundant in the tumor microenvironment promoting both immune suppression and resistance to anti-cancer therapy [43]. Signaling from cancer cells and immune cells in the tumor microenvironment induce and maintain constant immune suppression, successfully allowing tumors to escape immune surveillance by efficiently blocking anti-tumor immune activation [44].

Immune checkpoint inhibitors are a fairly recent discovery and a trending research in the field of cancer immunotherapy. The first immune checkpoint described and characterized as important in anti-tumor immune evasion is *cytotoxic T-lymphocyte-associated protein 4* (CTLA4) [45]. In order to prime a T cell-mediated immune response, antigen-presenting cells (APC) interact with T cells by both display of antigens and engagement of CD28 receptors on T cells with CD80 and CD86 activating ligands, a critical step for T cell stimulation [46]. CTLA4 prevents this latter interaction through its higher affinity in binding CD80/86 compared to that of CD28, therefore sequestering an APC signal needed for T cell stimulation [47]. Regulatory T cells

recruited to the tumor microenvironment suppress T cell activation by abundant CTLA4 production and can be blocked by using antibodies targeting CTLA4 [48]. Recent work has shown that CTLA4 blockade using monoclonal antibody therapy enhances T cell priming by increasing the number of new tumor neoantigens detected by T cells. Interestingly, T cells already recognizing tumor antigens prior to anti-CTLA4 therapy did not increase in frequency, suggesting enhanced priming but not boosting of T cells [49]. A second checkpoint, *programmed cell death protein 1* (PD1), was found to be expressed on activated T cells and shown to "switch off" T cells when interacting with its ligand PDL1 in the context of autoimmunity [50]. Tumor cells have now been shown to respond to immune activation signals by expression of PDL1 on their cell membrane which allows them to escape immune-mediated clearance by inactivating T cells [51]. Detailed analyses at the single-cell level reveal how engaging immune checkpoint receptors on T cells leads to their exhaustion that is only partially reversible using checkpoint blockade [52]. Importantly, treating with antibodies against both checkpoints simultaneously is synergistic in some tumor types [53]. Presence of checkpoints and other immune-suppressing mechanisms in the tumor creates an environment highly susceptible to viral replication [54].

Oncolytic viruses can reverse cancer-induced immune-suppression

OVs have been recently shown to strongly synergize with immune checkpoint blockade (e.g. HSV [55], VSV [56] and VacV [57]). When OVs replicate in the tumor and lyse cancer cells, this triggers an immunogenic response both in tumor cells and infiltrating immune cells. Firstly, tumor and immune cells can sense OVs through a

pathogen-associated molecular pattern (PAMPs). For instance, dsRNA in the cytoplasm made by replicating oncolytic rhabdoviruses is sensed by RIG-I and other RIG-I like receptors (RLR) [58], while dsDNA in the cytoplasm of Vaccinia infected cells is sensed by cyclic GMP-AMP synthase (cGAS) and can induce type I IFN response through IRF3 activation [59]. Secondly, infected cells produce damage-associated molecular patterns (DAMPs), such as Calreticulin exposure or HMGB1 and ATP release [60]. These are sensed by infiltrating immune cells which produce type I and II IFNs, along with other activating cytokines [61]. Consequently, OVs change the immune composition and function of the tumor microenvironment through recruitment of activated immune cells, such as APCs and T cells, and the impairment of T_{reg} and MDSC mediated suppression [62]. These mechanisms have been shown to promote synergy of OVs with immune checkpoints [63].

Other strategies to overcome the immunosuppressive tumor microenvironment include encoding immune-stimulating transgenes in viruses. One transgene, *Fms-like tyrosine kinase 3 receptor ligand* (FLT3LG), when administered systemically as a recombinant protein has been shown to induce dendritic cell differentiation and recruitment to the tumor [64]. An HSV virus encoding FLT3LG has shown increased therapeutic potency compared to HSV without transgenes in a murine established glioma model [65]. A different study has shown that Adenovirus expressing FLT3LG works well as a vaccine adjuvant, but not as a treatment of pre-established tumors in a murine colon cancer model [66]. Similarly, Vaccinia expressing E6/E7 HPV neo-antigens has been found to induce a stronger response against E6/E7 when also expressing FLT3L, better controlling E6/E7 tumor growth [67]. Interestingly, a study found VSV to infect

dendritic cells (DC), an important class of antigen-presenting cells when administered systemically. The authors argued that encoding FLT3LG in VSV restores DC activity, showing better survival in a few murine models using the transgene expressing virus [68]. Although OVs expressing FLT3LG have overall been shown to have improved activity, the mechanism and context of this improvement seem to be model-dependent (virus and tumor type).

Another example of overcoming cancer-induced immune suppression is through the use of cytokines, such as Interleukin 12 (IL12). Interleukins are cytokines secreted as signaling molecules by immune cells, with recombinant IL12 being used clinically and shown to activate both Natural Killer (NK) and T cells in the tumor microenvironment [69]. Encoding IL12 into an HSV backbone has been shown to be beneficial in several models where OV treatment of tumor-bearing mice with this virus has shown better T cell activity and less suppressive T_{reg} activity as a mechanism for better survival in a glioblastoma model [70]. Similarly, an oncolytic measles virus expressing IL12 has shown better T cell activation and potent induction of anti-tumor immunity in a murine colon cancer model [71]. Combining HSV expressing IL12 with immune checkpoint blockade further shows synergy in terms of cancer therapy [55].

Vaccinia as a therapeutic agent

Vaccinia is a poxvirus from a large family of DNA viruses encoding an arsenal of immune-modulating genes that can overcome multiple anti-viral strategies. Differences in virulence genes between poxviruses decide host tropism post viral entry, allowing some poxviruses to replicate and persist in a human tumor microenvironment

[72]. An advantage of using VacV as a therapeutic is its replication life cycle. Vaccinia viruses encode their own genome replication and RNA transcription machinery, which functions in the cytoplasm. This minimizes the risk of viral DNA integration in the host [73]. Furthermore, several different methods have been recently developed to engineer recombinant Vaccinia viruses expressing transgenes or deletions of viral genes [74].

Vaccinia virus is globally famous for being used by the World Health Organization as the first successful vaccine in the eradication of smallpox in 1977. This successful campaign saw the use of different Vaccinia strains in people worldwide, attributing a relatively safe profile to this virus [75]. Of course, different Vaccinia strains were not equally safe, some causing major side effects and rarely fatalities in immunocompromised people [76]. For this reason, smallpox vaccinations have stopped after the eradication of the disease, which paves the way for Vaccinia to be used as systemic therapy, with little prevalence of antibodies in the general population [77].

Aside from their use as smallpox vaccines, Vaccinia and other poxviruses have also been used therapeutically as vaccines against non-poxviral antigens [78] and as oncolytic agents for the treatment of aggressive tumors [79]. A very popular Vaccinia used as a vaccine in pre-clinical and clinical studies is Modified Vaccinia Ankara (MVA) [80]. MVA is the result of 500 passages of a virus called Chorioallantois Vaccinia virus Ankara (CVA) in chicken embryonic fibroblast tissue for the purpose of creating a smallpox vaccine in the 1970s [81]. The creation of MVA through serial passaging lasted decades and resulted in a virus with restricted tropism, poorly replicating in mammalian cells [82] thus limiting it’s use as an OV. MVA has also been shown to trigger potent immune responses and is currently tested as a vaccine for a wide

variety of pathogens, including HIV [83]. Other uses of Vaccinia include its study as a model pathogen in anti-viral innate and adaptive immune response formation [84].

Life cycle and biology

Vaccinia is the prototype *orthopoxvirus* studied for its biology and therapeutic potential. The dsDNA genome is approximatively 200kb encoding for roughly 200 genes (1 gene/kb). The middle of the genome is highly conserved among poxviruses, encoding genes needed for virion morphogenesis and transcription, while the extremities of the genome are more variable and typically encode virulence factors determining host range [85]. A diagram representing the structure of the Vaccinia genome in terms of location and known function of Vaccinia genes can be found in Figure 1.1.

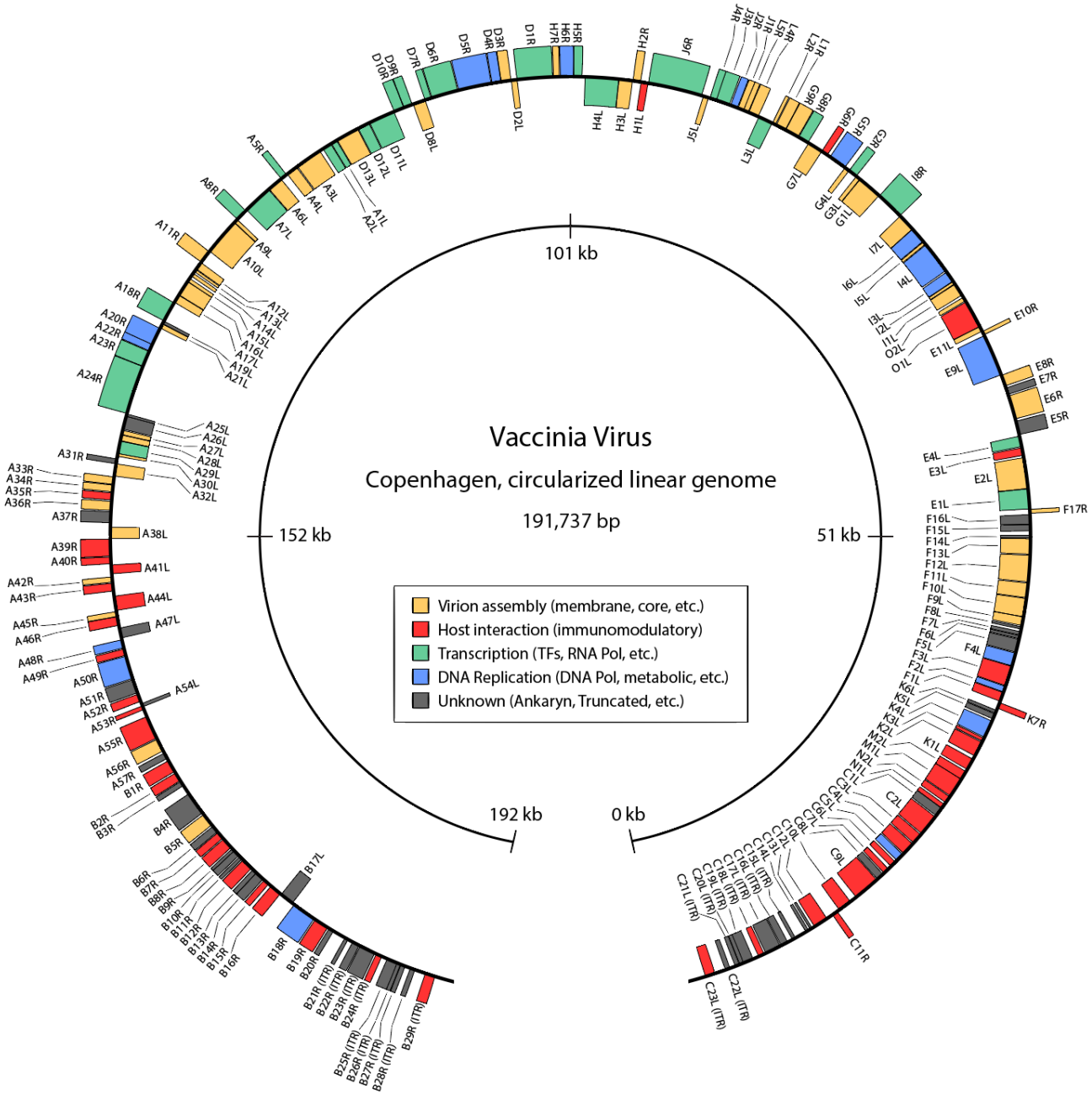

Vaccinia Virus
Copenhagen, circularized linear genome
191,737 bp
101 kb
51 kb
0 kb
192 kb
152 kb
Virion assembly (membrane, core, etc.)
Host interaction (immunomodulatory)
Transcription (TFs, RNA Pol, etc.)
DNA Replication (DNA Pol, metabolic, etc.)
Unknown (Ankaryn, Truncated, etc.)

Figure 1.1: Overview of Vaccinia genome features.

Circular representation Vaccinia Copenhagen strain genome (GenBank Accession # M35027). Genes are color-coded according to their functional role curated from the literature [86]. Forward sense genes are showing on the outside of the circle and end in "R" while reverse sense genes are shown on the inside of the circle and end in "L".

Structurally, virions are typically 200-300nm in size and are composed of 80 different viral proteins, 30 of which are on the surface of the virion [87]. There have been several entry receptors identified [88], however recent work also shows the virus to be capable of cell entry without the use of a receptor through macropinocytosis [89]. Analysis of Vaccinia transcriptome dynamics has revealed two classes of early genes, a class of intermediary genes and a class of late genes [86]. Upon entry, early viral mRNAs begin to be transcribed by early transcription factors packaged in the virion [90]. Most of these genes are known to suppress innate anti-viral response [91] and skew translation from host mRNA to viral mRNA [92]. Some of the protein products of early genes are late transcription factors which begin their activity during viral DNA replication and encode mostly virion membrane proteins, but also early transcription factors which then get packaged into the virion [93]. The structure of early and late promoters has been defined which allows engineering of transgenes expressed either early, late or throughout the viral lifecycle [94]. Virions bud out of infected cells in two forms, as mature virions (MV) that spread to nearby cells and enveloped extracellular virions (EEV), which can spread between tissues as well as from host to host [95]. The latter form accounts for a small percentage of virion progeny and can be increased by introducing a few mutations [96]. EEVs are less susceptible to immune recognition of antibody neutralization as they have a second envelope formed from the host plasma membrane, having fewer virion proteins compared to the MV form [97].

Virulence factors

One of the most striking features of Vaccinia is that roughly one-quarter of its 200kb genome which encodes for virulence factors expressed immediately after viral

infection [86]. These genes which are found at the genome extremities are incredibly redundant, such that removing one or a few of these genes may not result in an *in vitro* phenotype [98]. This is likely due to several genes targeting the same anti-viral pathway. For instance, initiation of interferon signaling, the main cell-intrinsic anti-viral response, is blocked at the level of cytosolic viral DNA sensing by *stimulator of interferon genes* (STING) [99] and DNA-PK [100]. If this strategy fails, the downstream effect of cellular sensing of the virus is interferon regulatory factor 3 (IRF3) phosphorylation, which can be blocked by a different set of virulence genes [101-103]. If this fails too, phosphorylated-IRF3 will translocate to the nucleus and begin transcription of type I interferon, a secreted cytokine meant to alert uninfected cells of viral presence, for which Vaccinia encodes a soluble inhibitor [104]. Lastly, Vaccinia virions are pre-packaged with proteins able to stop active interferon signaling upon infection [105].

Another common anti-viral immune signaling pathway is through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor [106]. Similar to inhibition of interferon signaling, NF-κB activation is blocked by Vaccinia virulence factors at the Toll-like receptor (TLR) sensing stage [107, 108]. Other genes block NF-κB activation sterically [109], block NF-κB translocation to the nucleus [110], and yet unknown genes can block NF-κB activity even post-nuclear translocation [111]. The above literature on Vaccinia genes inhibiting host anti-viral factors is summarized in Figure 1.2.

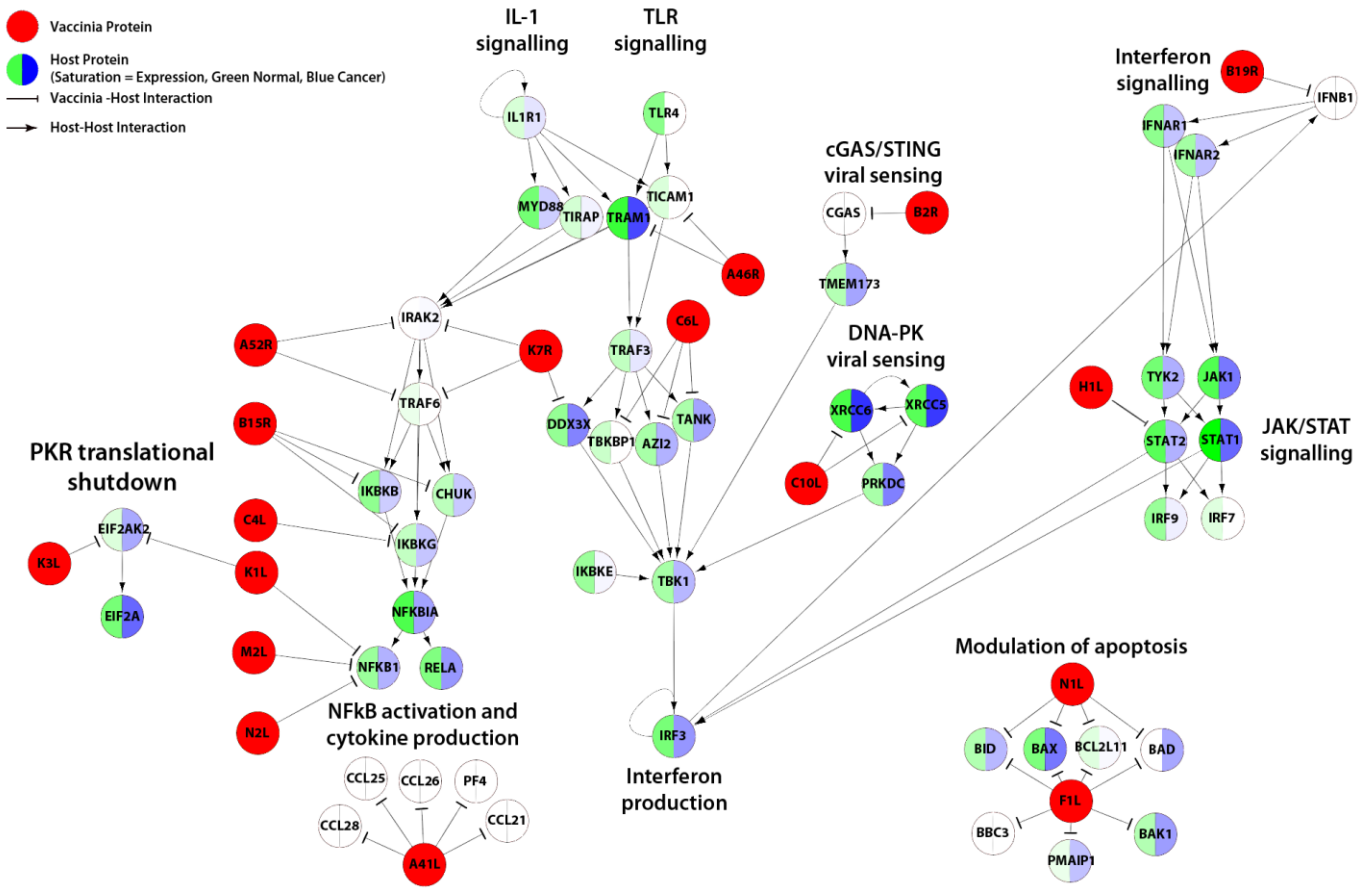

Vaccinia Protein
Host Protein
(Saturation = Expression, Green Normal, Blue Cancer)
Vaccinia -Host Interaction
Host-Host Interaction
IL-1 signalling
TLR signalling
cGAS/STING viral sensing
Interferon signalling
DNA-PK viral sensing
JAK/STAT signalling
PKR translational shutdown
NFkB activation and cytokine production
Interferon production
Modulation of apoptosis
IL1R1
TLR4
MYD88
TIRAP
TRAM1
TICAM1
A46R
CGAS
B2R
TMEM173
B19R
IFNB1
IFNAR1
IFNAR2
IRAK2
A52R
K7R
C6L
TRAF3
TRAF6
B15R
DDX3X
TBKBP1
AZI2
TANK
XRCC6
XRCC5
C10L
PRKDC
H1L
TYK2
JAK1
STAT2
STAT1
IRF9
IRF7
IKBKB
CHUK
C4L
IKBKG
EIF2AK2
K3L
K1L
EIF2A
IKBKE
TBK1
NFKBIA
M2L
NFKB1
RELA
N2L
IRF3
CCL25
CCL26
PF4
CCL28
CCL21
A41L
N1L
BID
BAX
BCL2L11
BAD
F1L
BBC3
BAK1
PMAIP1

Figure 1.2: Overview of Vaccinia genes known to counter anti-viral response.

Summary of Vaccinia genes shown in the literature [28] to inhibit various steps of interferon and NF-κB signaling. Vaccinia genes are shown in red while host genes shown in green and blue circles, where green is indicative of expression in normal tissues while blue in cancer tissues. Host genes with white circles (e.g. cGAS) have very low baseline expression in either normal or cancer cells.

Aside from the multitude of mechanisms that counter anti-viral response, Vaccinia, like other viruses also modulates host transcription and translation [112]. One strategy the virus employs is mRNA degradation via two virally-encoded decapping enzymes D9 and D10. These not only prevent viral dsRNA accumulation, which can activate protein kinase R (PKR) mediated translational shutdown, but also induce host mRNA degradation [113]. Namely, this impairs global host translation by increasing the turnover rate of host and virus mRNA. Ultimately, this strategy is short-lived as D9 decapping enzyme is only expressed early during infection [114] while D10 activity is inhibited by the accumulation of uncapped mRNA [115]. Past the early stage at 4h post Vaccinia infection, viral mRNA accounts for up to half of all mRNAs [116]. During this time, viral transcription switches to intermediary and late viral mRNA that are poly-adenylated at their 5' end [117]. The difference between 5' end of early and late viral mRNA highlights a second strategy the virus employs to control cellular translation at a late stage of viral lifecycle. It has recently been reported that a highly conserved kinase in poxviruses, B1, phosphorylates serine/threonine residues in the human small ribosomal subunit protein, the receptor for activated C kinase (RACK1). As a consequence, RACK1 has increased affinity towards mRNA with poly-A sequences in their 5' UTRs, namely late viral mRNAs [92]. While Vaccinia uses decapping to prevent translation of host mRNA early during infection, it then shifts ribosome affinity away from host mRNA and towards late viral transcripts.

Unsurprisingly, the ability of this virus to block different steps of the same pathway makes most virulence genes redundant and lacking in phenotype when deleted alone *in vitro* where infection conditions are optimal. However, bigger differences are

noticed *in vivo* in murine models where single gene deletions may have an impact on virulence and adaptive immune response formation [118-120]. Furthermore, although different Vaccinia strains are highly similar to each other at the nucleotide and gene content level [121], their virulence in mice differs substantially [122]. Lastly, the high level of redundancy is likely contributing to the broad host tropism of Vaccinia since there are differences in anti-viral response strategies among different species [123]. Perhaps the offensive arsenal of Vaccinia virulence factors is a beneficial characteristic in terms of infecting highly heterogeneous tumors discussed in previous sections.

Oncolytic Vaccinia

Vaccinia is an orthopoxvirus which has advanced to a Phase III clinical trial (PHOCUS) for the treatment of liver cancer (Clinical Trial NCT02562755). It is an attractive oncolytic virus (OV) candidate since relatively few modifications are required to make the virus safe for use in human trials [77]. The use of this virus as a tumor-killing virus dates back decades ago when it was shown that melanoma tumors can regress when treated directly with Vaccinia [124].

Targeting Vaccinia to the tumor

Although the Vaccinia virus has a natural affinity to infect cancer cells due to their active metabolism, further modifications are required to promoter onco-selectivity. A popular strategy for generating oncolytic Vaccinia virus relies on the deletion of metabolic genes [125] which increases selectivity for highly metabolic tissues such as tumors. The most popular choice is the deletion of viral TK, which is currently the Vaccinia candidate in a Phase III clinical trial [126] (of note: an August 2nd, 2019 news

release by SillaJen revealed a futility analysis recommended discontinuation of the clinical trial). TK is involved in nucleotide metabolism and high pools of this enzyme are found in replicating tissues, which are found in cancer cells. Therefore, the deletion of Vaccinia TK restricts viral replication to cancer cells. The drawback of this strategy is the presence of highly metabolic non-cancerous tissues, such as hepatocytes, as well as low metabolic activity, such as hypoxic cancer niches. Furthermore, some patients treated with higher doses with Vaccinia TK- have developed pox lesions indicating the ability of this attenuated virus to replicate off-target [34]. Aside from TK but in a similar manner, other Vaccinia nucleotide metabolism genes (ex. RR) have been inactivated in an attempt to promote cancer cell selectivity [127].

In addition to metabolism genes, both TK and Vaccinia Growth Factor (VGF) have been deleted in Vaccinia virus WR generating an OV known as vvDD, currently used both pre-clinically [128] and clinically [129]. VGF is an Epidermal Growth Factor homolog, thought to be secreted by the virus in order to stimulate DNA synthesis and cell proliferation through EGF receptor (EGFR) binding in a both autocrine and paracrine manner [130]. Since cancer cells proliferate and exhibit active EGF-EGFR signaling [131], deletion of Vaccinia VGF was thought to further enhance viral onco-selectivity. Recently, however, VGF was also implicated in viral spread by promoting cell motility and its deletion was shown to attenuate viral plaque size and spread in cancer cells [132], decreasing OV potency. Furthermore, although VGF deletion does increase onco-selectivity, vvDD is also commonly detected in mouse ovaries after systemic administration [133]. This suggests that targeting Vaccinia based on both metabolism and proliferative signaling can still fail to avoid off-target effects. Lastly,

similarly to TK targeting, not all tumors are positive for EGFR signaling due to cancer heterogeneity [134].

More recently, deleting Vaccinia mRNA decapping enzymes have been proposed as a method to generate a tumor-selective Vaccinia OV [135]. Vaccinia decapping enzymes D9 and D10 play an important role in de-stabilizing host mRNA and promoting viral mRNA translation [136]. Deleting these enzymes from Vaccinia impairs viral spread even in cancer cells and induces anti-viral response [113].

To create a more robust virus, a better understanding of the poxvirus genome is needed for novel strategies in engineering tumor-specific Vaccinia OV. Like most viruses, Vaccinia has developed its gene repertoire to infect non-cancerous tissue and spread from host to host [137]. Targeting the virus with the methods discussed above are not only failing to reduce off-target replication but also impair viral dissemination in cancerous tissue. Since OVs can also be used to encode therapeutic cargo, OVs need to be able to spread well and persist in the tumor microenvironment. A better understanding of Vaccinia can help increase its efficacy and appeal for clinical use.

Enhancing Vaccinia immunogenicity

Traditionally, the first OVs were generated for the sole purpose of tumor cell lysis [138]. As the field of immunotherapy evolves, OVs are increasingly viewed as tools that can stimulate the immune system and synergize with other immunotherapeutic agents [139]. It is becoming clear that OVs armed with therapeutic payloads can stimulate the immune system and lead to long-term remissions [140]. Vaccinia is inherently immunosuppressive, encoding genes which can block immune cell signaling,

thereby allowing the virus to remain stealthy [28]. To further explore Vaccinia immune suppresion, a version of vvDD was further deleted for B18R (type I interferon suppressor) and A48R (NF-κB suppressors). The resulting virus, WR delta-4, has been shown to be more immunogenic and better at controlling tumor growth [141]. Unfortunately, there is a great deal of redundancy in the Vaccinia genome, with multiple viral genes targeting the same pathway, making it complicated to study gene knock-outs [98].

A trending approach to enhance Vaccinia anti-tumor capabilities involves encoding therapeutic payloads in the virus. For instance, the Vaccinia virus currently in Phase III clinical trials encodes the *Granulocyte-macrophage colony-stimulating factor* (GM-CSF) [126]. GM-CSF is a cytokine that promotes differentiation of myeloid cells and has been used as a therapy in cancer patients being administered as a recombinant protein, which resulted in immune-activation, but also immune-suppression [142]. Recent studies on kidney cancer showed this GM-CSF encoding Vaccinia to prime the tumor microenvironment for immunotherapy [143]. Similarly, a vvDD encoding IL-15 has been used in various pre-clinical models and shown to have superior induction of adaptive anti-tumor immunity [144]. Clinically, systemic administration of cytokines has shown promise at the expense of toxicity in a dose-dependent manner [145]. In order to prevent a secreted cytokine encoded in vvDD from leaking systemically, a vvDD version encoding membrane-bound IL-2 was tested. When compared with vvDD encoding secreted IL-2, the virus encoding membrane-bound IL-2 was less toxic, had similar efficacy and localized the IL-2 cytokine in the tumor microenvironment [146].

Other cytokines, such as CXCL11, have also been encoded into a Vaccinia backbone and shown to induce the recruitment and activation of tumor-specific T cells [147].

Combination of Vaccinia with other therapies

A large portion of patients enrolled in OV clinical trials have previously undergone some form of standard cancer therapy [148]. To evaluate the effect of such therapies on viral replication, Vaccinia virus has been tested in various murine pre-clinical models in combination with other therapeutic agents. Using radiotherapy in combination with Vaccinia proved beneficially synergistic, possibly due to cancer cells being sensitized to ionizing radiation by Vaccinia anti-apoptotic factors [149]. However, *in vitro* chemotherapeutic agents nab-paclitaxel and gemcitabine in combination with Vaccinia showed mixed results with some cell lines exhibiting synergistic cancer cell lysis while in others the combination had no effect. In cell lines that did not respond, viral replication was shown to be severely impaired (more than 100-fold), implying chemotherapy can also have anti-viral effects [150].

More recently, immune checkpoints have been described as an important mechanism of cancer immune evasion. Cancer cells are able to upregulate PDL1, which by interacting with PD1 receptors on activated T cells switches them off inducing T cell exhaustion [151]. This led to the engineering of monoclonal antibodies against these targets, also known as immune checkpoint inhibitors (ICIs) [152]. ICIs have shown the best results in terms of extending patient survival in melanomas with complete response achieved in 22.2% of patients receiving a combination of two ICIs [153]. Lack of patient response to ICIs may be attributed to tumors not expressing immune checkpoints or

simply not being infiltrated with T cells [154]. Recently, Vaccinia was shown to induce PDL1 expression in anti-PDL1 resistant murine cancer models, synergizing with PDL1 blockade [57]. The combination of Vaccinia with a different ICI targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA4) showed timing of both virus and ICI to be critical for synergistic effects [155]. Lastly, cytokine encoding Vaccinia viruses have been shown to synergize further with ICI [146, 156]. Mechanistically, Vaccinia infection of the tumor microenvironment causes induction of type I IFN, which leads to recruitment of T cells and upregulation of PDL1 on cancer cells, sensitizing the tumor to ICI therapy [157].

Shortcomings and thesis rationale

Despite encouraging clinical and pre-clinical success, the full potential of Vaccinia virus remains to be uncovered as over half of the 200 genes that it enocdes remain poorly described with unknown functions. Furthermore, it is unclear which genes are required and which are counter-productive for oncolytic virotherapy. For instance, current Vaccinia clinical candidates encode a wide variety of immune-modulatory genes that suppress the innate immune axis [158]. These genes undoubtedly allow for viral replication and persistence in the tumor microenvironment. When thinking of using OVs for direct oncolysis of cancer cells as well as vectors for immunotherapeutic transgene delivery, production of transgenes will be affected by viral clearance. Deletion of some immunomodulatory viral genes like E3L significantly impairs viral replication [159], making such a virus a poor choice for cargo delivery to the tumor microenvironment. On the other hand, deleting another viral gene F1L leads to faster cell death with minimal

impact on replication [160]. These examples suggest that the presence of some genes is likely important while other genes are dispensable during replication in an immune-suppressed tumor microenvironment. A better understanding of viral gene contribution to replication in cancer cells on a genome-wide scale would be useful in designing a better Vaccinia OV.

A second issue is strain selection. With over a dozen Vaccinia strains identified [121], groups conducting pre-clinical or clinical research often focus on only one strain without rational justification for their choice. Furthermore, the natural host of this virus remains unknown with current strains having broad tropism [161]. Differences among Vaccinia strains include levels of virulence [76]. This parameter is important when considering the safety profile of the virus and whether virulence correlates with anti-tumor activity. Another difference between Vaccinia strains is their method of spread; for instance, some strains produce more EEV-like particles allowing for distant dissemination [162]. The effect of EEV formation on the therapeutic potential of an OV would need to be further explored. Lastly, the most popular Vaccinia strain for pre-clinical investigation is a mouse-adapted WR strain [163]. Although advantageous for testing in syngeneic animal models, it is important to consider host adaptations when moving from mouse pre-clinical studies to human clinical trials.

Objectives

Several features distinguish cancer cells from healthy cells. Some of the differences discussed in this chapter revolve around cancer cells being impaired in interferon signaling and creating an immune-suppressing microenvironment favorable to viral replication. The underlying hypothesis governing the work described in this thesis

is that some Vaccinia genes are not necessary for replication in cancer cells and deletion of these genes will increase Vaccinia tumor selectivity without hindering the ability of the OV to replicate and spread in the tumor microenvironment.

In the first chapter of my thesis, I evaluate the deletion of F1L from Vaccinia in the context of OV selectivity and immunogenicity. Study of F1L has shown its role in the suppression of apoptosis, a feature common in cancer cells. I therefore hypothesized and tested the benefits of F1L deletion in the context of cancer therapy.

The next chapter explores differences between various Vaccinia strains in their ability to replicate in human cancer cells. To answer this question experimentally, various assays were designed to test the ability of different strains to spread in several human cancer models. I then set to determine in an unbiased manner whether individual Vaccinia genes are needed for replication in human cancer cells. A genome-wide gene interruption assay was used to target genes of all known functional categories for a functional knockout. Viruses harboring gene interactions were then passaged several times in cancer cells to test for their ability to replicate. This work has led to the identification of two Vaccinia clones harboring major genomic deletions (CopMD5p and CopMD3p) which retain an ability to replicate in cancer cells compared to wild-type levels. These were further characterized and the successful generation of a recombinant Vaccinia clone missing two large areas of its genome, CopMD5p3p, was accomplished. Interestingly, unlike in the case of MVA and NYVAC which also have large genomic deletions, the deleted genes in CopMD5p3p allow the virus to maintain it's ability to replicate in mammalian cells (Figure 1.3). Experiments carried out tested the ability of CopMD5p3p to replicate and lyse cancer tumors in a variety of *in vitro* tumor models as

well as *ex vivo* patient cancerous tissue. *In vivo* testing included tumor selectivity, tumor control and the ability to stimulate the immune system in both human xenografts as well as syngeneic mouse tumor models.


C23L_ITR_Host_interaction
C22L_ITR_Pseudogene
C21L_ITR_Unknown
C20L_ITR_Pseudogene
C19L_ITR_Pseudogene
C18L_ITR_Host_interaction
C17L_ITR_Unknown
C15L_ITR_Unknown
C14L_Pseudogene
C13L_Unknown
C12L_Host_interaction_IL−18_blocker
C9L_Host_interaction_IFN−inhibitor
C7L_Host_interaction
C6L_Unknown
C5L_Unknown
C4L_Unknown
C3L_Host_interaction_Compliment_binding
C2L_Host_interaction
C1L_Unknown
N1L_Host_interaction_Anti−apoptosis
N2L_Unknown
M1L_Unknown
M2L_Host_interaction_NF−kB_inhibitor
K1L_Host_interaction_NF−kB_inhibitor
K2L_Host_interaction_Cell−fusion−suppresor
K3L_Host_interaction_PKR−inhibitor
K4L_DNA_replication
K5L_Pseudogene
K6L_Truncated
K7R_Host_interaction_NF−kB−and−IRF3_inhibitor
F1L_Host_interaction_Anti−apoptosis
F2L_DNA_replication
F3L_Host_interaction
F5L_Unknown
F11L_Host_interaction
O1L_Unknown
I4L_DNA_replication_Ribonucleoside−reductase
J2R_DNA_replication_Thymidine−kinase
A25L_Virion_association
A26L_Virion_association
A39R_Truncated
A52R_Host_interaction_IL−1_blocker
A54L_Unknown
A53R_Pseudogene
A55R_Host_interaction
A56R_Virion_association
B2R_Truncated
B4R_Unknown
B8R_Host_interaction_IFN−g_blocker
B13R_Host_interaction
B14R_Pseudogene
B15R_Unknown
B16R_Host_interaction_IL−1−beta−inhibitor
B17L_Unknown
B18R_Unknown
B19R_Host_interaction_IFN−a/b_blocker
B20R_Host_interaction
B21R_ITR_Unknown
B23R_ITR_Unknown
B24R_ITR_Host_interaction
B25R_ITR_Pseudogene
B26R_ITR_Pseudogene
B27R_ITR_Unknown
B28R_ITR_Pseudogene
B29R_ITR_Host_interaction
CopMD5p3p
MVA
NYVAC
Gene present
Gene absent

Figure 1.3: Comparison of deleted genes between CopMD5p3p, MVA and NYVAC.

Summary of Vaccinia gene deletions in viruses missing significant portions of their genome. Red rectangles indicate a deleted gene while green rectangles indicate presence of a gene in that particular Vaccinia virus.

Chapter 2 – Deletion of apoptosis inhibitor F1L in vaccinia virus increases safety and oncolysis for cancer therapy

Preface

Work described in this chapter has been previously published [164] as an article in the journal for Molecular Therapy Oncolytics. The published paper is titled "Deletion of apoptosis inhibitor F1L in vaccinia virus increases safety and oncolysis for cancer therapy" and has the following authors: Adrian Pelin, Johann Foloppe, Julia Petryk, Ragunath Singaravelu, Marian Hussein, Florian Gossart, Victoria A. Jennings, Lawton J. Stubbert, Madison Foster, Christopher Storbeck, Antonio Postigo, Elena Scut, Brian Laight, Michael Way, Philippe Erbs, Fabrice Le Boeuf and John C. Bell.

As I joined the Bell lab work on this project was underway, supervised by Dr. Bell, Dr. Le Boeuf and Dr. Storbeck. I have helped conduct *in vivo* experiments carried out at OHRI. I wrote the first draft of the manuscript. After initial submission I performed additional experiments asked by reviewers.

Abstract

Vaccinia virus (VACV) possesses a great safety record as a smallpox vaccine and has been intensively used as oncolytic virus against various type of cancer over the past decade. Different strategies were developed to make vaccinia virus safe and selective to cancer cells. Leading clinical candidates, such as Pexa-Vec, are attenuated through deletion of the viral thymidine kinase (TK) gene, which limits virus growth to replicate in cancer tissue. However, tumors are not the only tissues whose metabolic activity can overcome the lack of viral TK. In this study we sought to further increase the tumor specific replication and oncolytic potential of Copenhagen strain VACV ΔTK. We show that deletion of the anti-apoptosis viral gene F1L not only increases the safety of the Copenhagen ΔTK virus, but also improves its oncolytic activity in an aggressive glioblastoma model. The additional loss of F1L does not affect vaccinia virus replication capacity, yet its ability to induce cancer cell death is significantly increased. Our results also indicate that cell death induced by the Copenhagen ΔTK/F1L mutant releases more immunogenic signals, as indicated by increased levels of IL-1B production. A cytotoxicity screen in NCI-60 panel shows that the ΔTK/F1L virus induces faster tumor cell death in different cancer types. Most importantly, we show that compared to the TK-deleted virus, the ΔTK/F1L virus is attenuated in human normal cells and causes fewer pox lesions in murine models. Collectively, our findings describe a new oncolytic vaccinia deletion strain that improves safety and increases tumor cell killing.

Introduction

Vaccinia virus (VACV) and closely related poxviruses have been used for centuries as a vaccine against the smallpox virus, culminating in the eradication of smallpox in the late 1970's. The eradication campaign showed that VACV has an impressive safety profile, the reasons for which are now better understood. To further improve on this attractive vaccine backbone, techniques were developed to engineer and rescue recombinant VACV [165]. Yet to-date, more than half of genome's 200 genes have unknown or incompletely understood functions, leaving much be learned about the intricate host-pathogen interaction of this virus. Interestingly, it was shown that several VACV genes can be knocked out with no observable in vitro or in vivo phenotype, revealing a complex genome with redundant and non-essential elements [98].

In addition to its application as a vaccine vector, VACV also has intrinsic oncolytic properties [166]. In particular, it shows preferential replication in tumor cells that display activated EGFR/Ras/MAPK pathways [167], which are observed in many cancer types [168, 169]. Beyond use as a potential mono-therapy agent, VACV has also long been used in combination with other therapy agents [170, 171]. For instance, oncolytic VACV strains was recently found to synergize well with immunotherapy [57, 157] as well as induce anti-tumor immunity when encoding immune-stimulatory transgenes [172] or tumor-associated antigens [173].

The most advanced clinical VACV candidate is Pexa-Vec (JX-594). Pexa-Vec is a strain that has its viral thymidine kinase gene deleted, thus requiring the virus to utilise nucleotide pools produced in metabolically active tumor cells. The virus additionally expresses the GM-CSF cytokine to activate immune cells at the tumor site [174]. Pexa-

Vec was shown in a Phase I and II clinical trial studies to be safe after intravenous delivery and capable of expressing the immunostimulatory gene [77]. Currently, this virus is being assessed in a Phase III clinical trial, testing its efficacy by intra-tumoral injection of patients suffering from hepatocarcinoma (ClinicalTrial NCT02562755). Although Pexa-Vec has shown minimum adverse side effects, the thymidine kinase (TK) deletion may not fully preclude virus replication in non-cancerous metabolically active tissues, such as the liver. Furthermore, this attenuation may also hinder the virus's capacity to proliferate in metabolically inactive tumor niches present in heterogeneous tumors [175]. Therefore, there remains room for improved engineering of the virus as well as a need to seek alternative ways to increase the therapeutic window in a way that would target the widest possible range of cancer cells.

F1L is a VACV gene that inhibits infection-induced apoptosis and inflammation. F1L binds the pro-apoptotic Bcl-2 family members Bak and Bim [176], thus preventing apoptotic stimulus-induced loss of the inner mitochondrial membrane potential and the subsequent cytochrome c release [160, 177]. The structure of F1L resembles a dimer-swapped Bcl-2 homolog with an atypical amino-terminal extension [178, 179]. The amino-terminus of F1L binds to and inhibits caspase-9 and the inflammasome component NLRP1 [178]. Overall, virulence of a F1L-deleted virus was attenuated in a mouse model, and this was attributed to the loss of NLRP1 activity of F1L [178]. Deletion of F1L from the Copenhagen strain has shown induction of apoptosis in fibroblast cells [160].

Because non-cancerous cells use apoptosis as an anti-viral defense mechanism, we hypothesized that disabling anti-apoptosis mechanisms in VACV would be beneficial

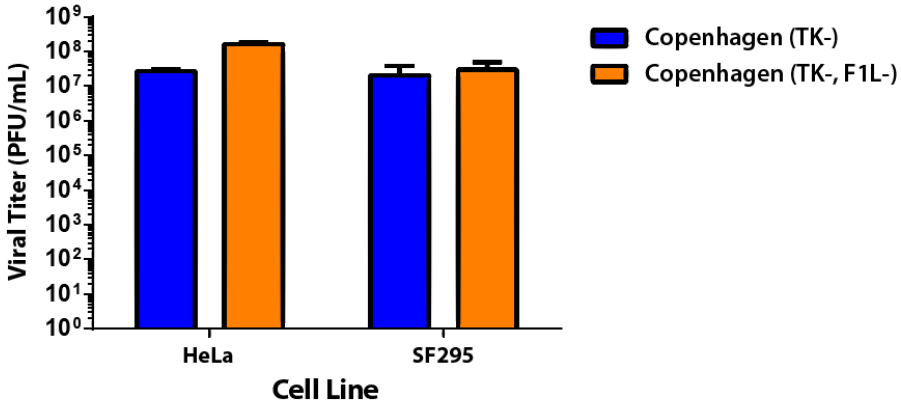
for tumor selectivity. Furthermore, since apoptosis is only partially functional in tumor tissues, we ventured that our F1L-deleted Copenhagen can both replicate and induce faster cell death in cancer cells. In this study, we show that removing the F1L gene increases vaccinia's ability to induce immunogenic apoptosis and oncolytic activity in cancer cells

Results

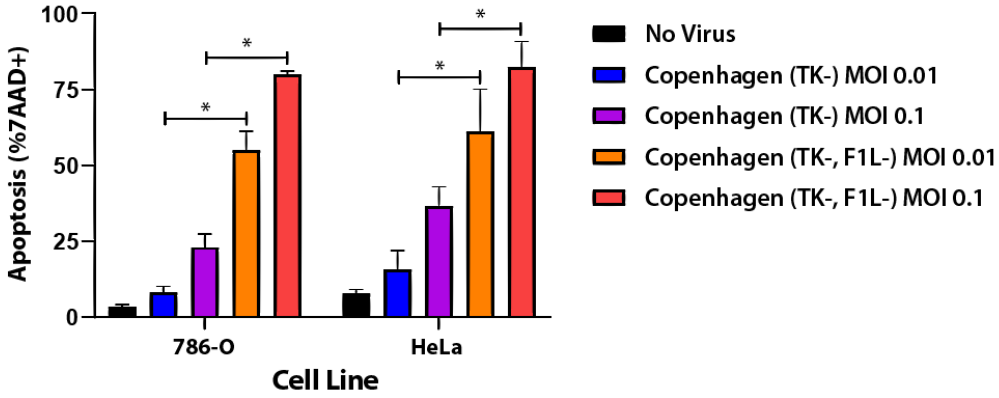
F1L KO increases immunogenic apoptosis

Here, we rescued an F1L deletion in a VacV Copenhagen ΔTK strain (Cop-ΔTK/F1L). Testing the ability of the virus to replicate, we found Cop-ΔTK/F1L to generate ten-fold more progeny virus compared to Cop-ΔTK in HeLa cells (production cell line) but not SF295 (Figure 2.1A). Both 786-O and HeLa cells show higher percent of dead 7AAD positive cells when infected with Cop-ΔTK/F1L compared to Cop-ΔTK (Figure 2.1B). Furthermore, unlike Cop-ΔTK, Cop-ΔTK/F1L induces secretion of IL-1□ cytokines in a variety of glioblastoma cells (Figure 2.1C). To assess if these phenotypes are specific to certain tumor types, we screened 40 of the NCI-60 human cancer cell lines for virus-induced cell death. We found that except for blood cancer, Cop-ΔTK/F1L induced faster cell death in all tumor types when compared to Cop-ΔTK (Figure 2.2).

A.


Viral Titer (PFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
HeLa
SF295
Cell Line
Copenhagen (TK-)
Copenhagen (TK-, F1L-)

B.


Apoptosis (%7AAD+)
100
75
50
25
0
*
*
*
*
786-O
HeLa
Cell Line
No Virus
Copenhagen (TK-) MOI 0.01
Copenhagen (TK-) MOI 0.1
Copenhagen (TK-, F1L-) MOI 0.01
Copenhagen (TK-, F1L-) MOI 0.1

C.

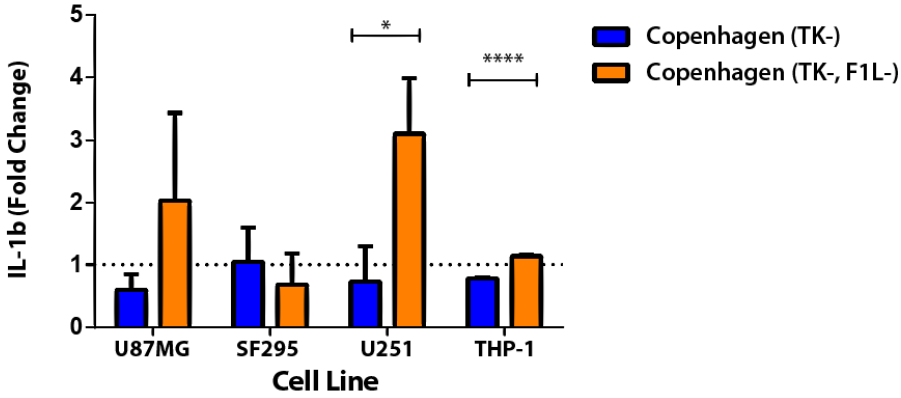

IL-1b (Fold Change)
5
4
3
2
1
0
*
****
U87MG
SF295
U251
THP-1
Cell Line
Copenhagen (TK-)
Copenhagen (TK-, F1L-)

Figure 2.1: Deletion of F1L increases cancer cell death without affecting replication.

(A) Viral replication of F1L deleted virus assessed through plaque assay in HeLa and SF295 cell lines at MOI 0.1 48h post infection. (B) Flow cytometric analysis of 7-AAD apoptosis marker in 786-O and HeLa cells at various MOIs 24h post infection. (C) Induction of IL-1β levels as measured by ELISA in various cancer cell lines infected for 48h at MOI 0.1. * = $p < 0.05$; **** = $p < 0.0001$.

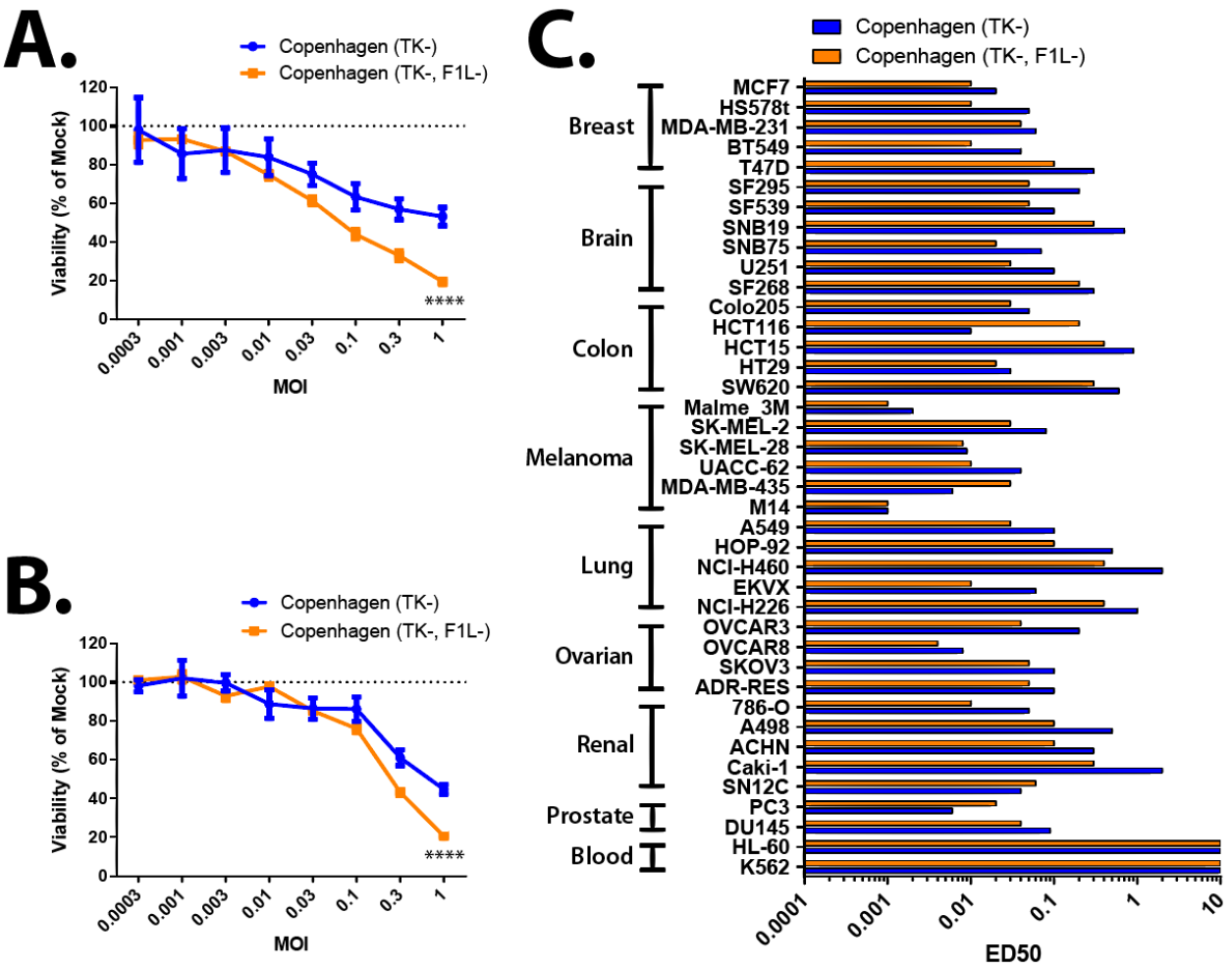

A.
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
Viability (% of Mock)
120
100
80
60
40
20
0
****
0.0003
0.001
0.003
0.01
0.03
0.1
0.3
1
MOI
B.
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
Viability (% of Mock)
120
100
80
60
40
20
0
****
0.0003
0.001
0.003
0.01
0.03
0.1
0.3
1
MOI
C.
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
Breast
MCF7
HS578t
MDA-MB-231
BT549
T47D
Brain
SF295
SF539
SNB19
SNB75
U251
SF268
Colon
Colo205
HCT116
HCT15
HT29
SW620
Melanoma
Malme_3M
SK-MEL-2
SK-MEL-28
UACC-62
MDA-MB-435
M14
Lung
A549
HOP-92
NCI-H460
EKVX
NCI-H226
Ovarian
OVCAR3
OVCAR8
SKOV3
ADR-RES
Renal
786-O
A498
ACHN
Caki-1
SN12C
Prostate
PC3
DU145
Blood
HL-60
K562
0.0001
0.001
0.01
0.1
1
10
ED50

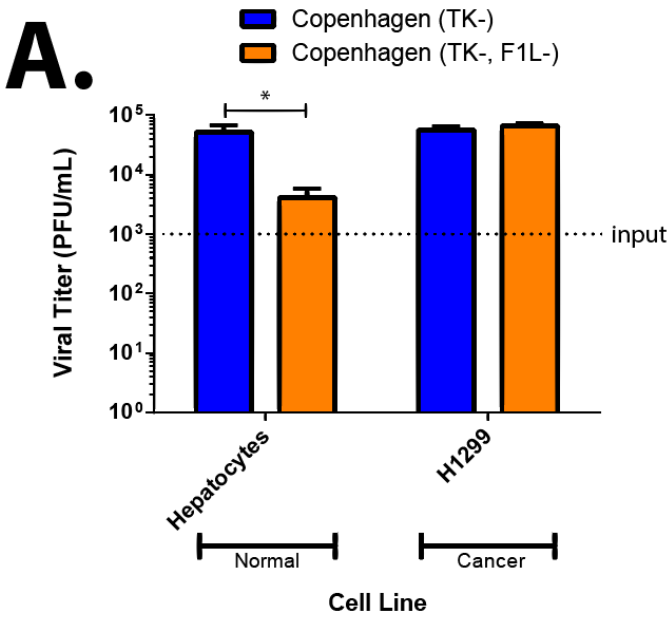
Figure 2.2: Vaccinia Delta F1L induces rapid cancer killing in a wide variety of tumour types.

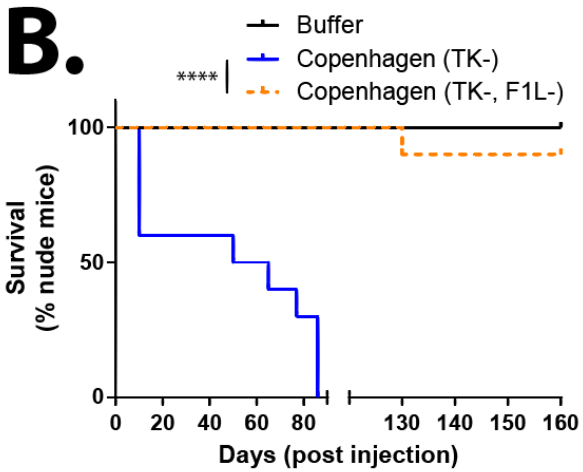
(A-B) Alamar blue viability of U87MG (A) and SF295 (B) cell lines measured as percent of non-infected cells at various MOIs for a 48h infection. (C) Alamar blue viability of various NCI-60 cell lines infected with either Vaccinia for 48h.
**** = $p < 0.0001$.

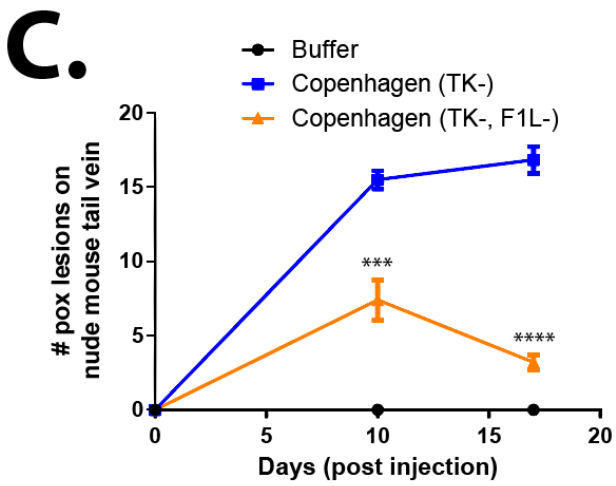
Removal of F1L increases VACV safety

Interestingly, when we compared the two different Copenhagen mutant strains in normal human hepatocyte cells, we found Cop-ΔTK/F1L ten-fold less able to replicate than Cop-ΔTK/F1L (Figure 2.3A). These results suggest a role of F1L in VACV virulence and ability to replicate in normal tissue. To evaluate whether this difference is significant in the presence of an immune system, we tested Cop-ΔTK/F1L activity in two mouse models. Strikingly, challenging immune-deficient mice intravenously at 1 X 108 pfu had a 90% survival rate for Cop-ΔTK/F1L and 0% for Cop-ΔTK (Figure 2.3B). Interestingly, injecting systemic Copenhagen in the tails of nude mice produced significantly more pox lesions at the site of injection by the Cop-ΔTK virus compared to the double Cop-ΔTK/F1L (Figure 2.3C). Lastly, when deleting F1L in the WR neuro-adapted Vaccinia, we see less viral titer in the brain of nude mice after systemic injection of WR ΔTK/ΔF1L (Figure S2.1).

One disadvantage with VACV is the inability to use higher therapeutic doses of the virus when administering systemic intravenous injections to treat tumors. Indeed, injection of 1 X 108 pfu of Cop-ΔTK systemically induces viral toxicity and mice succumb within 4 days (Figure 2.3D). However, same dose of Cop-ΔTK/F1L does not induce fatal toxicity and results in a survival rate of 100% (Figure 2.3D). Therefore, data suggests that Cop-ΔTK/F1L has higher safety profile and is more efficacious.


A.
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
*
Viral Titer (PFU/mL)
10^5
10^4
10^3
10^2
10^1
10^0
input
Hepatocytes
H1299
Normal
Cancer
Cell Line


B.
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
****
Survival
(% nude mice)
100
50
0
0 20 40 60 80 130 140 150 160
Days (post injection)


C.
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
pox lesions on
nude mouse tail vein
20
15
10
5
0
***
****
0 5 10 15 20
Days (post injection)

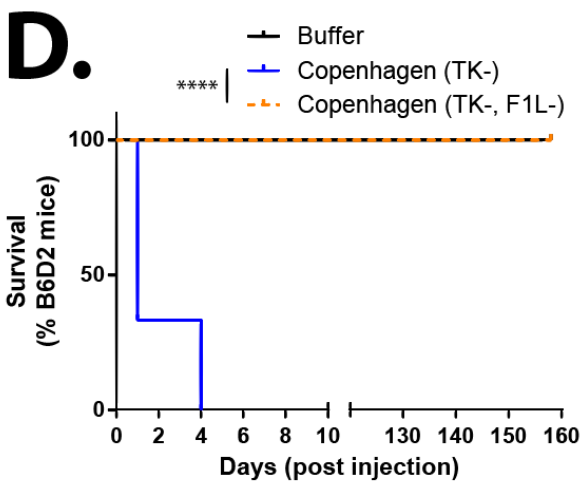

D.
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
****
Survival
(% B6D2 mice)
100
50
0
0 2 4 6 8 10 130 140 150 160
Days (post injection)

Figure 2.3: Deletion of F1L increases the safety and tumour selectivity of Vaccinia.

(A) Viral replication in primary hepatocytes and H1299 cancer cells during a 72h infection at MOI 0.001 as determined by plaque assay. Nude mouse (B) and immunocompetent B6D2 mouse (D) survival monitoring following a 1 X 108 pfu dose of either VACV intravenously. (C) Amount of pox lesions on mouse tails quantified at two timepoints during the experiment done in panel B.
* = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$.

Cop-ΔTK/F1L is more efficacious in glioblastoma tumors

We have identified that Cop-ΔTK/F1L kills tumor cells faster and displays less off-target activity, even in mouse brain tissue. Since most oncolytic viruses can't be administrated intra-cranially at therapeutic doses without causing sickness and mouse death [180], the Cop-ΔTK/F1L safety profile makes it an appealing therapeutic candidate for glioblastoma treatment. To test the efficacy of Cop-ΔTK/F1L in a challenging glioblastoma model, we treated luciferase expressing U87 xenografts implanted in CD-1 nude mouse brains. We observed the more tumor inhibition in Cop-ΔTK/F1L treated mice as compared to Cop-ΔTK (Figure 2.4A), indicating improved oncolysis and tumor control. Immunohistochemical analysis also revealed increased caspase-3 straining in Cop-ΔTK/F1L treated mice (Figure 2.4B). This suggests higher induction of tumor cell death in vivo, and is consistent with the higher rates of apoptosis observed in vitro (Figure 2.1B). Indeed, Cop--ΔTK/F1L mediated oncolysis translates to higher survival (85% vs 25% compared to Cop-ΔTK) when treating U87 xenografts intra-cranially (Figure 2.4C). In line with these results, we also found that Cop-ΔTK/F1L administrated intravenously extended mouse survival in the same model (Figure 2.4D).

In order to test whether F1L deletion enhances the immunogenicity of the virus we have tested our virus in a syngeneic model. Upon treatment of CT26 tumours with either of our viruses we find Cop--ΔTK/F1L control tumour growth rates better (Figure 2.4E). Interestingly vaccinia-mediated tumor control in this model was shown to be TLR4 dependant [157]. Taken together, our data demonstrates that the F1L deletion in Cop-ΔTK improves efficacy of the oncolytic virus.

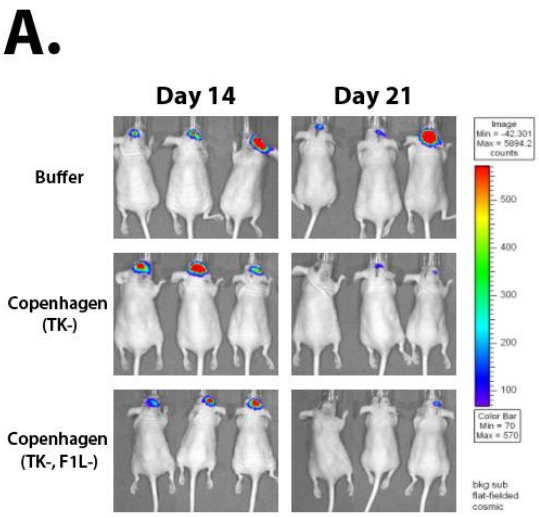

A.
Day 14
Day 21
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
B.
Vaccinia antibody
Caspase 3 antibody
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
C.
Percent Survival
Days (post IT injection)
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
D.
Percent Survival
Days (post IV injection)
*
Buffer
Copenhagen (TK-)
Copenhagen (TK-, F1L-)
E.
Tumor Volume (mm³)
Day (Post-Seeding)
*
***
Buffer
Copenhagen (TK-)
Copenhagen (TK-; F1L-)

Figure 2.4: Treatment with Vaccinia delta F1L extends survival in a glioblastoma model and delays tumor growth in a syngeneic mouse colon model.

(A) IVIS imaging of U87 Luciferase expressing tumors seeded intracranially in Nude mice after one intracranially injection of either vaccinia or PBS vehicle. (B) IHC on slices of U87 tumors treated intracranially with either vaccinia virus and stained with both vaccinia virus anti-body and anti-body for caspase-3. (C-D) Survival of Nude mice seeded with U87 Luciferase expressing tumors seeded intracranially and treated with one intracranial (C) or intravenous (D) dose of 1e7 pfu of either Vaccinia. (E) Colon CT26-LacZ tumours were seeded subQ at 5e5 cells in Balb/C mice in a syngeneic model. Two weeks later mice were treated with one dose 1e7 pfu intra-tumoral injection with either Copenhagen ΔTK or Copenhagen ΔTK/F1L virus. Tumours measurements are displayed. * = $p < 0.05$, *** = $p < 0.001$.

Discussion

VACV and other poxviruses have large genomes, encoding approx. 200 genes – of which ~25% are considered virulence factors [28]. Mammalian cells' first line of defense against viral infection is interferon stimulated antiviral signaling and apoptosis, both of which are targeted by VACV genes [158]. Although these genes increase the pathogenic fitness of the virus, they have somewhat redundant functions, and their expression may compromise the oncolytic virotherapy's safety. Cancer is a characterised by a series of defects, one of which is impaired interferon signaling and upregulation of endogenous anti-apoptosis genes. The rationale of our study was to delete a major anti-apoptotic virulence factor in VACV due to the inherent ability of target cancer cells to evade apoptosis. We hypothesized that normal tissues would be better able to fight off viral infection, while cancerous tissue would succumb to oncolysis more efficiently.

This is the first study, to the best of our knowledge, to report improved anti-tumor efficacy of VACV-based OV with a double deletion of both TK and F1L. The leading VACV-based clinical OV candidate, Pexa-Vec encodes solely a TK deletion to drive tumor selectivity. This strategy aims to restrict VACV growth to metabolically active cells expressing high levels of thymidine kinase, such as metabolically active cancerous tissue [30]. This approach has some shortfalls however, for instance some non-cancerous tissues such as immune cells and hepatocytes are continuously undergoing turnover – resulting in off-target effects. This is consistent with reported clinical incidences of Pox lesions in patients receiving high doses of Pexa-Vec [34]. This suggests that increased attenuation of the virus is required to improve VACV's safety profile. Our addition of an F1L deletion significantly improved in vivo safety profile of the virus, as demonstrated by less pox lesions and increased survival in nude mice

(Figure 3C). Collectively, our data suggest that the double deletion may improve safety in vivo.

In addition to increased safety, our study demonstrates the F1L deletion conveys improved anti-tumor efficacy. The Cop-ΔTK/F1L virus maintained its ability to replicate in vitro, trigger higher rate of apoptosis, and cause cytokine release upon cell death (Figure 2.1). An increased levels of cytokines is positive in the context of a tumor microenvironment, as they can attract more lymphocytes to the tumor or activate already existing one [181]. This is consistent with a previous study reporting that deleting F1L in a non-replicating poxvirus vaccine vector increased its immunogenicity generating larger immune responses to encoded HIV antigens [182]. Furthermore, F1L deletion delays tumor growth in a syngeneic colon cancer murine model (Figure 2.4E). This particular model has been previously tested with other Vaccinia, where control of tumor growth was shown to occur via TLR4 stimulation by HMGB1 release from infected cells [157]. Interestingly, TLR4 stimulation results among many things in production of IL-1β [183] which we show our F1L deleted virus can induce in some cancer cells (Figure 2.1C). The advantage of the Cop-ΔTK/F1L virus is the ability to combine immunogenicity with oncolysis.

Several poxviruses encode anti-apoptotic factors to counteract cell death and increase viral persistence [184]. Our work suggests that deletion of these factors may improve safety and anti-tumor efficacy of poxvirus-based OVs. Our results are consistent with the enhanced tumor selectivity of a VACV Western Reserve strain harboring a double deletion of two anti-apoptosis genes (SPI-1 and SPI-2) [185]. The authors similarly reported enhanced tumor selectivity and safety for their mutant strain.

Other VACV strains encode other virulence factors with anti-apoptotic activity [186]. Future investigations should examine the potential of deleting additional anti-apoptotic factors to improve poxvirus vectors as OVs.

Overall, our study has shown that the deletion of the F1L gene in VACV increases safety profile and efficacy of the oncolytic potential of an existing clinical platform. We demonstrated that a single TK knockout is insufficient as a tumor targeting mechanism. Our work highlights the need for more research into the therapeutic properties of additional VACV genes with unknown function as their loss may further improve the use of VACV in oncolytic therapy.

Materials and Methods

Cell Lines

Cells were obtained from the American Type Culture Collection (ATCC) and passaged in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

Construction of plasmids

Shuttle plasmid for deleting F1L was constructed using the DNA from the VV Copenhagen strain (GenBank Accession Number M35027). The DNA flanking regions of F1L were amplified by PCR. Primers of the F1L downstream flanking region (F1L-L sequence) were 5'- CGC GGA TCC ATC ATT TTT TCA CCA TTA CTT CT -3' (BamHI site underlined) and 5'- CCG GAA TTC TTG TAG ATT ATA TAA CGG ACA TT -3' (EcoRI site underlined). Primers for the F1L upstream region (F1L-R sequence) were 5'- GCC TGG CCA TTA GTG GAC TTG TCA AAT CTA T – 3' (MscI site underlined) and 5' – GCC CAG CTG ATG TTA TAT ACG TAA TGG GAG G – 3' (PvuII site underlined). The amplified DNA fragments were digested with restriction enzymes BamHI/EcoRI or MscI/PvuII then ligated into the corresponding sites in the PpolyIII plasmid [187]. A repeat region of the downstream flanking region of F1L (F1L-L sequence) was amplified by PCR using the primers 5'- TCC CCC GGG TGA TGA TAT AGG GGT CTT CAT A - 3' (SmaI site underlined) and 5'- GCC GCA TGC TTG TAG ATT ATA TAA CGG ACA TT – 3' (SphI site underlined) and inserted in the pPolyIII plasmid. The repeat region was used to eliminate the selection cassette during the production of deleted viruses. The selection cassette GFP/GPT, a fusion of the gene encoding the green fluorescent protein (GFP) and the gene encoding guanine phosphoribosyltransferase (GPT) was positioned under the control of pH5R vaccinia

promoter and inserted into the SmaI/SphI site in the pPolyIII plasmid. The resulting plasmid is a shuttle plasmid named pΔF1L for the deletion of F1L gene.

Generation of recombinant Vaccinia

All recombinant VACVs were derived from the Copenhagen strain. Thymidine kinase gene-deleted VACV expressing the fusion gene FCU1 (VVTKko-FCU1) under the control of the p11k7.5 promoter have been constructed and characterized previously [188] For generation of the double deleted VVTKkoF1Lko-FCU1, CEF cells were infected with VVTKko-FCU1 at a MOI of 0.01 and incubated at 37°C for 1 h, then transfected with a CaCl2 precipitate of the shuttle plasmid pΔF1L (0.2 µg). The cells were incubated for 48 h at 37°C. Double recombination occurs between homologous regions (F1L-L and F1L-R) in the shuttle plasmid and the virus, resulting in the insertion of the gene cassette into the F1L-L locus of VACV. Recombinant VACV expressing GPT are isolated in a selective medium containing hypoxanthine (15 µg/ml), xanthine (250 µg/ml) and mycophenolic acid (250 µg/ml). GPT+ plaques that displayed GFP-fluorescence (easily detectable with a Leika MZ16 fluorescence binocular stereomicroscope) were isolated and submitted to several additional rounds of selection on CEF cells in presence of GPT selection medium. To confirm that the VVTKko-FCU1 was deleted of the targeted F1L regions, 40 PCR cycles were performed using primers within the F1L deleted region and in the flanking region, external to the site of recombination. The doubly deleted virus was then used to infect CEF without any GPT selection to eliminate the GFP/GPT marker obtained after intragenic homologous recombination between the two F1L-L sequences flanking the GFP/GPT gene cassette. Non-fluorescent plaques were isolated and submitted to 2 additional plaque purification cycles in CEF. Virus structure was confirmed by multiple PCRs. Recombinant VACVs

were amplified in CEF, purified over sucrose gradients and virus stocks were titrated on CEF by plaque assay.

In vitro virus yield

To evaluate viral replication between human tumor cells and human primary cells, human tumoral cells H1299 and human primary hepatocytes were infected in 12-well plates VVTKko-FCU1 and VVTKkoF1Lko-FCU1 at a MOI of 0.001 (1000 PFU/well). At 72 h post-infection, supernatant and cells were collected, freeze-thawed and sonicated and viral progeny were quantified on CEF by plaque assay.

Viral pathogenicity

Viral pathogenicity was assessed by survival studies done on both Swiss nude mice (female, 6 weeks old from Charles Rivers Laboratories) and immunocompetent B6D2 mice (female, 6 weeks old from Charles Rivers Laboratories). High dose (1x108 PFU) of the indicated vectors were injected intravenously by tail vein injection. Mice were observed daily throughout the course of the experiment. Pox lesions on tail were also measured and recorded after i.v. injection of 1x108 PFU of each virus in Swiss nude mice.

Mice and tumor models.

Mice were from Charles River Laboratories (Wilmington, MA). Imaging studies: U87 (1×106) tumors were established in the brain after surgery of 6-week-old CD1 female nude mice (N = 5). Vaccinia viruses were administered intratumor or intravenously (1×107 PFU). Survival was followed. All experiments were performed in accordance with institutional guidelines review board for animal care (University of Ottawa).

In vivo imaging

Mice were injected with D-luciferin (Molecular Imaging Products, Ann Arbor, MI) for firefly luciferase imaging. Mice were anesthetized under 3% isoflurane (Baxter, Deerfeld, IL) and imaged with IVIS200 Series Imaging System (Xenogen, Hopkinton, MA). Data acquisition and analysis were performed using Living Image v2.5 software. For each experiment, images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence is plotted on identical color scales.

Immunohistochemistry

Tissues were harvested, placed in OCT mounting media (Tissue-Tek, Sakura Finetek, Torrance, CA). Sectioned tissues were processed as previously described with anti-VV or Anti-Active Caspase 3. Tumor images were obtained with an Epson Perfection 2450 Photo Scanner (Epson, Toronto, Canada) whereas magnifications were captured using a Zeiss Axiocam HRM Inverted fluorescent microscope (Zeiss, Toronto, Canada) and analyzed using Axiovision 4.0 software.

Statistical analysis

Statistical significance is only shown when p-values < 0.05. All reported tests compare Copenhagen TK– and Copenhagen TK– F1L– groups and are two-tailed. All data are shown as mean and standard error (SEM). For survival analysis, Kaplan–Meier survival curves were compared using a log-rank Mantel-Cox test. All statistical analysis was done using GraphPad Prism 5 software.

Funding

JCB is supported by the Terry Fox Foundation (TFF), the Canadian Cancer Society Research Institute (CCSRI), the Ontario Institute for Cancer Research (OICR), the Canadian Institute for Health Research (CIHR), the Ottawa Regional Cancer Foundation and the Ottawa Hospital Foundation. AP was supported by Ontario Graduate Student scholarship (OGS).

Author contributions

AP, JF, CS, APo, MW, PE, FLB and JCB designed this study and planned the experiments. AP, JF, JP, FG, MF, CS and FLB performed animal experiments and helped with tissue processing. VA, LS and ES performed flow cytometry experiments. AP, JF, RS, MH, CS, ES, BL and FLB performed in vitro experiments. AP, RS, FLB and JCB wrote the manuscript.

Chapter 3 – Viral genome editing reveals non-essential genes in the development of a safe and efficient oncolytic platform

Preface

Work described in this chapter was already in progress at the start of my PhD thesis. Dr. Fabrice LeBeouf, Dr. Chris Storbeck, Dr. Jiahu Wang and Dr. Brian Keller were instrumental in helping me get started and carrying out these experiments. This manuscript is currently in preparation for submission.

I wrote the first draft, performed most of the work detailed in this manuscript and will be first author upon publication. Work done by others include the generation of the transposon insertion library in Vaccinia, which was accomplished by Dr. Jiahu Wang. Passaging the transposon library in cancer cells was accomplished by Dr. Brian Keller. Purification and expansion of transposon clones was done by Dr. Jiahu Wang, Dr. Fabrice LeBeouf, Dr. Chris Storbeck and Dr. Brian Keller. Ribosomal profiling was done by Dr. Tyson Graber and Dr. Hoàng Dũng at CHEO. All analysis including bioinformatic analysis were performed by me.

For all *in vivo* experiments I performed the Bell lab veterinary technician Julia Petryk was involved, performing all tumor seeding, all intra-venous injections and helping me overall wellness the mice. The flow cytometry component of various experiments was performed and analysed either by Elena Scut, Fanny Tzelepis, Leonard Angka or Victoria A. Jennings. Several *in vitro* experiments including plaque assays size (Figure 3.3) and cytotoxicity (Figure 3.6) were performed under my supervision by my summer student Jessie Duong. Other lab members not already mentioned which helped design or perform some experiments include: Ragunath Singaravelu, Stephen Boulton,

Mike Huh, Matt Tang, Michael Phan, Mohsen Hooshyar, Mohammed Selman, Justin Cowen, Katherine Baxter, Christiano Tanese de Souza, Brian Laight, Serge Neault, Marie-Eve Wedge, John Chmara, Catia Cemeus and Russell Barkley.

Abstract

Vaccinia virus has a large and still incompletely understood genome although several strains of this virus are already in clinical development. For the most part, clinical candidates have been attenuated from their wild type vaccine strains through deletion of metabolic genes like the viral thymidine kinase gene. We thoroughly examined the genetic elements of vaccinia which could be modulated to improve the oncolytic/therapeutic characteristics of the virus. Using a variety of cancer cell lines and primary tumor explants, we performed a fitness assay that directly compares multiple wild-type Vaccinia strains to identify the genetic elements that together create an optimal “oncolytic engine”. Using a transposon insertion strategy and deep sequencing of viral populations we systematically examined vaccinia genes that do or do not play a role in the therapeutic activity of the virus. Our studies allowed us to identify large areas of the vaccinia genome that when deleted, augment the oncolytic activity of a newly created recombinant virus (32 genes deleted). This novel virus was compared to vaccinia strains currently in the clinic and in a variety of assays, the deleted virus displayed superior therapeutic activity, immunogenicity and an enhanced safety profile.

Introduction

Poxviruses are a large family of DNA viruses used therapeutically as vaccines [78] and as oncolytic agents for the treatment of aggressive tumours [79]. Vaccinia is an orthopoxvirus which has advanced to a Phase III clinical trial (PHOCUS) for the treatment of liver cancer (Clinical Trial NCT02562755). Despite encouraging clinical and pre-clinical success, the full potential of these viruses remains to be uncovered as over half of their 200 genes remain poorly described with unknown function. Furthermore, it is unclear which genes are required and which are counter-productive for oncolytic virotherapy. In addition, with over a dozen Vaccinia strains identified [121], groups conducting pre-clinical or clinical research often focus on only one strain without rational justification for their choice.

Vaccinia is an attractive oncolytic virus (OV) candidate, since relatively few modifications are required to make the virus safe for use in human trials [77]. A popular strategy for generating oncolytic Vaccinia virus relies on the deletion of metabolic genes [126] which is argued to increase selectivity for highly metabolic tissues such as tumours. The drawback of this strategy is the presence of highly metabolic non-cancerous tissues, such as hepatocytes, as well as low metabolic activity in certain cancer niches, for instances hypoxic areas. To create a more robust virus, a better understanding of the poxvirus genome is needed for novel strategies in engineering tumour specific Vaccinia OV. Like most viruses, Vaccinia has developed its gene repertoire to infect non-cancerous tissue and spread from host to host [137]. A better understanding of this virus can help increase its efficacy and appeal for clinical use.

Our study aims to shed light on the unfamiliar aspects of the Vaccinia genome to rationally develop a next generation Vaccinia OV. Our first goal was to identify the Vaccinia strain with the most powerful "viral engine" in the context of tumour cells. To this end we have developed a novel competition assay which identified the strain that best replicates in tumour cells and has the highest fitness. Secondly, we aimed to remove genes in the Vaccinia genome that are unessential for oncolysis. We have used transposon mutagenesis, a common technique applied to bacterial genomes to identify essential genes [189] but scarcely used for viral genomes. We generated a library of gene knockouts which we passaged in cancer cells to identify non-essential genes in tumour oncolysis. Lastly, using next generation sequencing (NGS) we screened for naturally occurring large truncations of non-essential genes in viral clones. Combining two such truncations allowed us to generate a novel replicating Vaccinia virus with enhanced oncolytic features. We anticipate our screens and assays to have widespread applications in tailoring other viruses for clinical use.

Results

Identifying which strain replicates best in cancer cells

Many genetically different Vaccinia strains exist which form a monophyletic group with up to 11.5% nucleotide divergence (Figure 3.1). To determine which of these would serve best for oncolysis we have focused on the replication of five strains currently in the clinic: Copenhagen (NCT03294486), Western Reserve (NCT00574977), Lister (NCT01766739), Wyeth (NCT02562755) and Tian Tan (NCT01705223). In our first assay we pooled equal amounts of each wild-type strain together and passaged this mixture in cancer cells and patient tumour samples (Figure 3.2). Using NGS with qPCR validation on passaged virus population, we determined the Copenhagen strain was present in the greatest abundance (Figure 3.3A, Figure S3.1) and makes the largest plaques (Figure 3.3B).

In addition to replication and spread, we looked at the production of extracellular virus (EEV) which is thought to be important in spread to distant sites of the virus. Production of EEV during Vaccinia infection can be measured using a comet assay, in which convection currents drive EEV spread in a comet shape form as shown for the Vaccinia IHD strain [190]. We therefore performed comet assays on cancer cell lines which showed us the Copenhagen strain to be the only one with a visible comet tail (Figure 3.4), suggesting Copenhagen makes more EEV in cancer cells than the other Vaccinia strains we tested.

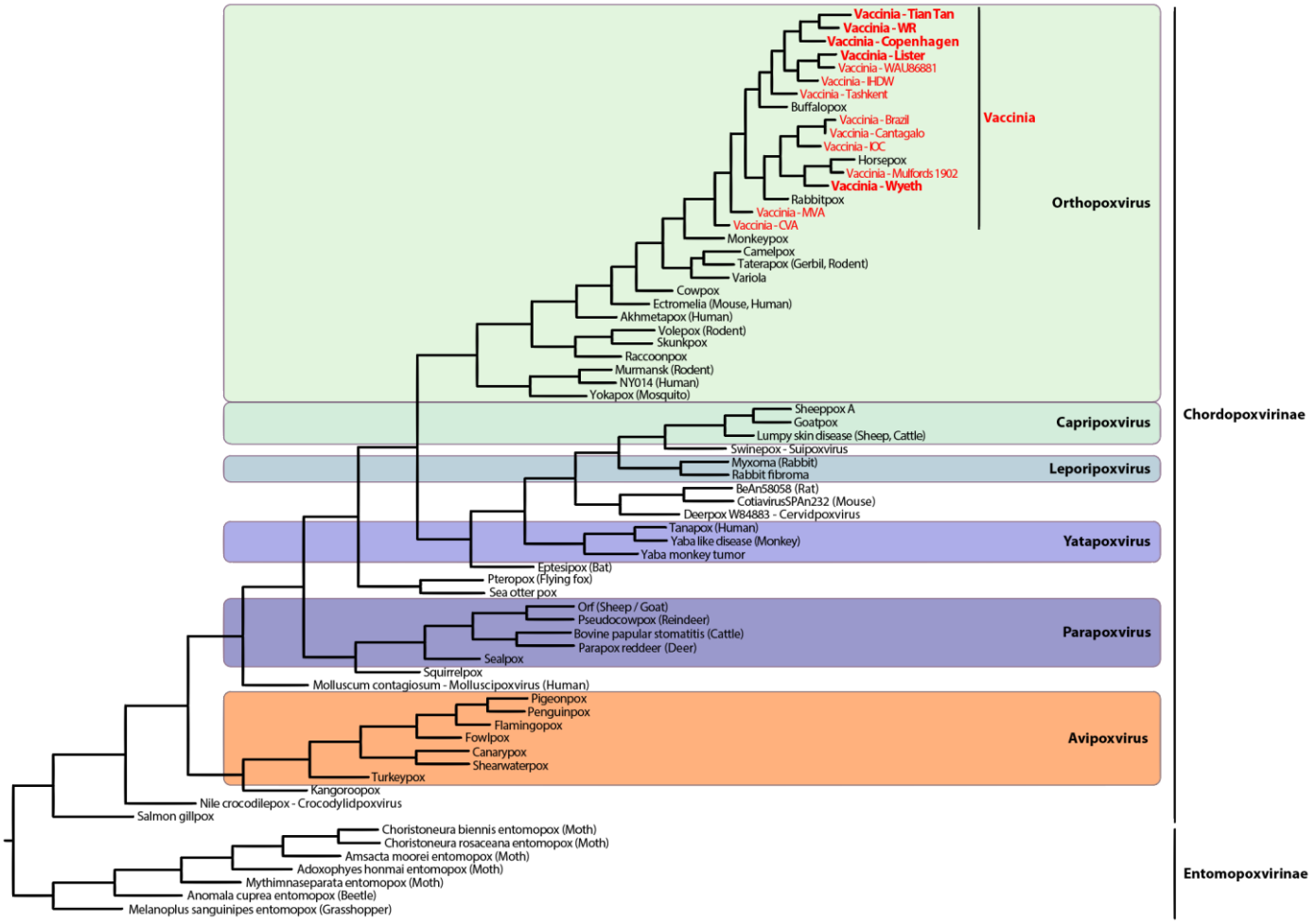

Vaccinia - Tian Tan
Vaccinia - WR
Vaccinia - Copenhagen
Vaccinia - Lister
Vaccinia - WAU86881
Vaccinia - IHDW
Vaccinia - Tashkent
Buffalopox
Vaccinia - Brazil
Vaccinia - Cantagalo
Vaccinia - IOC
Horsepox
Vaccinia - Mulfords 1902
Vaccinia - Wyeth
Rabbitpox
Vaccinia - MVA
Vaccinia - CVA
Monkeypox
Camelpox
Taterapox (Gerbil, Rodent)
Variola
Cowpox
Ectromelia (Mouse, Human)
Akhmetapox (Human)
Volepox (Rodent)
Skunkpox
Raccoonpox
Murmansk (Rodent)
NY014 (Human)
Yokapox (Mosquito)
Vaccinia
Orthopoxvirus
Sheeppox A
Goatpox
Lumpy skin disease (Sheep, Cattle)
Capripoxvirus
Swinepox - Suipoxvirus
Myxoma (Rabbit)
Rabbit fibroma
Leporipoxvirus
BeAn58058 (Rat)
CotiavirusSPAn232 (Mouse)
Deerpox W84883 - Cervidpoxvirus
Tanapox (Human)
Yaba like disease (Monkey)
Yaba monkey tumor
Yatapoxvirus
Eptesipox (Bat)
Pteropox (Flying fox)
Sea otter pox
Orf (Sheep / Goat)
Pseudocowpox (Reindeer)
Bovine papular stomatitis (Cattle)
Parapox reddeer (Deer)
Parapoxvirus
Sealpox
Squirrelpox
Molluscum contagiosum - Molluscipoxvirus (Human)
Pigeonpox
Penguinpox
Flamingopox
Fowlpox
Canarypox
Shearwaterpox
Turkeypox
Avipoxvirus
Kangoroopox
Nile crocodilepox - Crocodylidpoxvirus
Salmon gillpox
Chordopoxvirinae
Choristoneura biennis entomopox (Moth)
Choristoneura rosaceana entomopox (Moth)
Amsacta moorei entomopox (Moth)
Adoxophyes honmai entomopox (Moth)
Mythimnaseparata entomopox (Moth)
Anomala cuprea entomopox (Beetle)
Melanoplus sanguinipes entomopox (Grasshopper)
Entomopoxvirinae

Figure 3.1: Bayesian analysis of completely sequenced Poxvirus genomes.

Conserved genes among all poxviruses were aligned at the amino acid level (translational alignment) and concatenated into a matrix file. Evolutionary relationships among the different poxviruses were phylogenetically computed using Bayesian analysis. The resulting consensus tree is displayed with branch length representing evolutionary distance on a log2 scale. Vaccinia viruses are colored in red and major known groups of poxviruses are highlighted and labeled.

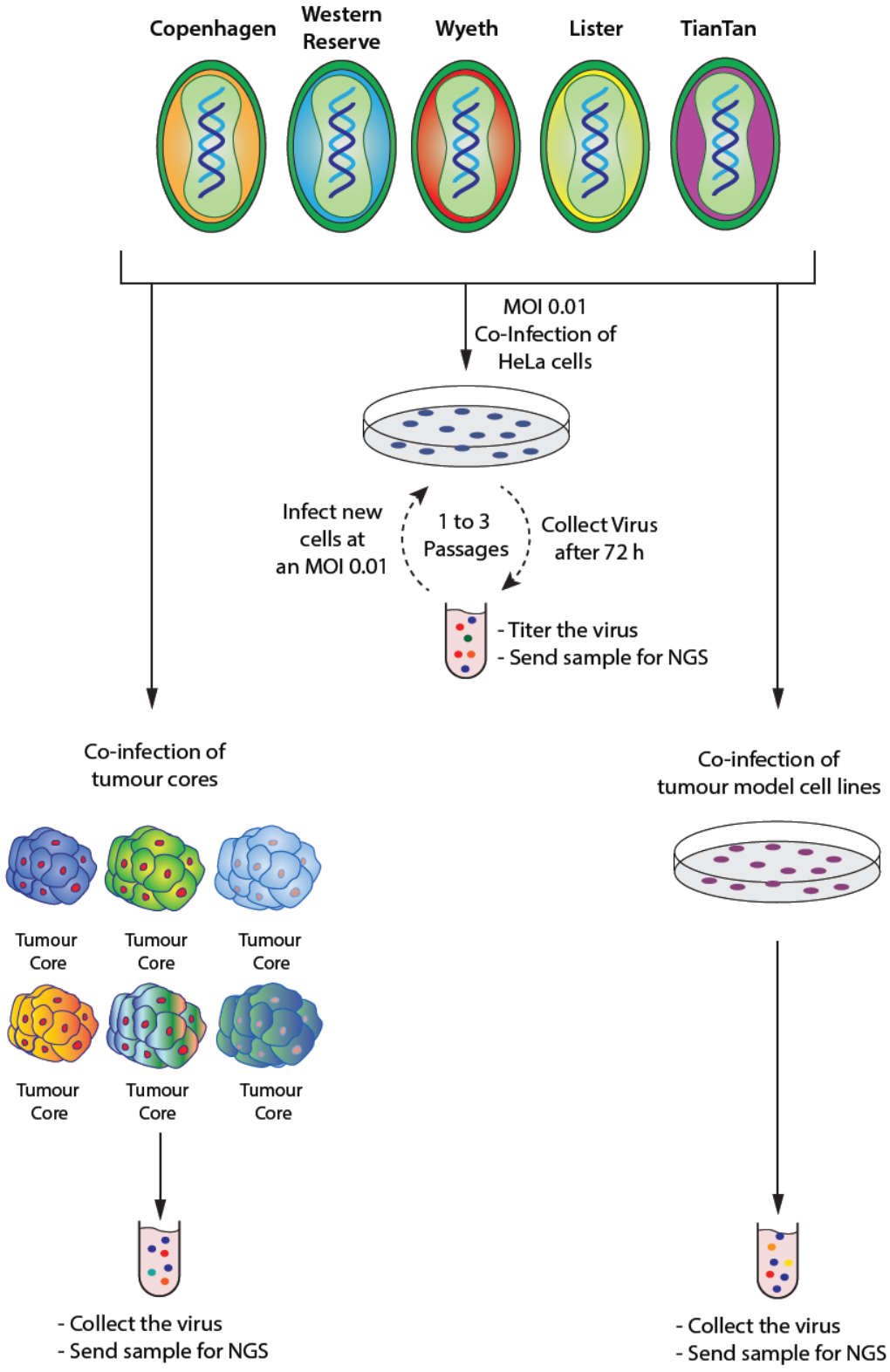

Copenhagen
Western Reserve
Wyeth
Lister
TianTan
MOI 0.01
Co-Infection of HeLa cells
Infect new cells at an MOI 0.01
1 to 3 Passages
Collect Virus after 72 h
- Titer the virus
- Send sample for NGS
Co-infection of tumour cores
Tumour Core
Tumour Core
Tumour Core
Tumour Core
Tumour Core
Tumour Core
- Collect the virus
- Send sample for NGS
Co-infection of tumour model cell lines
- Collect the virus
- Send sample for NGS

Figure 3.2: Schematic representation of the fitness assay.

Five Vaccinia strains were pooled together in equimolar rations. This pooled population was passaged in cancer cell lines, patient primary cell lines as well as human cancer xenografts. Abundance of each strains after passaging was determined using NGS sequencing of viral genomes.

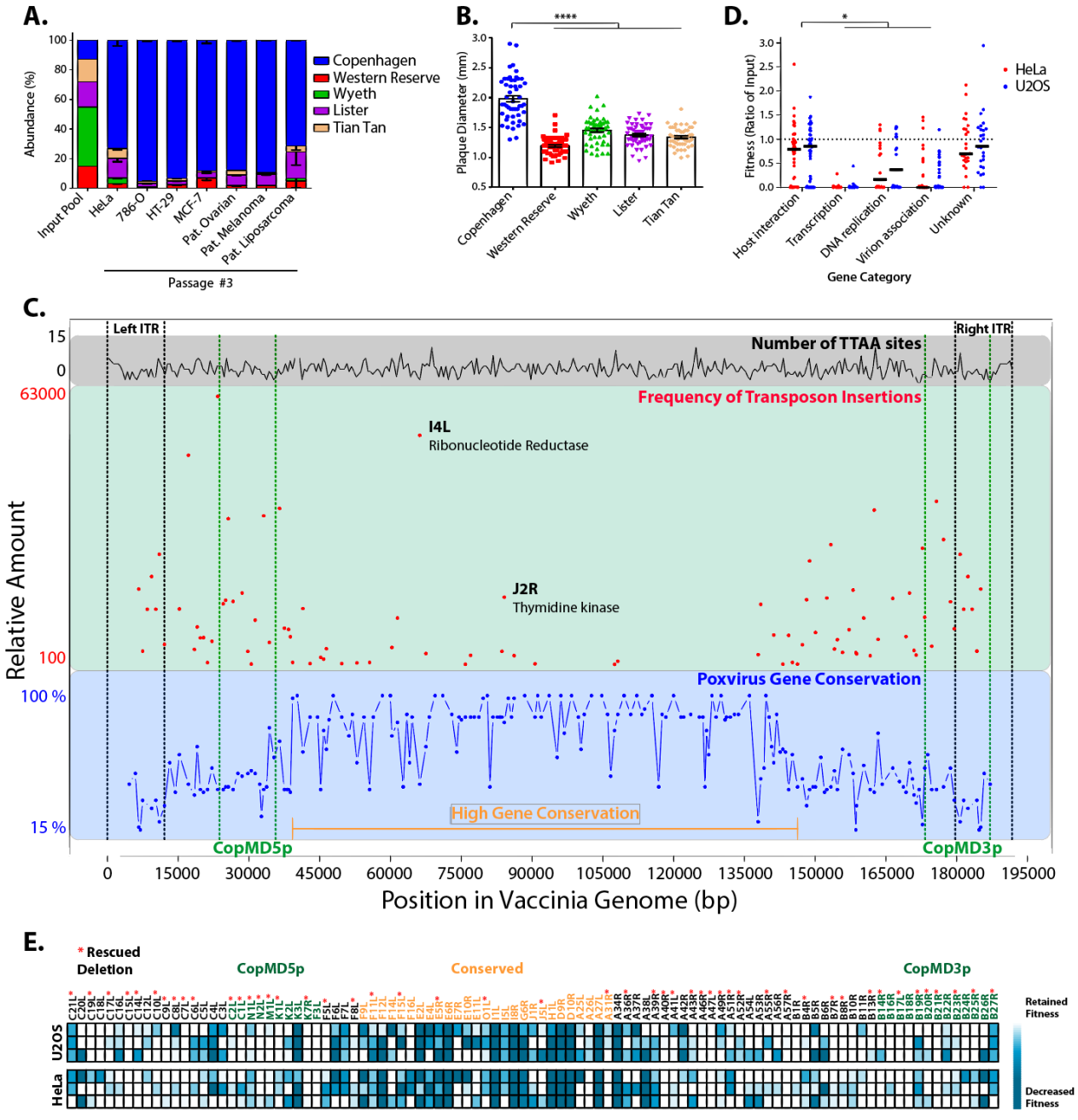

A.
Abundance (%)
Copenhagen
Western Reserve
Wyeth
Lister
Tian Tan
Input Pool
HeLa
786-O
HT-29
MCF-7
Pat. Ovarian
Pat. Melanoma
Pat. Liposarcoma
Passage #3
B.
****
Plaque Diameter (mm)
Copenhagen
Western Reserve
Wyeth
Lister
Tian Tan
D.
*
Fitness (Ratio of Input)
HeLa
U2OS
Host interaction
Transcription
DNA replication
Virion association
Unknown
Gene Category
C.
Left ITR
Right ITR
Number of TTAA sites
Frequency of Transposon Insertions
I4L
Ribonucleotide Reductase
J2R
Thymidine kinase
Poxvirus Gene Conservation
High Gene Conservation
CopMD5p
CopMD3p
Relative Amount
Position in Vaccinia Genome (bp)
E.
* Rescued Deletion
CopMD5p
Conserved
CopMD3p
U2OS
HeLa
Retained Fitness
Decreased Fitness

Figure 3.3: Copenhagen is the Vaccinia strain which replicates the fastest in cancer cells.

(A) Relative fitness of five Vaccinia wild-type strains after passaging (cummulative MOI 0.1) three times in various cancer cell lines including two patient derived cancer cell lines. Viral populations were sequenced using NGS after which virus sequences were assigned to each strain to determine abundance.
(B) Plaque sizes (n=50) was measured for each of the five Vaccinia strains in U2OS cells 72 hpi. One-way ANOVA parametric test was used to compare Copenhagen plaque sizes to those of other Vaccinia (**** p-value < 0.0001).
(C) Distribution of TTAA transposon insertion sites in the Copenhagen genome (top). Frequency of transposon insertion in Vaccinia genes (middle) in HeLa cells. Conservation of Copenhagen genes among all Poxviruses (bottom). Two major deletions, CopMD5p and CopMD3p are shown with dotted green lines.
(D) Relative fitness of interrupted viral genes of different functional categories quantified by Tn-seq after three passages (MOI 0.1) in either HeLa or U2-OS cells. Fitness is represented as fold change from initial interruption frequencies shown in panel (C). Statistical analysis was performed used two-way ANOVA tests comparing the Host interaction category to that of Transcription, DNA replication and Virion association, lowest p-value shown (* p-value < 0.05).
(E) Relative fitness measurement of transposon interruptions shown for each viral gene sorted from the left side to the right side of the genome. Experiment done in HeLa and U2OS human cancer cells (n=3, rows are replicates).

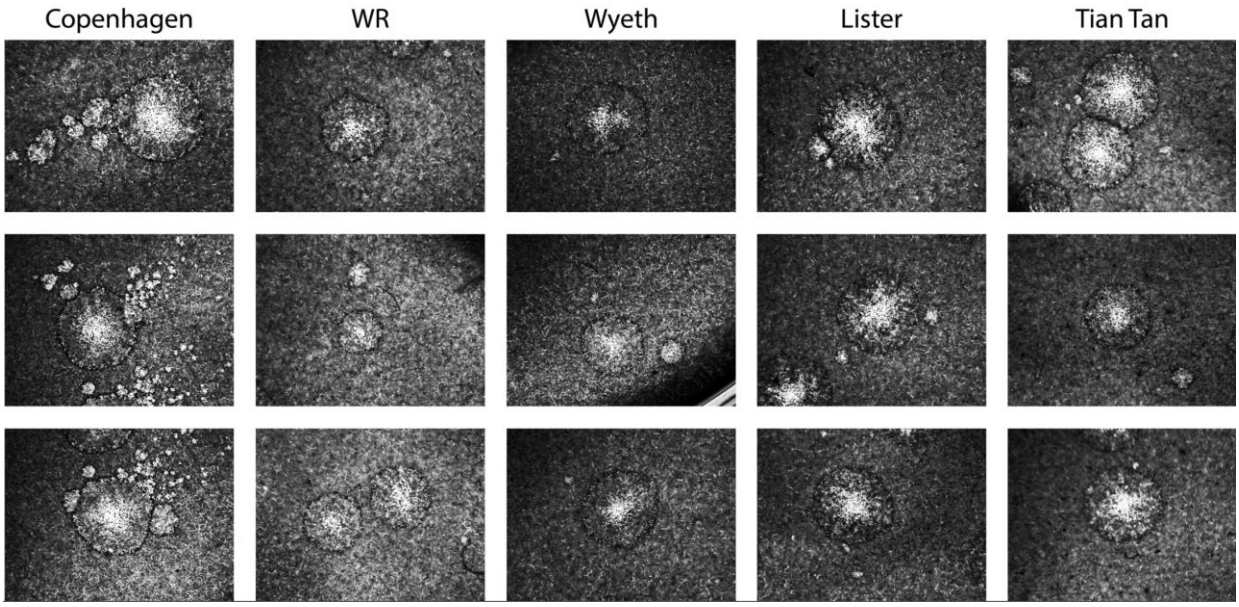

Copenhagen
WR
Wyeth
Lister
Tian Tan

Figure 3.4: Vaccinia Copenhagen produces more EEV particles compared to other wild-type strains.

Confluent U2-OS monolayers were infected with 10-30 PFU units of five Vaccinia wild-type strains for 48h. The 6 well plates were then stained with crystal violet and pictures taken with brightfield microscopy.

Genome-wide analysis reveals non-essential genes for oncolysis

Poxviruses have between 150-250 genes involved in a wide variety of functions such as genome replication [94], virion components [91] and immune evasion genes [158]. In order to identify genes that do or do not play a role in the therapeutic activity of the virus, we used transposon mutagenesis to randomly knock out genes. To this end, a piggyback transposase was used to insert the mCherry genes randomly into TTAA sites of the Copenhagen genome. Despite ubiquitous presence of TTAA sites in the genome, Tn-seq of our knockout population suggests preferential interruption of poorly conserved poxvirus genes (Figure 3.3C).

Passaging our knockout population in permissive U2OS and HeLa cells shows little change in fitness when interrupting Host interaction and Unknown genes. On the other hand, there is great fitness reduction for virions with interrupted genes involved in housekeeping function such as Translation, DNA replication and Virion association (Figure 3.3D). Indeed, the middle of the genome enriched in conserved housekeeping genes has a negative impact on fitness when interrupted (Figure 3.3E). This is likely due to the importance of those genes in the life cycle of the virus, without which virion replication is impaired.

Since interrupted viruses express mCherry under a synthetic Vaccinia early-late promoter, we have used flow cytometry and plaque purification to successfully isolate and expand 52 Vaccinia viruses with individual gene knockouts (Table 3.1). Unsurprisingly, the viruses we managed to isolate were those with interruptions in poorly conserved genes located at genome extremities (Figure 3.3E).

Table 3.1: List of transposon mediated gene interruptions rescued in Vaccinia Copenhagen. Information includes genomic position (GenBank M35027) of transposon insertion, insert index (relative location of insert to the entire CDS of the gene) as well as functional category of the affected gene.

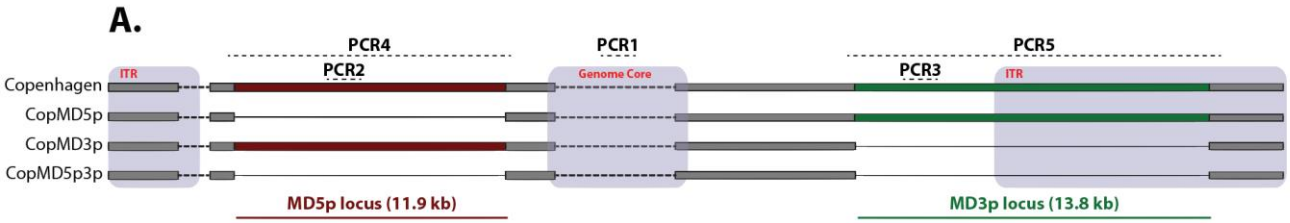
Gene	Position	Insertion Index	Category
C21L	6589	0.7	Unknown
C19L	7584	0.3	Pseudogene
C17L	9378	0.5	Unknown
C15L	11121	0.1	Unknown
C14L	12126	0.3	Pseudogene
C10L	15665	0.1	Host interaction
C9L	18015	0.1	Host interaction
C8L	18545	0.3	Unknown
C7L	18807	1	Host interaction
C6L	19814	0.3	Host interaction
C2L	23654	0.3	Host interaction
C1L	24415	0.7	Unknown
N1L	25169	0.2	Host interaction
N2L	25545	0.6	Host interaction
M1L	26519	0.6	Host interaction
K1L	28634	0.4	Host interaction
K5L	32274	0.5	Pseudogene
K7R	33032	0.3	Host interaction
F2L	34514	0.1	DNA replication
F4L	36031	1	DNA replication
F5L	37753	0.2	Unknown
F8L	38729	0.8	Unknown
F11L	41685	0.3	Virion association
F15L	45925	0.3	Unknown
E5R	52400	0	Unknown
O1L	62013	0.2	Host interaction
I4L	65350	0.9	DNA replication
J2R	84281	0.8	DNA replication
J5L	86021	1	Virion association
A31R	141381	0.9	Unknown
A36R	144647	0.9	Virion association
A39R	146865	0.1	Host interaction
A40R	148246	0.6	Host interaction
A41L	148916	0.4	Host interaction
A43R	149965	0.3	Host interaction

A46R	152552	0.6	Host interaction
A47L	153616	0.1	Unknown
A48R	154341	0.9	DNA replication
A49R	154579	0.3	Host interaction
A50R	155176	0.1	DNA replication
A51R	157201	0.5	Unknown
A52R	158261	0.9	Host interaction
A55R	159523	0	Host interaction
A57R	162524	0.5	Pseudogene
B4R	166682	0.6	Unknown
B7R	169069	0.1	Host interaction
B8R	169729	0.2	Host interaction
B13R	172760	0.6	Pseudogene
B14R	172926	0.1	Pseudogene
B17L	175536	0.7	Unknown
B19R	178742	0.6	Host interaction
B20R	179372	0.2	Unknown

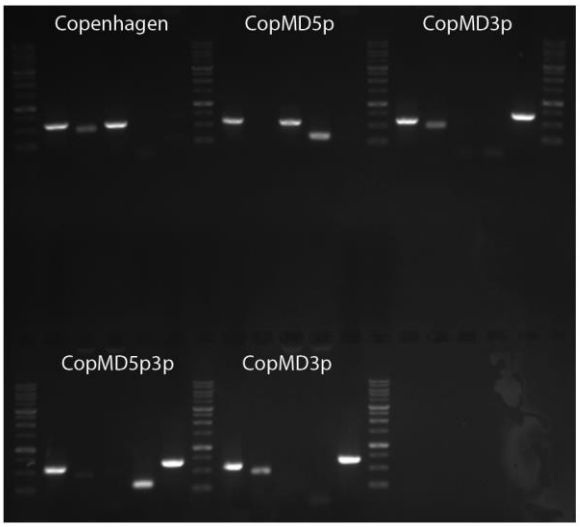
Naturally occurring deletions of non-essential genes

Our Tn-seq data suggests dozens of genes are dispensable for oncolysis. This data is supported by the existence of poxviruses such as MVA which have accumulated multiple large deletions through passaging and can still replicate albeit with a very restricted host range [191]. In order to screen for replicating viruses with large deletions of dispensable genes, we have infected cancer cell lines with our wild-type Copenhagen at low MOI (0.0001), picked and sequenced 200 Vaccinia plaques. After assembling all viral genomes, we have found 2 replicating viruses CopMD5p and CopMD3p to harbour deletions larger than 10kb in non-essential regions (Figure 3.3C). We were able to isolate these viruses and recombine them into a double-deleted virus (Figure 3.5) which we will refer to as CopMD5p3p (32 gene KO; Table 3.2).

Characterizing this virus revealed it is able to induce much faster cell death than its parental counterpart in 32 human cell lines (Figure 3.6A). Despite killing faster, we found no replication disadvantage *in vitro* or *ex vivo* when tested on patient tumour cores (Figure 3.6B-C).



B.



Lane:
1 - PCR1 - Vaccinia control
2 - PCR2 - MD5p locus present
3 - PCR3 - MD3p locus present
4 - PCR4 - MD5p locus deleted
5 - PCR5 - MD3p locus deleted

Figure 3.5: PCR strategy to screen recombinants for a double deleted virus.

(A) Genomic schematic of wild-type Vaccinia Copenhagen, the single major deletions CopMD5p and CopMD3p as well as an expected double deleted CopMD5p3p. Also shown are PCR products expected from the designed PCR primers. Product PCR1 is a positive control and should always give product. Product PCR2 will only give a product when MD5p is present (Copenhagen, CopMD3p). Product PCR3 will only give a product when MD3p is present (Copenhagen, CopMD5p). For PCR products PCR4 and PCR5 the expected amplicon length on Copenhagen DNA is >10kb which would not amplify with a standard Taq polymerase and 1 min extension time. Product PCR4 will only give a product when MD5p is deleted (CopMD5p, CopMD5p3p). Product PCR5 will only give a product when MD3p is deleted (CopMD3p, CopMD5p3p).
(B) PCR gel showing validation of CopMD5p3p deletion. Top row are Copenhagen, CopMD5p and CopMD3p expected patterns. Bottom left row is CopMD5p3p, since only control PCR1 and presence of deletion products PCR4 and PCR5 give a band.

Table 3.2: List of genes and their respective function deleted in CopMD5p, CopMD3p and CopMD5p3p viruses

Name	Category	Function	Virus Deletions	
C2L	Host interaction	Inhibits inflammation; Kelch-like [192]	CopMD5p	CopMD5p3p
C1L	Unknown	Unknown		
N1L	Host interaction	Inhibits NF-κB and Apoptosis [193]		
N2L	Host interaction	Inhibits IRF3 [103]		
M1L	Unknown	Unknown [194]		
M2L	Host interaction	Inhibits NF-κB and Apoptosis [110]		
K1L	Host interaction	Inhibits PKR and NF-κB [195]		
K2L	Host interaction	Prevents cell fusion [196]		
K3L	Host interaction	Inhibits PKR [197]		
K4L	DNA replication	DNA modifying nuclease [198]		
K5L	Pseudogene	Pseudogene		
K6L	Pseudogene	Pseudogene		
K7R	Host interaction	Inhibits NF-κB and IRF3 [118]		
F1L	Host interaction	Inhibits Apoptosis [178]		
F2L	DNA replication	Deoxyuridine triphosphatase [199]		
F3L	Host interaction	Virulence factor [200]		
B14R	Pseudogene	Pseudogene	CopMD3p	
B15R	Host interaction	Inhibits NF-κB [109]		
B16R	Host interaction	IL-1-beta-inhibitor [201]		
B17L	Unknown	Unknown		
B18R	Unknown	Genome uncoating [202]		
B19R	Host interaction	Secreted IFNa/b sequester [203]		
B20R	Unknown	Ankyrin-like [204]		
B21R-ITR*	Unknown	Unknown		
B22R-ITR*	Unknown	Unknown		
B23R-ITR*	Unknown	Unknown		
B24R-ITR*	Unknown	Unknown		
B25R-ITR*	Unknown	Unknown		
B26R-ITR*	Unknown	Unknown		
B27R-ITR*	Unknown	Unknown		
B28R-ITR*	Pseudogene	TNF-a receptor [205]		
B29R-ITR*	Host interaction	Secreted CC-chemokine sequester [206]		

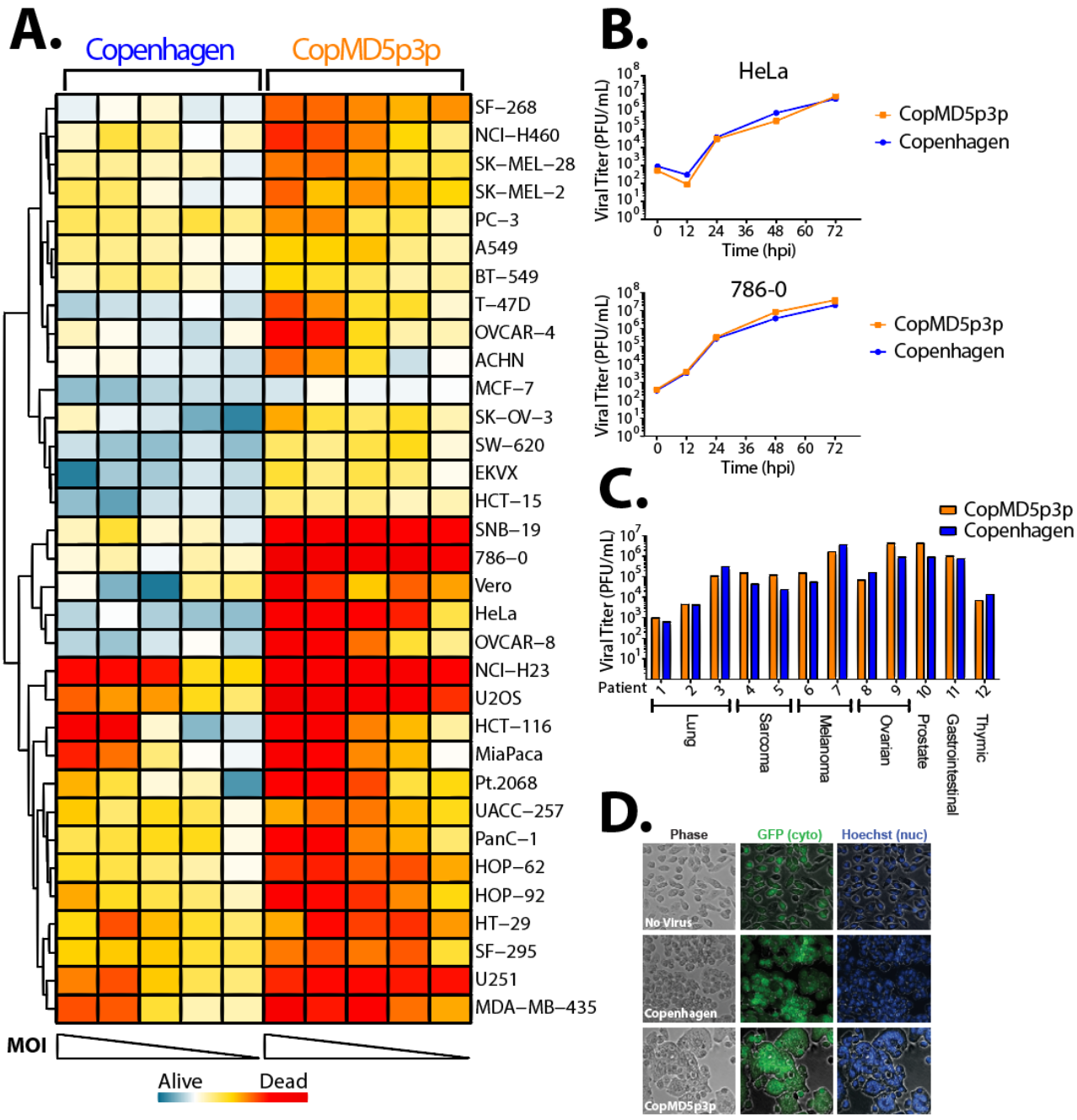
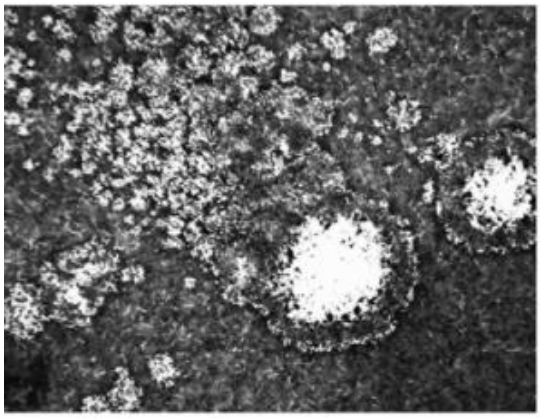

A.
Copenhagen
CopMD5p3p
SF–268
NCI–H460
SK–MEL–28
SK–MEL–2
PC–3
A549
BT–549
T–47D
OVCAR–4
ACHN
MCF–7
SK–OV–3
SW–620
EKVX
HCT–15
SNB–19
786–0
Vero
HeLa
OVCAR–8
NCI–H23
U2OS
HCT–116
MiaPaca
Pt.2068
UACC–257
PanC–1
HOP–62
HOP–92
HT–29
SF–295
U251
MDA–MB–435
MOI
Alive
Dead
B.
HeLa
Viral Titer (PFU/mL)
Time (hpi)
CopMD5p3p
Copenhagen
786-0
Viral Titer (PFU/mL)
Time (hpi)
CopMD5p3p
Copenhagen
C.
CopMD5p3p
Copenhagen
Viral Titer (PFU/mL)
Patient
Lung
Sarcoma
Melanoma
Ovarian
Prostate
Gastrointestinal
Thymic
D.
Phase
GFP (cyto)
Hoechst (nuc)
No Virus
Copenhagen
CopMD5p3p

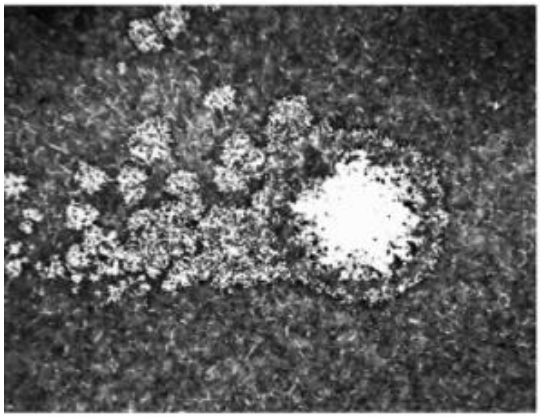
Figure 3.6: CopMD5p3p kills cancer cells faster maintaining replication levels compared to parental Copenhagen.

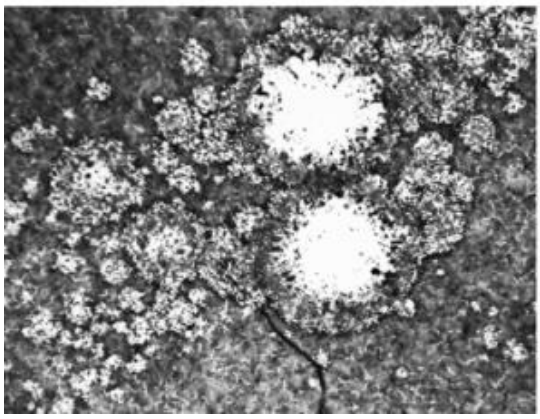
(A) Cytotoxicity of Parental and Deleted CopMD5p3p in a wide variety of NCI-60 human cancer cell lines. Statistical analysis was performed used two-way ANOVA tests comparing the various MOIs (1, 0.3, 0.1, 0.03 and 0.01) between the two viruses, lowest p-value shown (**** p-value < 0.0001).
(B) Multi-step growth curve of Parental and Deleted Copenhagen in HeLa and 786-0 cell lines at MOI 0.001.
(C) Growth of Parental and Deleted Copenhagen in ex-vivo patient tumours after 72h infected with 1e4 PFU).
(D) GFP expressing 786-Os where infected at MOI 0.01 for 48h and stained for nuclei with Hoechst to observe syncytia formation.

Looking at cytopathic effects and plaque formation, we noticed cells underwent fusion (syncytia) when infected by CopMD5p3p (Figure 3.6D), This is likely due to CopMD5p3p missing K2L, an SPI-3 gene which in cowpox has been shown to form complexes with A56R that prevent cell fusion [207]. CopMD5p3p also forms larger comets (Figure 3.7) and plaques (Figure 3.8) compared to its parental virus. The former is indicative of greater EEV release while the latter is affective by replication, spread and cell killing. Lastly, the ability of CopMD5p3p to express a functional transgene was assessed by encoding Firefly Luciferase in the TK locus and assaying for luciferase activity. This experiment revealed comparable luciferase levels between CopMD5p3p and Copenhagen (Figure 3.9), suggesting a retained ability to express virally encoded functional transgenes.

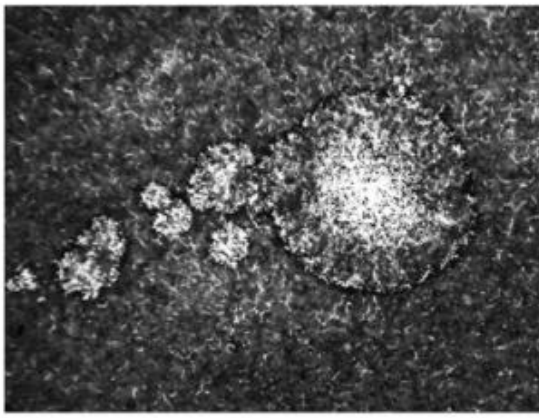
CopMD5p3p

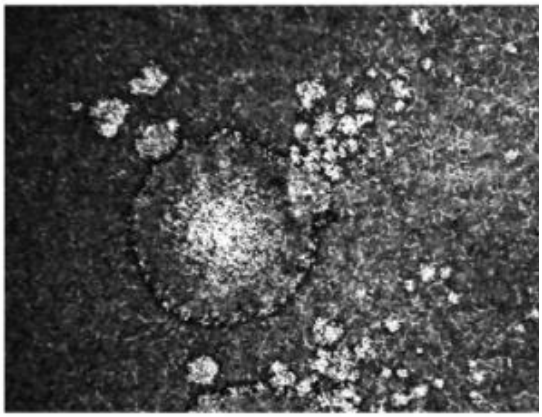






Copenhagen





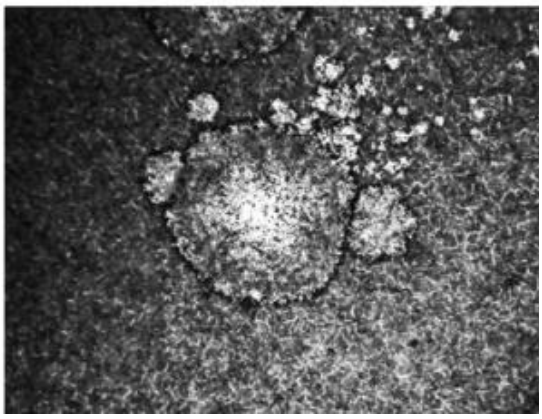


Figure 3.7: Vaccinia CopMD5p3p produces more EEV particles compared to parental wild-type Copenhagen.

Confluent U2-OS monolayers were infected with 10-30 PFU units of CopMD5p3p or Copenhagen Vaccinia wild-type for 48h. The 6 well plates were then stained with crystal violet and pictures taken with brightfield microscopy.

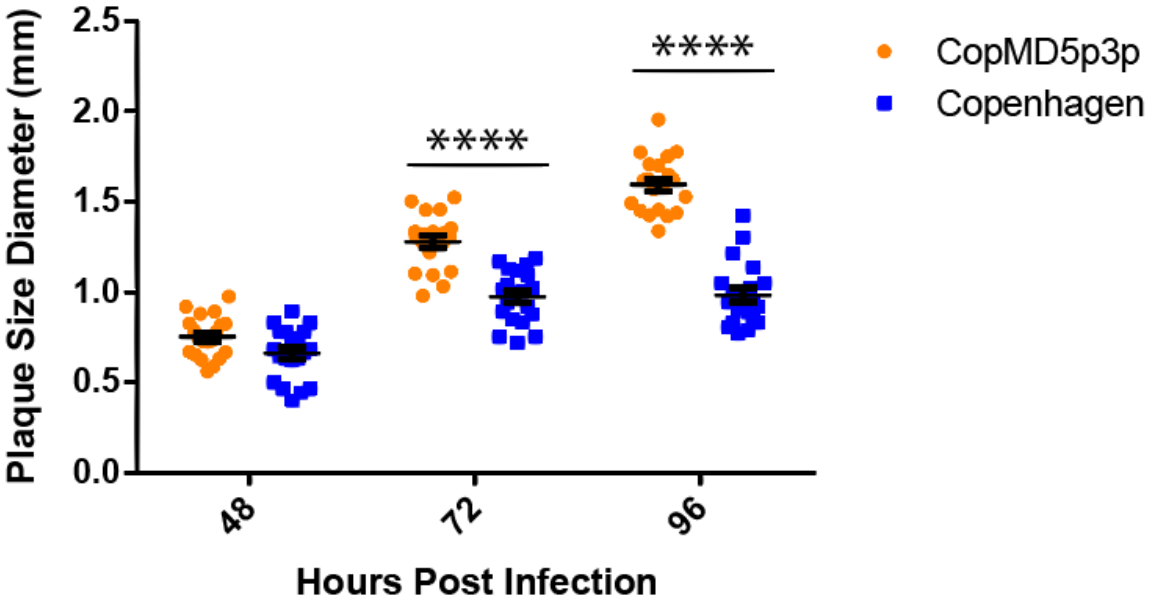
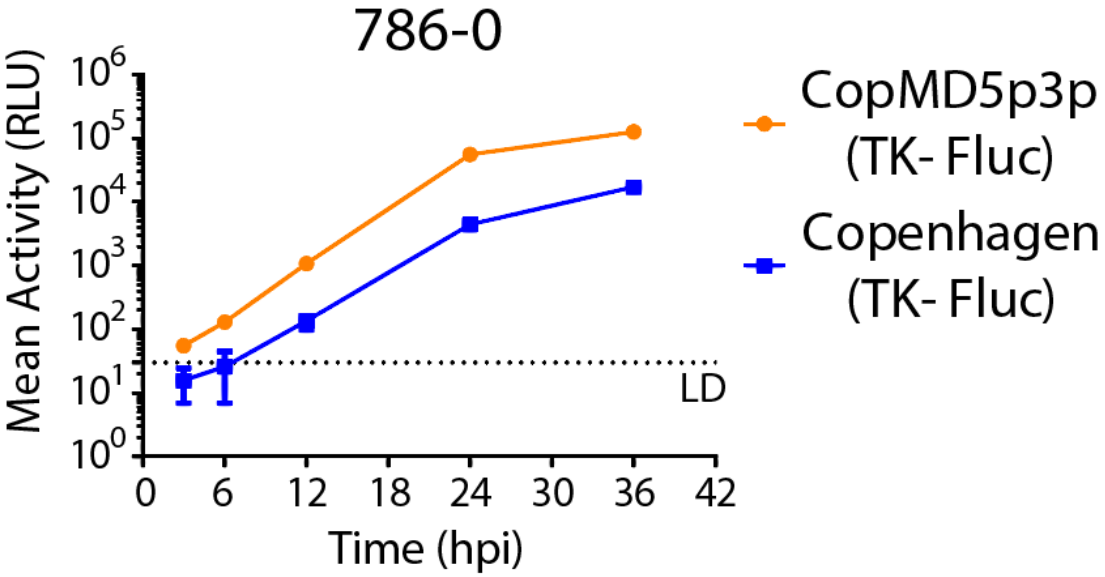

Plaque Size Diameter (mm)
2.5
2.0
1.5
1.0
0.5
0.0
****
****
48
72
96
Hours Post Infection
CopMD5p3p
Copenhagen

Figure 3.8: Vaccinia CopMD5p3p forms larger plaques compared to parental wild-type Copenhagen.

Confluent monolayers of 786-O cells were infected with 10-30 PFU units of CopMD5p3p or Copenhagen Vaccinia wild-type for various time points. The 6 well plates were then stained with crystal violet, dried and scanned. Pictures were analyzed with ImageJ for plaque diameter. (**** p-value < 0.0001)


786-0
Mean Activity (RLU)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LD
0 6 12 18 24 30 36 42
Time (hpi)
CopMD5p3p (TK- Fluc)
Copenhagen (TK- Fluc)

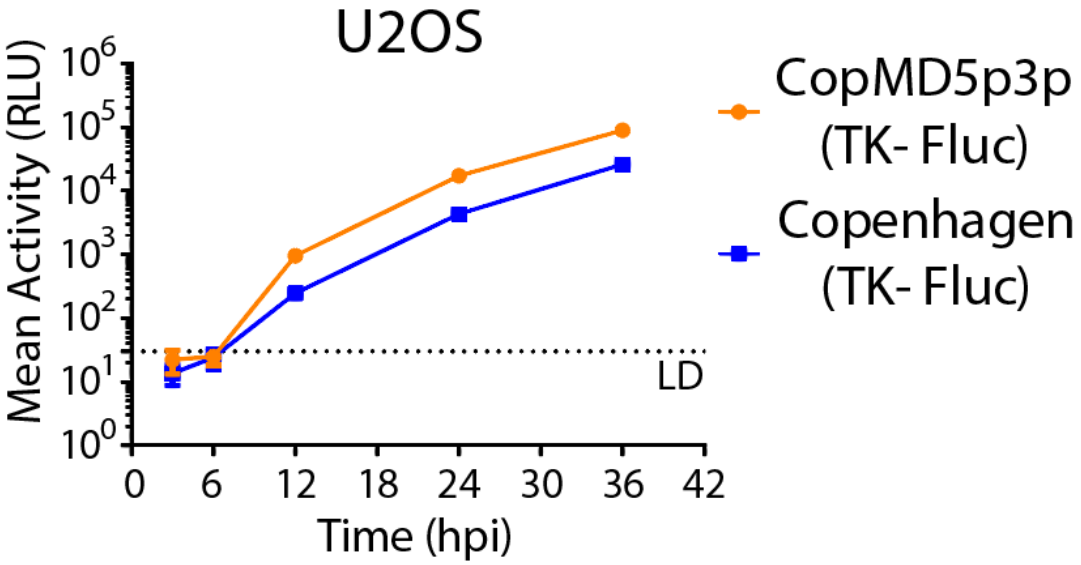

U2OS
Mean Activity (RLU)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LD
0 6 12 18 24 30 36 42
Time (hpi)
CopMD5p3p (TK- Fluc)
Copenhagen (TK- Fluc)

Figure 3.9: Vaccinia CopMD5p3p can express transgenes at levels comparable to parental virus.

Confluent monolayers of 786-O or U2OS cells were infected at an MOI of 0.1 with either CopMD5p3p or Copenhagen Vaccinia wild-type. Luciferase activity was assessed at various time points.

CopMD5p3p is more tumor selective

Historically Vaccinia strains in their wild-type form have shown adverse effects when used at small doses to vaccinate against smallpox [208]. Despite being attenuated, current Vaccinia agents in the clinic induce pox lesions in some patients, suggesting off-target replication [34]. Most often, viral infection induces interferon signaling which leads to immune cell recruitment and viral clearance. Although Vaccinia encodes multiple inhibitors of interferon and anti-viral response [28], CopMD5p3p is missing some of these inhibitors (Table 3.2). Transcriptome analysis of infected cells show CopMD5p3p to induce interferon in normal peripheral blood mononuclear cells (PBMCs) immune cells but not cancer cells (Figure 3.10). For comparison, we chose to evaluate Copenhagen deleted for TK as a clinical surrogate having a similar attenuation to Pexa-Vec currently in clinical trials. This analysis showed Copenhagen ΔTK to fully suppress anti-viral signaling in both immune cells and cancer cells (Figure 3.10). Lastly, a CopMD5p3p infection of PBMCs failed to show expression of genes involved in virion assembly at a late time point (18h). These genes are typically expressed during the late stages of the lifecycle and show high expression in cancer cells (Figure 3.11). This observation is consistent with previous reports of Vaccinia undergoing abortive replication in immune cells [209].

Altogether, these results suggest CopMD5p3p may be a safer virus as it does not impair anti-viral response in normal cells and does not replicate in immune cells.

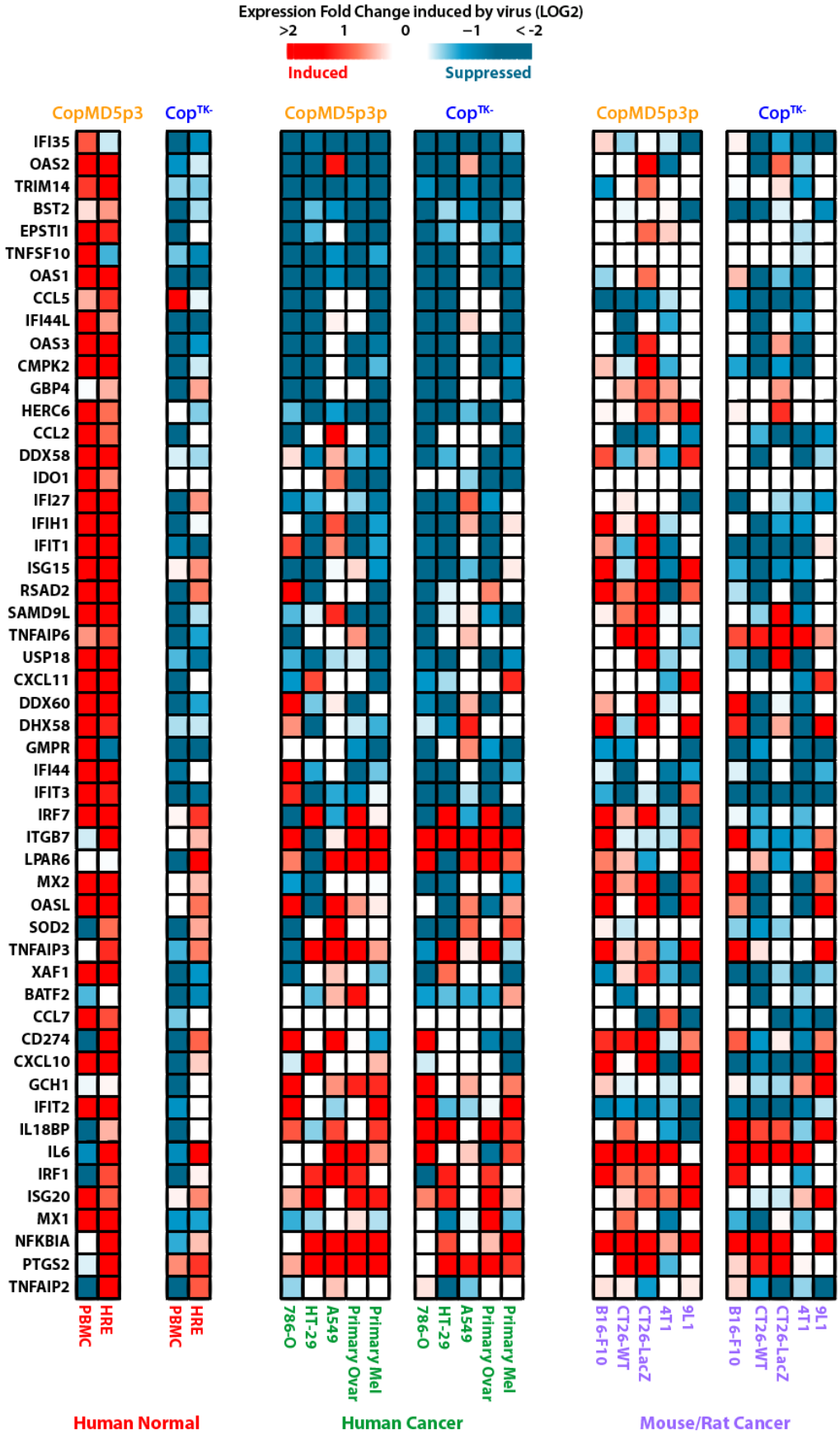

Expression Fold Change induced by virus (LOG2)
>2
1
0
−1
< -2
Induced
Suppressed
CopMD5p3
Cop^TK-
CopMD5p3p
Cop^TK-
CopMD5p3p
Cop^TK-
IFI35
OAS2
TRIM14
BST2
EPSTI1
TNFSF10
OAS1
CCL5
IFI44L
OAS3
CMPK2
GBP4
HERC6
CCL2
DDX58
IDO1
IFI27
IFIH1
IFIT1
ISG15
RSAD2
SAMD9L
TNFAIP6
USP18
CXCL11
DDX60
DHX58
GMPR
IFI44
IFIT3
IRF7
ITGB7
LPAR6
MX2
OASL
SOD2
TNFAIP3
XAF1
BATF2
CCL7
CD274
CXCL10
GCH1
IFIT2
IL18BP
IL6
IRF1
ISG20
MX1
NFKBIA
PTGS2
TNFAIP2
PBMC
HRE
PBMC
HRE
786-O
HT-29
A549
Primary Ovar
Primary Mel
786-O
HT-29
A549
Primary Ovar
Primary Mel
B16-F10
CT26-WT
CT26-LacZ
4T1
9L1
B16-F10
CT26-WT
CT26-LacZ
4T1
9L1
Human Normal
Human Cancer
Mouse/Rat Cancer

Figure 3.10: Vaccinia CopMD5p3p triggers an anti-viral response in normal cells.

ISG expression as quantified by RNA-seq of human normal, human cancer and mouse cancer cells after infection for 18h at MOI 3 with either CopMD5p3p or Copenhagen ΔTK. Heatmap shows fold change values on a $\log_2$ scale between infected and uninfected samples.

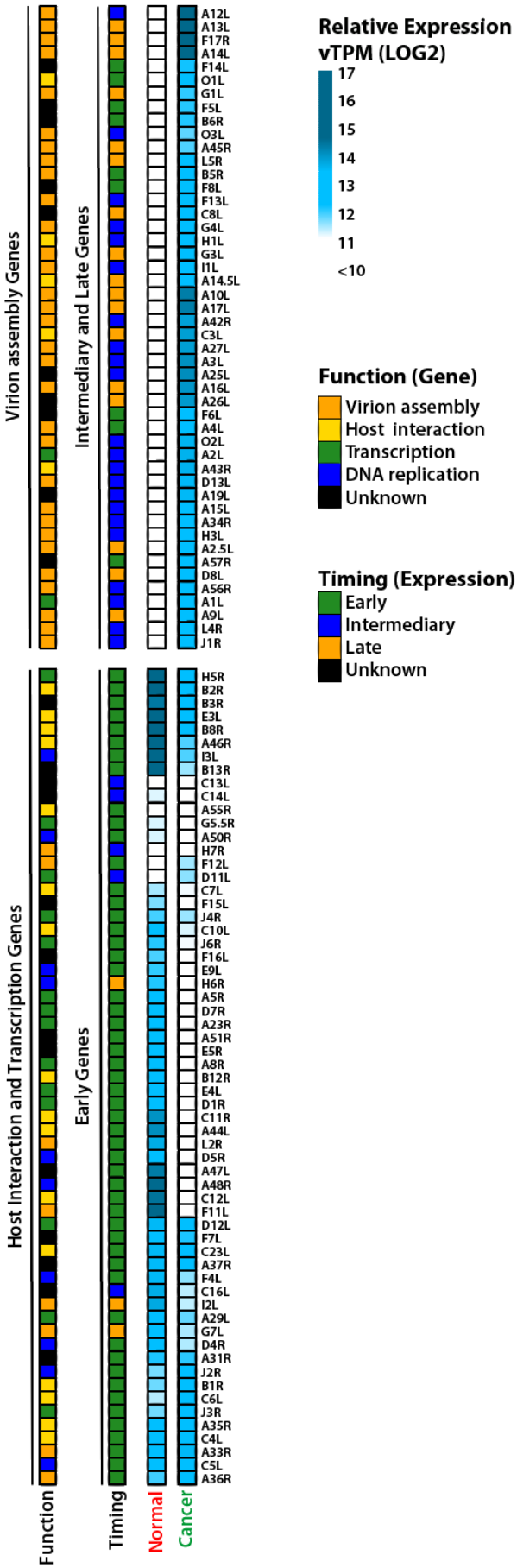

Virion assembly Genes
Intermediary and Late Genes
A12L
A13L
F17R
A14L
F14L
O1L
G1L
F5L
B6R
O3L
A45R
L5R
B5R
F8L
F13L
C8L
G4L
H1L
G3L
I1L
A14.5L
A10L
A17L
A42R
C3L
A27L
A3L
A25L
A16L
A26L
F6L
A4L
O2L
A2L
A43R
D13L
A19L
A15L
A34R
H3L
A2.5L
A57R
D8L
A56R
A1L
A9L
L4R
J1R
Host Interaction and Transcription Genes
Early Genes
H5R
B2R
B3R
E3L
B8R
A46R
I3L
B13R
C13L
C14L
A55R
G5.5R
A50R
H7R
F12L
D11L
C7L
F15L
J4R
C10L
J6R
F16L
E9L
H6R
A5R
D7R
A23R
A51R
E5R
A8R
B12R
E4L
D1R
C11R
A44L
L2R
D5R
A47L
A48R
C12L
F11L
D12L
F7L
C23L
A37R
F4L
C16L
I2L
A29L
G7L
D4R
A31R
J2R
B1R
C6L
J3R
A35R
C4L
A33R
C5L
A36R
Function
Timing
Normal
Cancer
Relative Expression
vTPM (LOG2)
17
16
15
14
13
12
11
<10
Function (Gene)
Virion assembly
Host interaction
Transcription
DNA replication
Unknown
Timing (Expression)
Early
Intermediary
Late
Unknown

Figure 3.11: Vaccinia CopMD5p3p does not express virion assembly genes in normal cells.

Expression levels of viral genes shown as log2 viral transcripts per million (vTPM). Normal (human PBMC) or cancer (786-O) cells were infected with CopMD5p3p virus at an MOI of 3 for 18h, after which RNA was extracted and transcript levels quantified using RNA-seq. Only viral genes which are expressed at high levels (> 4096 vTPM) in either normal or cancer cells are shown.

We have set to compare the safety profile of CopMD5p3p to replicas of three Vaccinia currently in the clinic. Upon treating CD-1 nude mice implanted with HT-29 xenografts with three 1e7 Vaccinia doses intravenously (IV), we find that unlike Copenhagen, our CopMD5p3p does not cause weight loss and controls tumour growth (Figure 3.12A). To investigate this further, we have encoded Firefly Luciferase in the TK locus of our Vaccinia viruses and imaged following one IV dose. We find that both, Wyeth TK- and Copenhagen TK- viruses are able to infect and replicate in murine healthy tissues while our CopMD5p3p TK- is tumor selective (Figure 3.12B). To confirm off-target replication in various organs we have performed a viral biodistribution and found less CopMD5p3p virus in normal tissues when compared with attenuations (i.e. TK-, VGF- targeting cellular replication) in current clinical candidates (Figure 3.12C). Infection of normal cells *in vitro* also shows a difference in killing between CopMD5p3p and Copenhagen ΔTK, most visibly in normal human prostate cells (Figure 3.12D). Consistent with IVIS imaging and biodistribution results, TK- viruses replicate in mouse tails and cause pox lesions while CopMD5p3p infected mice do not (Figure 3.12E-F). Overall these experiments indicate the genes deleted in CopMD5p3p attenuate viral replication in normal tissues but do not change the ability of the virus to replicate in tumors.

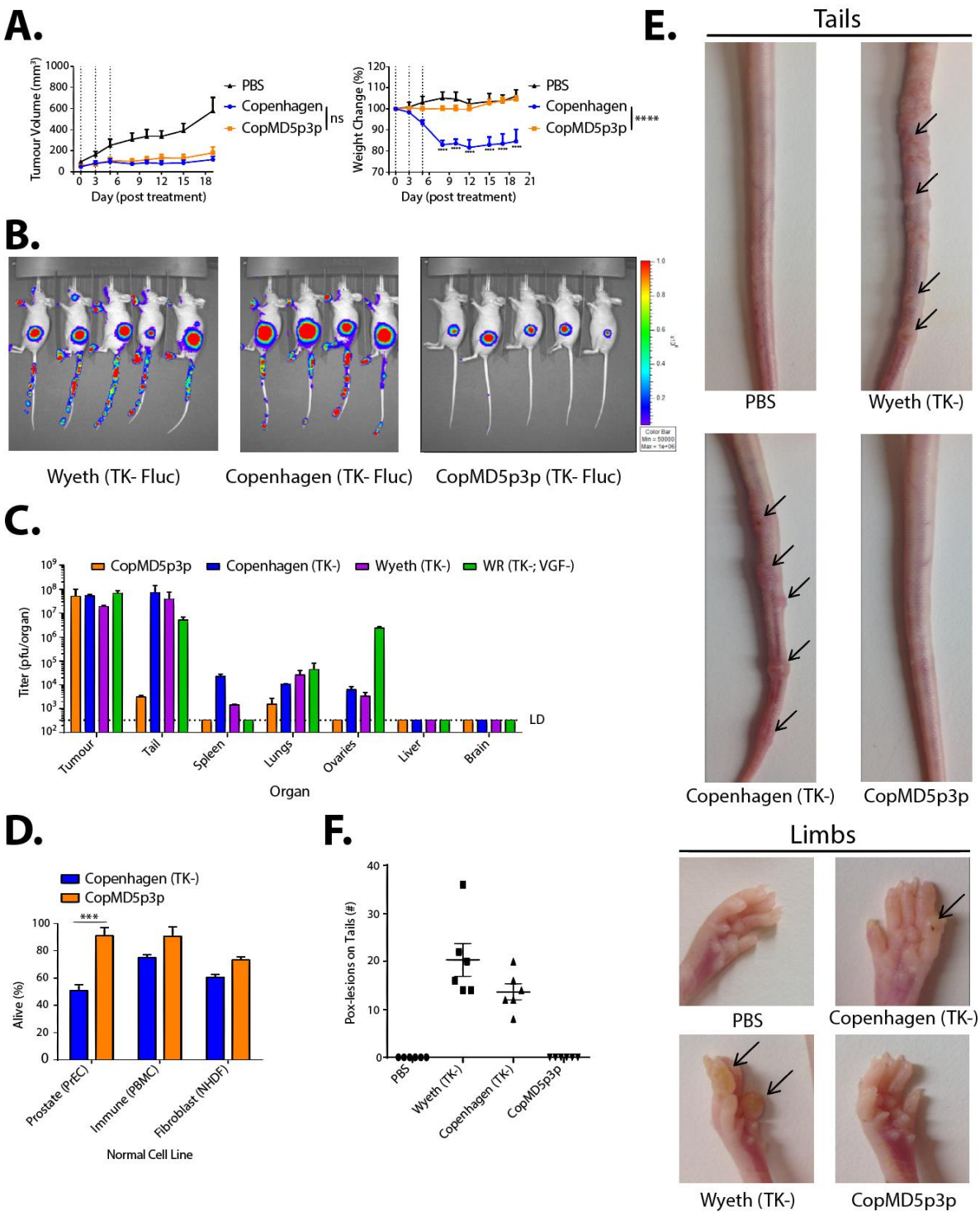

A.
Tumour Volume (mm³)
Day (post treatment)
PBS
Copenhagen
CopMD5p3p
ns
Weight Change (%)
Day (post treatment)
PBS
Copenhagen
CopMD5p3p
****
B.
Wyeth (TK- Fluc)
Copenhagen (TK- Fluc)
CopMD5p3p (TK- Fluc)
C.
CopMD5p3p
Copenhagen (TK-)
Wyeth (TK-)
WR (TK-; VGF-)
Titer (pfu/organ)
LD
Tumour
Tail
Spleen
Lungs
Ovaries
Liver
Brain
Organ
D.
Copenhagen (TK-)
CopMD5p3p
***
Alive (%)
Prostate (PrEC)
Immune (PBMC)
Fibroblast (NHDF)
Normal Cell Line
F.
Pox-lesions on Tails (#)
PBS
Wyeth (TK-)
Copenhagen (TK-)
CopMD5p3p
E.
Tails
PBS
Wyeth (TK-)
Copenhagen (TK-)
CopMD5p3p
Limbs
PBS
Copenhagen (TK-)
Wyeth (TK-)
CopMD5p3p

Figure 3.12: Increased tumour specificity and safety profile of CopMD5p3p virus.

(A) Tumour control in HT-29 xenograft (left panel) and weight loss after treatment with either Vaccinia (right panel). **** p-value < 0.0001
(B) IVIS imaging 14 days after systemic injection of 1e7 pfu Vaccinia encoding Fluc into CD1 nude mice bearing HT-29 xenografts.
(C) Biodistribution of Vaccinia 7 days after systemic injection of 1e7 pfu into CD1 nude mice bearing HT-29 xenografts.
(D) Viability of normal human cells after 48h infection at 0.3 MOI. (E) Representative pictures of pox lesion formation after systemic injection of 1e7 pfu Vaccinia into the tail vein (*** p-value < 0.001).
(F) Number of pox-lesions on tails after systemic injection of 1e7 pfu Vaccinia into the tail vein (n=6).

CopMD5p3p is more immune stimulating

Current attempts at making Vaccinia immunogenic are challenging, since the virus naturally encodes multiple factors suppressing immune signaling [210]. This is an obstacle for both, using the virus as a vaccine [211] and as an immunotherapeutic [172]. Some genes missing in CopMD5p3p are immunomodulatory (ex. B19R) and even shown to be NF-κB inhibitors (ex. N2L). Indeed, our RNA sequencing analysis of primary human PBMCs extracted from healthy donors and infected with Vaccinia showed induction of an anti-viral state with CopMD5p3p but not Copenhagen ΔTK (Figure 3.10). In a separate experiment, healthy PBMCs infected with either CopMD5p3p or Copenhagen ΔTK were evaluated using flow cytometry and shown higher expression of activation markers on immune cells during a CopMD5p3p infection (Figure 3.13A).

These results prompted us to further evaluate the immunogenicity of CopMD5p3p in a mouse *in vivo* system. To this end, tumor free mice were given 1e7 PFU of Vaccinia IV and sacrificed 24h later. Flow cytometry analysis on murine splenocytes showed a much higher levels of activation in mice treated with CopMD5p3p compared to Copenhagen ΔTK. This was true for both NK cells and as T cells (Figure 3.13B). Additionally, broad cytokine induction in the serum was noted in tumor bearing mice treated with the CopMD5p3p virus (Figure 3.14).

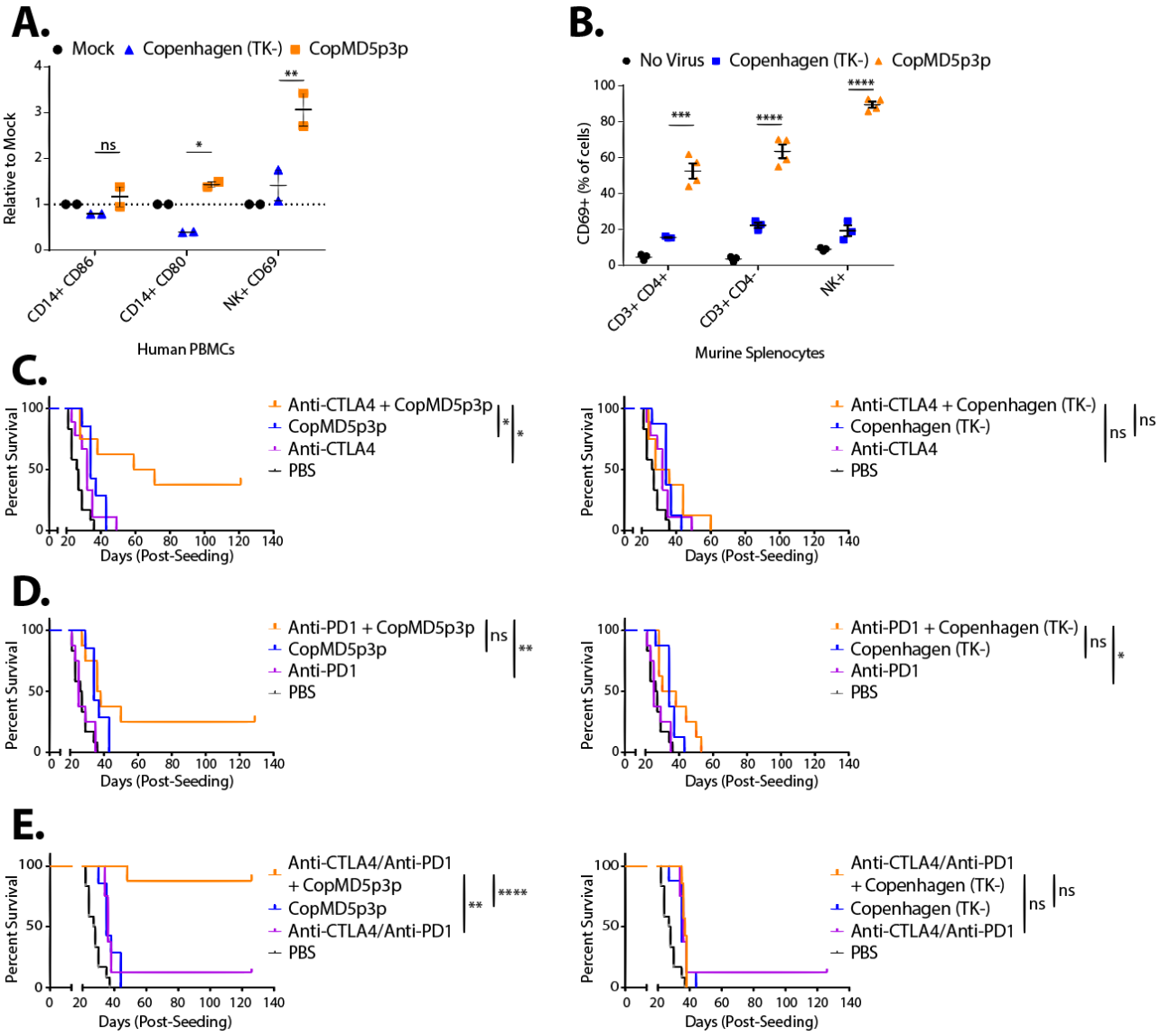

A.
Mock
Copenhagen (TK-)
CopMD5p3p
Relative to Mock
ns
*
**
CD14+ CD86
CD14+ CD80
NK+ CD69
Human PBMCs
B.
No Virus
Copenhagen (TK-)
CopMD5p3p
CD69+ (% of cells)
***
****
****
CD3+ CD4+
CD3+ CD4-
NK+
Murine Splenocytes
C.
Anti-CTLA4 + CopMD5p3p
CopMD5p3p
Anti-CTLA4
PBS
Percent Survival
Days (Post-Seeding)
Anti-CTLA4 + Copenhagen (TK-)
Copenhagen (TK-)
Anti-CTLA4
PBS
D.
Anti-PD1 + CopMD5p3p
CopMD5p3p
Anti-PD1
PBS
Anti-PD1 + Copenhagen (TK-)
Copenhagen (TK-)
Anti-PD1
PBS
E.
Anti-CTLA4/Anti-PD1 + CopMD5p3p
CopMD5p3p
Anti-CTLA4/Anti-PD1
PBS
Anti-CTLA4/Anti-PD1 + Copenhagen (TK-)
Copenhagen (TK-)
Anti-CTLA4/Anti-PD1
PBS

Figure 3.13: CopMD5p3p is immunogenic and synergizes with immune checkpoint inhibitors.

(A) Human PBMCs (n=2 donors) were infected with either Vaccinia for 24h at a MOI of 3. Change in activation markers of innate CD14 and NK cells after infection was evaluated with Flow Cytometry.
(B) Activation of murine splenocytes 24h after intravenous (IV) systemic administration of Vaccinia at 1e7 PFU.
(C-E) Mouse survival in a CT26-LacZ model implanted at 5e5 cells subQ and treated 14 days later three times with 1e7 PFU of Vaccinia intra-tumoral injection one day apart. Each panel is an independent experiment (n=10 per group).
(C-D) Mice received were given through intraperitoneal (IP) injections 100 ug of anti-body targeting immune checkpoints Anti-CTLA4 (C) or Anti-PD1 (D) in addition to Vaccinia virus.
(E) Mice received a simultaneous treatment of both Anti-CTLA4 and Anti-PD1, each at a dose of 50 ug given IP in addition to Vaccinia virus. (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001)

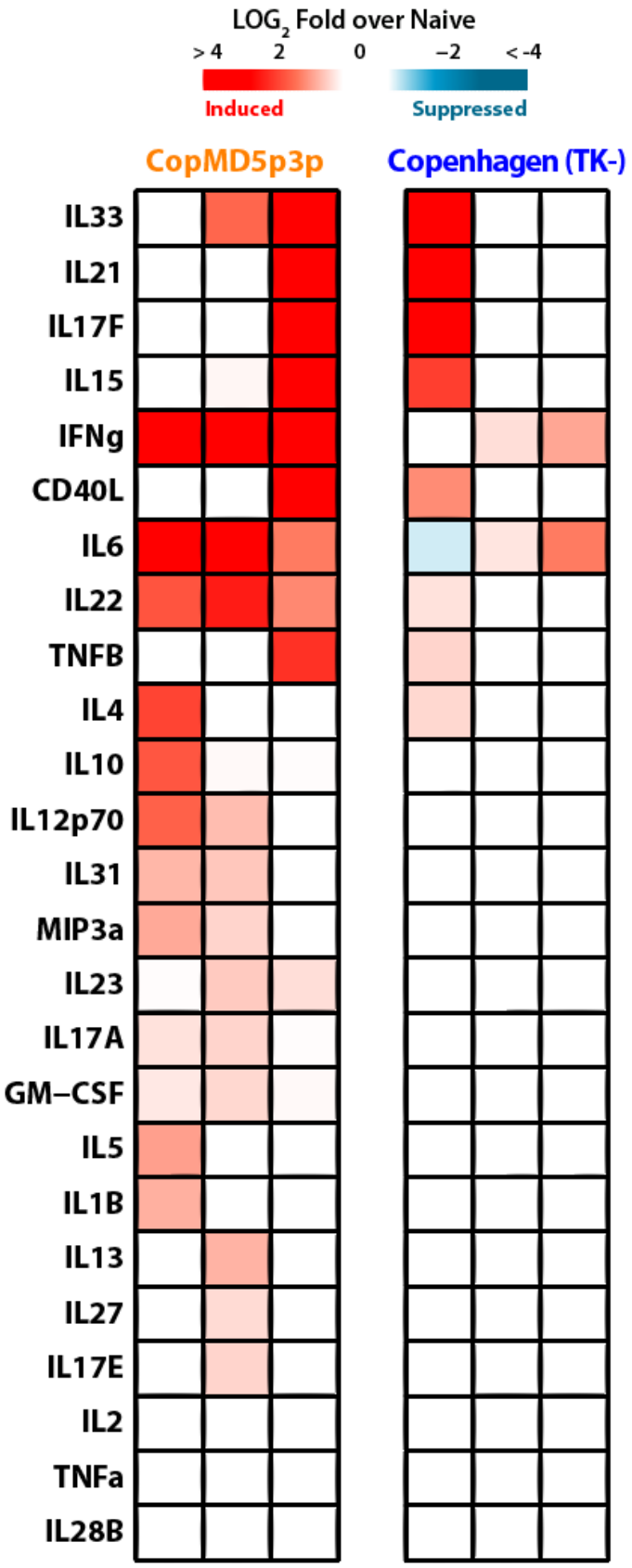

LOG2 Fold over Naive
> 4
2
0
–2
< -4
Induced
Suppressed
CopMD5p3p
Copenhagen (TK-)
IL33
IL21
IL17F
IL15
IFNg
CD40L
IL6
IL22
TNFB
IL4
IL10
IL12p70
IL31
MIP3a
IL23
IL17A
GM–CSF
IL5
IL1B
IL13
IL27
IL17E
IL2
TNFa
IL28B

Figure 3.14: Vaccinia CopMD5p3p induces a wide variety of cytokine release after systemic administration.

Naïve Balb/C mice (n=3 per group) were injected in the tail vein with 1e7 PFU of either Vaccinia. The next day 24h post inoculation mice were sacrificed and blood serum was obtained using cardiac puncture. Cytokine profiling was accomplished using a Milliplex Th17 cytokine array. Cytokine induction is shown as a normalization between virus treated mice and naïve mice, red indicating increased prevalence, white indicating no change and blue showing a decrease in cytokine levels.

CopMD5p3p synergizes with cancer immunotherapy

Immune activation is an advantageous quality for an oncolytic virus in the context of recent breakthroughs in immunotherapy [212]. Immune checkpoint inhibitors are antibodies which have been shown to block immune suppressing signals in the tumour microenvironment of patients [213, 214]. A few recent reports show some benefit for Vaccinia treatment of murine tumours with checkpoint inhibitors, either with non-replicating MVA [215], or with replicating WR in modified Fluc expressing MC38 [57].

Given the ability of CopMD5p3p to activate immune cells, we hypothesized that our virus will synergize with immune checkpoint inhibitors. To test this, we used the murine CT26-LacZ tumor model in immunocompetent Balb/C mice. We find that we are able to cure 20-30% of the tumours only when CopMD5p3p is combined with either Anti-CTLA4 (100 ug) or Anti-PD1 (100 ug) checkpoints (Figure 3.13C-D) Importantly, checkpoints alone or checkpoints combined with Copenhagen (TK-) do not achieve any durable cures.

As blocking checkpoint CTLA4 and PD1 have previously shown additive effects when used to treat murine tumors [216], we sought to test whether a combination of both checkpoints with CopMD5p3p oncolysis is synergistic. Although a low dose of 50ug per checkpoint alone isn't sufficient to cause significant durable remissions, the combination with CopMD5p3p treatments causes remission and long-term cures in 90% of mice (Figure 3.13E). Our results show CopMD5p3p but not parental Copenhagen ΔTK to synergize with checkpoint inhibitor immunotherapy. This hints at the immune stimulating properties of CopMD5p3p to have a role in driving anti-tumor immune response. No adverse effects were noticed in mice.

Expression of antigens, antibodies and other transgenes

Another important component of cancer immunotherapy is the development of tumor vaccines by targeting cancer neo-antigens [217]. Recently, immunogenic artificial antigens such as OVA have been targeted by the expression of OVA in Maraba virus, successfully inducing OVA response and tumor clearance [218]. We sought to test the ability of CopMD5p3p to initiate or boost an immune response towards OVA and compare it to that of Copenhagen ΔTK or Maraba. To this end we have performed a prime immunization of naïve mice with Adeno or either Vaccinia encoding the OVA transgene followed by a boost immunization with Maraba or either Vaccinia encoding the OVA transgene. To evaluate immune response against the OVA antigen following immunization, CD8+ T cells reactive to the OVA immunodominant epitope were quantified by both tetramer staining and peptide stimulation with intracellular cytokine staining (ICS). Previous literature shows heterologous Adenovirus prime followed by a Maraba virus boost to induce a strong response against a virally encoded antigen [219]. We were able to reproduce this result when using Adeno and Maraba (MG-1) virus encoding OVA proteins (Figure 3.15). Similar to this positive control group, mice primed with CopMD5p3p and boosted with Maraba or primed with Adenovirus and boosted with CopMD5p3p had high T cells responses versus the OVA antigen. This suggest that CopMD5p3p encoding an antigen can be used to initiate an immune response as a prime or boost a response in a heterologous prime/boost design. Importantly, the immune response generated by Copenhagen ΔTK groups was an order of magnitude smaller compared to either CopMD5p3p or Maraba groups (Figure 3.15).

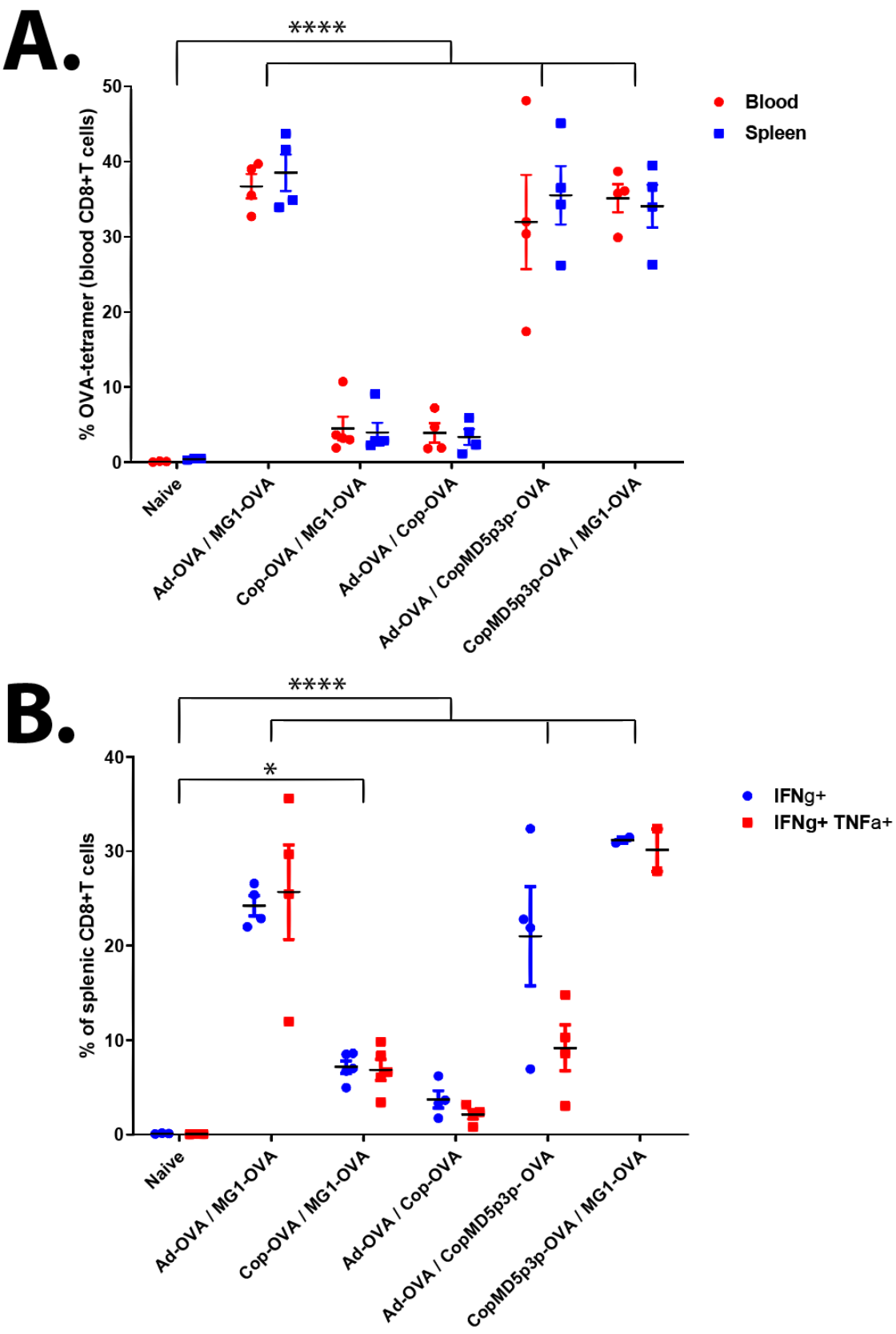

A.
****
% OVA-tetramer (blood CD8+T cells)
50
40
30
20
10
0
Blood
Spleen
Naive
Ad-OVA / MG1-OVA
Cop-OVA / MG1-OVA
Ad-OVA / Cop-OVA
Ad-OVA / CopMD5p3p- OVA
CopMD5p3p-OVA / MG1-OVA
B.
****
*
% of splenic CD8+T cells
40
30
20
10
0
IFNg+
IFNg+ TNFa+
Naive
Ad-OVA / MG1-OVA
Cop-OVA / MG1-OVA
Ad-OVA / Cop-OVA
Ad-OVA / CopMD5p3p- OVA
CopMD5p3p-OVA / MG1-OVA

Figure 3.15: CopMD5p3p generates a strong T cell response against an encoded antigen.

Naïve and tumor free C57BL/6 mice were immunized either IP with 2e8 PFU of Ad-OVA or IV with 1e7 PFU of either Vaccinia encoding OVA (CopMD5p3p / Copenhagen ΔTK). Fourteen days post first immunization mice received a boost immunization IV with 1e8 PFU of MG1-OVA or 1e7 PFU of either Vaccinia encoding OVA. Five days post boost immunization mice were sacrificed for immune analysis on blood and spleen. The x-axis conditions show first the prime treatment and second the boost treatment. The y-axis shows either tetramer OVA positive CD8+ T cells (A) or CD8+ that are IFNg+ or IFNg+ TNFa+ following peptide (OVA dominant CD8 epitope) stimulation (B). n=4 (* p-value < 0.05; **** p-value < 0.0001).

So far, we have shown the ability of CopMD5p3p to express transgenes such as firefly luciferase (Figure 3.12B) or ovalbumin (Figure 3.15) from it's TK locus. Both of these transgenes were expressed under a commonly used Vaccinia synthetic early/late promoter [220]. However, for some applications like expression of an antigen, timing and strength of the promoter have both been correlated with a better immune response against the antigen [221]. We have therefore tested the ability of CopMD5p3p to produce transgenes at various life cycle stages: early only (promoter B19R), late only (promoter synthetic late) and early/late (promoter H5R). To this end, we have encoded a full chain Anti-hCTLA4 antibody (Ipilimumab) in the deleted MD5p locus under the early/late H5R promoter. Additionally, we have replaced the B8R gene with a DC maturation ligand FLT3LG under an early B19R promoter and a membrane bound IL-12 cytokine under a late synthetic promoter. After rescuing the virus and validating the insertion of all three transgenes through DNA sequencing, we have performed ribosomal profiling in an attempt to sequence and quantify mRNA being translated by ribosomes [222]. As expected, Ipilimumab (labelled as Yervoy) is translated throughout the infection cycle while FLT3L and IL-12 are restricted to early and late translation respectively (Figure 3.16).

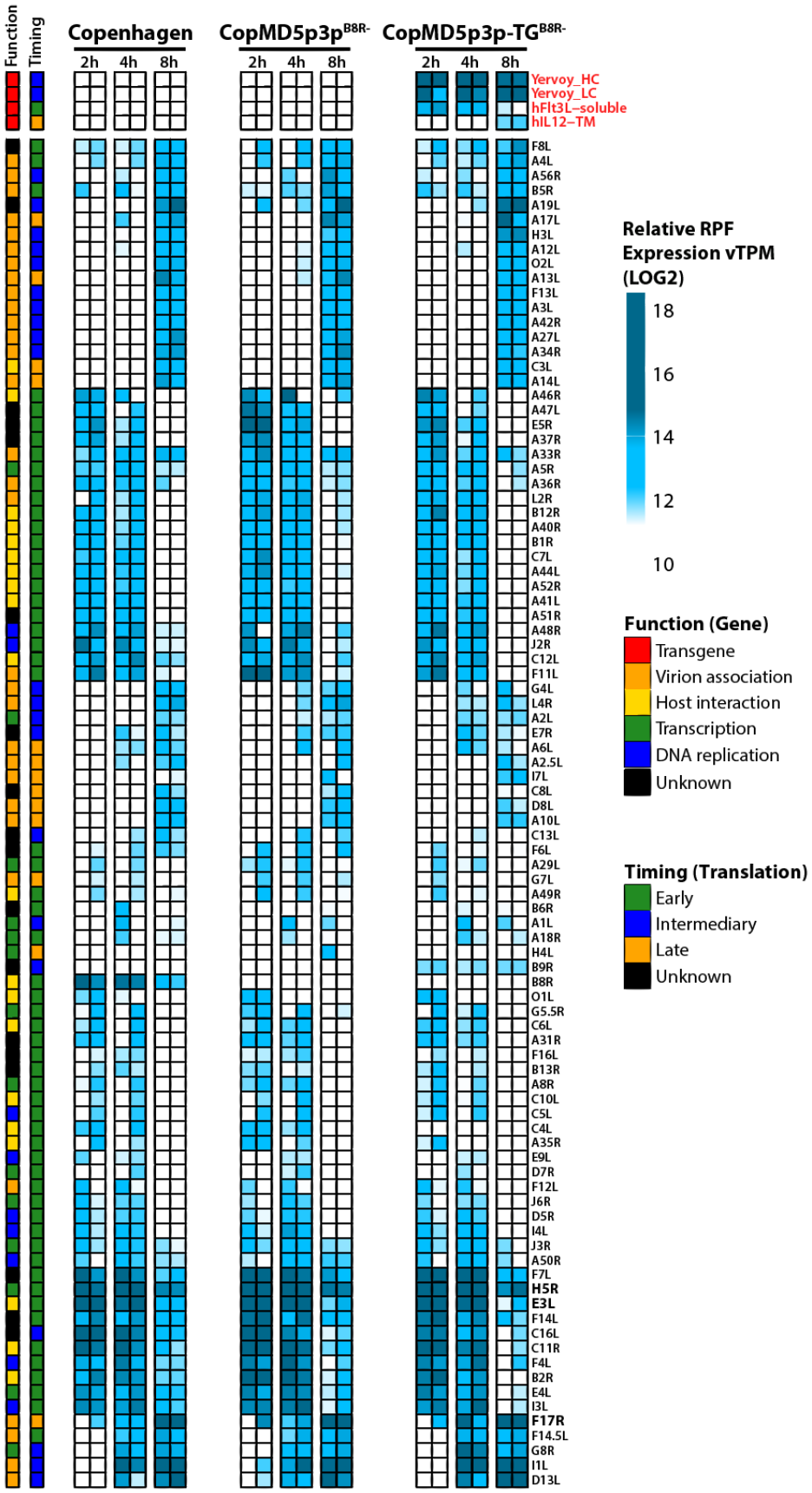

Function
Timing
Copenhagen
CopMD5p3p^{B8R-}
CopMD5p3p-TG^{B8R-}
2h
4h
8h
Yervoy_HC
Yervoy_LC
hFlt3L–soluble
hIL12–TM
F8L
A4L
A56R
B5R
A19L
A17L
H3L
A12L
O2L
A13L
F13L
A3L
A42R
A27L
A34R
C3L
A14L
A46R
A47L
E5R
A37R
A33R
A5R
A36R
L2R
B12R
A40R
B1R
C7L
A44L
A52R
A41L
A51R
A48R
J2R
C12L
F11L
G4L
L4R
A2L
E7R
A6L
A2.5L
I7L
C8L
D8L
A10L
C13L
F6L
A29L
G7L
A49R
B6R
A1L
A18R
H4L
B9R
B8R
O1L
G5.5R
C6L
A31R
F16L
B13R
A8R
C10L
C5L
C4L
A35R
E9L
D7R
F12L
J6R
D5R
I4L
J3R
A50R
F7L
H5R
E3L
F14L
C16L
C11R
F4L
B2R
E4L
I3L
F17R
F14.5L
G8R
I1L
D13L
Relative RPF Expression vTPM (LOG2)
18
16
14
12
10
Function (Gene)
Transgene
Virion association
Host interaction
Transcription
DNA replication
Unknown
Timing (Translation)
Early
Intermediary
Late
Unknown

Figure 3.16: Ribsosome profiling and RNA sequencing of transgene expressing CopMD5p3p.

Transcript levels of viral mRNA associated with ribosomes as quantified by RNA-seq. Infected 786-O were lysed at 2, 4 and 8h post infection with Vaccinia. Lysates were incubated with a cocktail of RNase T1 and RNase A to digest unprotected mRNA. Small 15-30nt RNA was then sequenced and mapped to the Vaccinia genome. Viruses used were wild-type Copenhagen, CopMD5p3p deleted for B8R (CopMD5p3p^{B8R-}) and CopMD5p3p expressing transgenes (CopMD5p3p-TG^{B8R-}). Transgenes encoded by the later virus are Yervoy (Anti-hCTLA4 Ipilimumab) under an H5R promoter, FLT3L under a B19R promoter and IL-12 under a synthetic late promoter.

Discussion

Although Vaccinia strains have already been shown to have differences in gene content and phenotype, there is currently a lack of justification for using any one particular strain as an oncolytic virus (OV). In our study we focused on the five popular strains, all of which are currently in pre-clinical or clinical trials. We show that the Copenhagen strain replicates favourably in a cancer setting (Figure 3.3A) and thus is the preferred platform for creating a next generation Vaccinia OV.

Current Phase I-II clinical trials show Vaccinia safety when the Thymidine Kinase (TK) [77, 223] or Vaccinia Growth Factor (VGF) [129] gene is interrupted while pre-clinical studies also focus on deleting Ribonucleotide Reductase (RR) for safety [224]. Thymidine Kinase and Ribonucleotide Reductase are involved in nucleotides metabolism that when deleted, targets the virus to replicating tissue. The other target for deletion, Vaccinia Growth Factor, is an Epidermal Growth Factor homolog promoting cell division [225]. Strategies targeting metabolically active tissues have been explored before in the context of chemotherapy, and have limited success as dysregulated metabolism in tumour cells leads to resistance [226]. Furthermore, some normal tissues like hepatocytes and keratinocytes have increased metabolic rates, perhaps explaining why some patients treated with Vaccinia develop pox lesions on their skin [34].

Because current strategies do not prevent off-target replication and put the virus at a disadvantage to colonize certain tumour niches we explored other avenues for creating a potent Vaccinia OV. Vaccinia has a large genome with over 200 protein coding genes about which little is known in terms of their contribution or lack-off to the safety and

oncolysis of the virus. We have answered this question by randomly knocking out Vaccinia genes using a transposase system and passaging them in cancer cells. We find that Copenhagen genes that are not present in most poxviruses (poorly conserved) are dispensable and have little effect on the ability of the virus to replicate. Genes we deem unnecessary for oncolysis are located at the extremities of the genome as opposed to the middle (Figure 3.3C). This is perhaps unsurprising, as the middle of poxvirus genomes is most strongly conserved while the extremities have rapidly evolving genes under positive selection [227]. Interestingly, dispensable genes seem to either have unknown function or are involved in virulence (Figure 3.3D). This provides an alternative opportunity to increase the safety of the virus, as it is known that tumours are largely impaired in their anti-viral response [14] and have highly immune suppressing environments [228].

Despite the comprehensive nature of our transposon mediated gene interruption analysis, it is limited in that it only looks at single gene knockouts. Since we have identified such a large number of candidates for knockout, it would neither make sense to delete just one, nor do we have any basis on which to delete multiple of these candidates. Deleting just one gene is unlikely to generate the best OV as Vaccinia genes have redundant functional equivalents [98]. On the other hand, the power of our non-biased analysis is the identification of genomic areas in which multiple genes do not hinder oncolysis.

To take advantage of this knowledge we hypothesized that selecting for naturally occurring deletions in genomic areas that do not contribute to oncolysis will make a

better Vaccinia OV. Previous research has shown that natural deletions can arise in poxviruses after lengthy passaging [191], however this approach does not guarantee deletions in specific areas and severely restricted the replication of the virus to cell line used for passaging [191]. An interesting observation was made when Qin L et al discovered naturally occurring deletions in Vaccinia when plaque picking recombinant viruses [229]. We have decided to pick a few hundred plaques after one round of Vaccinia infection and perform whole viral genome sequencing which allowed us to screen for naturally occurring large deletions. We have found two such deletions in opposing genomic areas which we were able to isolate and cross into a recombinant harbouring both large deletions of genes dispensable for oncolysis (Figure 3.5). Our rescued CopMD5p3p double deleted novel virus was able to replicate and spread both *in vitro* and *in vivo* similarly to its parental Copenhagen. CopMD5p3p is able to induce more cell death (Figure 3.6A) which could be explained by the lack of anti-apoptosis genes (F1L, N1L and M1L).

Lack of anti-apoptosis genes and other virulence factors help explain the safety profile of CopMD5p3p. We have shown through IVIS imaging (Figure 3.12B) and viral biodistribution (Figure 3.12C) that Vaccinia knocked out for TK and VGF replicates in off-target tissues and is inferior to the onco-selectivity of CopMD5p3p in immunocompromised mice. IVIS imaging and visual inspections also revealed pox lesion formation on mouse paws and tails (Figure 3.12E), consistent with reports of pox lesions forming in some patients treated with Vaccinia [34]. Importantly, CopMD5p3p encodes TK, RR and VGF, yet it does not produce pox lesions in nude mice (Figure

3.12F) and does not cause them to lose weight (Figure 3.12A), showing the successful creation of a non-metabolically targeted tumour specific OV. Furthermore, tumor targeting allows transgenes expressed by CopMD5p3p to be localized in production in the tumor microenvironment (Figure 3.16).

Deletion of anti-apoptosis and virulence genes (Table 3.2) not only increased the tumor specificity but also the immunogenicity of the virus. A portion of previously reported Vaccinia immune suppressing genes [210] are missing from CopMD5p3p, leading this virus to induce an anti-viral state when infecting normal immune cells but not cancer cells (Figure 3.10). We have also shown that compared to Copenhagen ΔTK, CopMD5p3p is able to induce an active immune state in both human and murine PBMCs (Figure 3.13). This may be a very important feature since tumor microenvironments are already very immunosuppressive and OVs have been shown to alleviate the immunosuppression thus promoting immune cell recruitment and activation [230]. CopMD5p3p's immunogenicity is also highlighted by its ability to initiate a T cell response against an encoded antigen (Figure 3.15).

Due to the increased ability of CopMD5p3p to activate immune cells, we have looked at its effectiveness as an immunotherapy combined with monoclonal antibody immune checkpoint blockade. Our results show CopMD5p3p is able to synergize with both Anti-CTLA4 and Anti-PD1 antibodies individually and in combination. Furthermore, we have also shown that the synergy with checkpoints can happen at a lower dose of antibody (50 ug) where only CopMD5p3p administered with both checkpoints results in

long lasting tumour cures (Figure 3.13). Furthermore, CopMD5p3p but not Copenhagen ΔTK was able to induce an immune response against an encoded artificial antigen (Figure 3.15). This is suggestive that some degree of immune stimulation (lacking in Copenhagen ΔTK) is needed to induce an immune response. The ability to act as a vaccine and to synergize with checkpoint inhibitors show CopMD5p3p's ability to immunologically prime the tumour microenvironment for immunotherapy [230].

In summary, we have rationally selected for an oncolytic Vaccinia virus with novel features beneficial for cancer therapy. Our virus can replicate as well as parental wild-type Copenhagen but has the advantage of being a faster and more efficient at oncolysis (Figure 3.6A). The large genomic deletions have removed genes which were counter-productive to oncolytic activity and were involved in suppressing the immune system allowing Vaccinia to replicate in healthy tissue. CopMD5p3p is safe, efficacious and can synergize with immunotherapy to increase its therapeutic value. Although other Vaccinia with large deletions have been shown to be safe and immunogenic (i.e. MVA, NY-VAC, vP811), CopMD5p3p has the major advantage of being able to replicate both *in vitro* and *in vivo* selectively in cancer cells.

Materials and Methods

Cell Lines

All cell lines used in this manuscript were obtained from the American Type Culture Collection (ATCC) and passaged in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS).

Viruses

Wild-type Vaccinia WR, Wyeth and Lister strains were purchased from ATCC. Vaccinia TianTan is the DTH14 clone identical to TianTan TP5 [229] and was a generous gift from Dr. David Evans. Vaccinia virus Copenhagen was a generous gift from Dr. Grant McFadden.

CopMD5p and CopMD3p were identified after inspecting Vaccinia genome coverage of 200 Copenhagen picked plaques sequenced using Illumina NextSeq whole virus genome sequencing at the Ottawa Hospital Research Institute's StemCore sequencing facility. Reads from each sample were mapped to the Copenhagen genome (GenBank accession M35027) and coverage distribution was analysed using Geneious v10 [231]. The deletion spanning C2L to F3L was termed CopMD5p and B14R to B29R was termed CopMD3p. Both CopMD5p and CopMD3p were amplified and used in equal amounts to co-infect monolayers of HeLa cells at an MOI of 10, with a PBS was and media change 2h post infection. The next day the infection lysate was collected and used for a plaque assay on U2OS cells for 48h. A total of 12 plaques were picked and screened for recombinants using PCR primers flanking the C2L-F3L and B14R-B29R genes. A double positive plaque was amplified and sent for whole genome sequencing,

confirming the presence of both C2L-F3L and B14R-B29R deletions and absence of any wild-type Copenhagen virus.

Generation of Fluc expressing virus in the TK locus of CopMD5p3p, Copenhagen and Wyeth viruses has been done according to a previously described method [165].

Sequencing and assembly of virus genomes

For virus DNA extraction, monolayers of U2OS cells were infected at an MOI of 1 for 24h or until significant cytopathic effects was observed. Cells were collected and incubated at 37 ºC with SDS Cell Lysis Buffer and Proteinase K (100 μg/mL) for 15 min. DNA was precipitated with isopropanol and washed twice with ethanol. DNA was resuspended in RNAse free water.

Approximatively 1 ug of DNA was sent to FASTERIS for Illumina HiSeq 4000 paired-end 150bp read sequencing. The resulting fastq files were trimmed for adapter removal using Trimmomatic [232]. Trimmed reads were assembled denovo using SPAdes v 3.13.0 [233] default parameters. Resulting assembled contigs were probed for homology to Vaccinia genomes using the BLAST algorithm [234]. The largest Vaccinia contigs were aligned against the closest Vaccinia genome using mafft [235] and GenBank annotations were transferred using Geneious v10 [231].

Fitness Assay

Equal amounts of Copenhagen, Western Reserve, Lister, Wyeth and Tian Tan were mixed in one tube to make our input. This input population was used to infect at an MOI of 0.01 various cancer cell lines for 72h. After infection, the output was tittered and

fresh monolayers were infected for a total of three infections (passages). The third passage for each cell line, along with the input population and each of the individual five wild-type Vaccinia were sent for Illumina NextSeq whole virus genome sequencing at the Ottawa Hospital Research Institute's StemCore sequencing facility. The complete genomes of the wild-type strains were assembled into single contigs using SPAdes v3.11.1 [233]. The strain of each genome was confirmed using blastn function Megablast [234]. Reads of sequenced passaged virus and input virus populations were mapped with BBMap v37.68 (bbsplit.sh http://sourceforge.net/projects/bbmap) simultaneously to all five wild-type genomes, which produces a refstats file containing the percent of unique mapping reads to each strain corresponding to strain abundance.

Transposon Library Generation

Sequence of IFT2 (TTAA insertion) Piggybac transposase (a generous gift from Prof. M. J Fraser, Jr. of University of Notre Dame) was cloned into a pcDNA3.1 mammalian express vector. Transposon donor consists of mCherry under a synthetic early late Vaccinia promoter sandwiched by two ITRs was assembled by PCR and then cloned into a high-copy pUC57-based vector. Sub-confluent U2OS cells (70-80% confluency) were transfected by the transposase construct using Lipofectamine 2000 (Invitrogen). Next day post transfection, cells were infected with Vaccinia Copenhagen strain at a multiplicity of infection (MOI) of 3 and transfected with the transposon plasmid. Cells were incubated at 37ºC for 4 hr after which the medium replaced followed by a 2 day incubation. After incubation this became our transposon library from which we were able to purify mCherry expressing plaques, indicative of successful transposon insertion into Vaccinia genome. To characterise this library further and identify insert locations,

we performed Tn-seq using Illumina HiSeq performed by FASTERIS. Briefly, total DNA was extracted and physically sheared after which a single P7 adapter was ligated to the ends of the fragments. This was followed by amplification with a custom primer consisting of illumine P5 sequence and transposon insert (underlined) with the following sequence 5' AATGATACGGCGACCAC CGAGATCTAACGCATGATTATCTTTAACGTACGTC 3'. Finally, a specific sequencing primer was used which binds the end of the transposon insert (ITR underlined) 5' CTTTAACGTACGTCACAATATGAT TATCTTTCTAGGG 3'. As a result of this strategy the sequenced reads mapped to the location of transposon insertions. Read mapping was done with BBMap v37.68 against the Vaccinia Copenhagen genomes and insertion sites and their frequencies were determined using bedtools genomecov [236].

Transcriptomic and RPF analysis

Cancer (HT-29) or normal (PBMC) cells were infected at a MOI of 3 for 18h with either CopMD5p3p or Copenhagen ΔTK. Cells were lysed with RLP buffer supplemented with 2-Mercaptoethanol (1% v/v). Lysates were processed through a QIAshredder column (Qiagen Cat # 79654) to shred DNA and prevent its excessive column binding. RNA was then isolated using the RNeasy Mini kit (Qiagen Cat # 74104) following manufacturer's protocol. Approximatively 1 ug of RNA was sent to FASTERIS for Illumina HiSeq 4000 paired-end 150bp read sequencing. For Ribosome profiling, lysates were first digested with a cocktail of RNase T1 (Thermofisher; 10 U/uL) and RNase A (Thermofisher; 20 ng/uL) prior to RNA extraction. Sequencing of 15-30 nt size selected

RNA fragments was performed by FASTERIS using Illumina HiSeq 4000 single-end 50bp read sequencing.

The resulting fastq files were trimmed for adapter removal using Trimmomatic [232]. Trimmed reads were then mapped to the latest Ensembl human genome (v95) using HISAT2 [237]. Relative mRNA levels of annotated protein coding genes were then estimated as transcripts per million (TPM) using StringTie [238]. In order to calculate fold change between uninfected and infected samples, a pseudoTPM values of 1 was added to all genes in all conditions to prevent the impossible division by zero in some cases. Data was processed using the R statistical software [239] and displayed as a heatmap using the pheatmap package developed by Raivo Kolde.

Cell Viability Assay

Cells were seeded in 24-well plates at a concentration of 2e5 cell per well. The following day, virus was added at a range of 1-0.1 MOIs in threefold dilutions. Two days post infection media was aspirated, cells were washed once with PBS and stained with Crystal Violet. After scanning, plates were de-stained in 1% SDS mixture for 24h after which 100 uL of destined sample was transferred to a 96-well plate from which absorbance was read at 570 nm using a Multiscan-Ascent machine. Values of untreated samples were used as a reference for 100% viability.

Flow cytometry

Collected samples were analysed by flow cytometry on a BD LSRFortessa (data analysed with the FlowJo software). The spleens were mashed and passed through

100µm cell strainers (Fisher) in cRPMI (RPMI+10% FBS). Red blood cells were lysed with ACK lysis buffer. Antibodies, fluorochromes and sources used are: Live/Dead (BV510; BD Cat. # 564406), CD3 (AP700; BD Cat. # 557984), CD8 (PE-CD594; BD Cat. # 562283), IFNg (APC; BD Cat. # 554413), TNFa (PE-Cy7; BD Cat. # 557644), OVA Tetramer (APC; Pro Immune Cat. # F093-4A).

Mice and tumor models

Mouse strains BALB/c, C57BL/6, NOD-SCID and CD-1 (nude mice) were purchased from Charles River Laboratories (Wilmington, MA). Syngeneic BALB/C models 4T1 and CT26 were seeded at 1e5 and 5e5 respectively subQ. Syngeneic C57BL/6 models MC38 and B16 were seeded at 1e5 cell subQ. Xenograft HT-29 and A549 models were seeded at 3e6 cell subQ in CD-1 and NOD-SCID mice respectively.

Vaccinia viruses were administered intratumor (IT) or intravenously (IV) at a dose of 1e7 PFU in 100 uL. Antibodies purchased from BioXCell targeting checkpoint inhibitors PD1 (clone RMP1-14) or CTLA4 (clone 9D9) were given IP at a dose of either 100ug or 50ug.

All experiments were performed in accordance with institutional guidelines review board for animal care (University of Ottawa).

Bioluminescent *in vivo* imaging

Mice were injected with D-luciferin (Molecular Imaging Products, Ann Arbor, MI) for firefly luciferase imaging. Mice were anesthetized under 3% isoflurane (Baxter,

Deerfeld, IL) and imaged with IVIS200 Series Imaging System (Xenogen, Hopkinton, MA). Data acquisition and analysis were performed using the Living Image v2.5 procedure part of the Igor Pro software. For each experiment, images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence is plotted on identical color scales.

Virus plaque assay and biodistribution

Mice harboring subQ tumors were tail injected intravenously with 1e7 pfu of Vaccinia virus when tumors were palpable. At various time points, mice were sacrificed, organs including tumors were dissected and mechanically homogenized. Organ homogenized lysate was then titered for Vaccinia using a starting volume of 100 uL with 1 in dilutions on U2-OS confluent monolayers. Overlay was supplemented with Penicillin-Streptomycin antibiotic (5 % v/v). Similarly, for growth curves, plaque assays were performed on U2-OS confluent monolayers.

For growth in patient tumor cores, three 2mm x 2mm cores were randomly distributed in 24-wells with 500 uL of DMEM + 10% FBS media. Infections were done at 1e4 pfu per well, after 2 h washing with PBS and replacing media. After 72h post infection, plaque assays were performed to assess viral titers and growth.

General discussion, future directions and concluding remarks

The work described in this thesis explores genetic alterations to Vaccinia virus in an attempt to create a tumor-selective oncolytic virus. The first chapter of this thesis provides a general overview of cancer biology, which informs the treatment of tumors with oncolytic viruses, including Vaccinia virus. The second chapter explores the deletion of a single viral gene, the apoptosis inhibitor F1L in an TK^- attenuated Vaccinia, showing better oncolytic properties. The third chapter shows Vaccinia Copenhagen strain replicates best in human tumors. Furthermore, a transposon gene interruption assay revealed several genes in Vaccinia can be knocked out (i.e. inserted into) with a limited impact on replication in cancer cells. Lastly, a Vaccinia Copenhagen with a 10% genomic deletion, CopMD5p3p, shows improved oncolytic activity through faster cancer cell oncolysis, enhanced tumor selectivity and synergy with cancer immunotherapy.

Choosing a Vaccinia strain as the backbone of a therapeutic virus

The decade long smallpox eradication campaign saw the use of multiple Vaccinia strains as smallpox vaccines. Similarities and differences among strains need to be better understood before choosing a backbone for a novel therapeutic agent. Convincing evidence shows that Copenhagen is the Vaccinia strain which replicates best in human cancer cell lines, including primary human tumors is shown (Figure 3.3AB). This is an important finding as all five strains tested in the assays are currently evaluated both pre-clinically and clinically as cancer therapeutics. As an important aspect to consider, Vaccinia viruses have a broad host tropism and being able to infect and

replicate in several mammalian hosts as well as other vertebrates [240]. This is crucial in the context of treating human tumors and using models available to test oncolytic viruses. Different Vaccinia strains may have different hosts in which they replicate preferentially. This is supported by studies which show the WR strain (passaged in mouse brains multiple times) to be more virulent compared to the Wyeth strain in mice [241]. A competition experiment may reveal a different Vaccinia strain to replicate better than others in mouse cancer cell lines. It is therefore difficult to compare the oncolytic performance of different Vaccinia strains, such as Pexa-vec (Wyeth) to vvDD (WR) in mouse syngeneic models. It is also difficult to foresee a Vaccinia strain's therapeutic potential in human patients based on mouse data alone. The rationale of performing the screen in human cancer cells was based on the end goal of selecting a Vaccinia backbone tailored to perform well in human patients.

Targeting Vaccinia to the tumor

The ability of a particular Vaccinia strain to outperform the others is perhaps tied to its virulence in that particular host. Indeed, Copenhagen was one of the vaccines with the most severe side effects during the smallpox eradication campaign [76]. Thus, we reasoned that Copenhagen had the superior "oncolytic engine" that needed to be modified to limit its selectivity to tumors. The random transposon-mediated viral gene interruption allowed us to query the Copenhagen genome for elements dispensable for oncolysis. Due to the functional redundancy of Vaccinia genes [98], it is perhaps unsurprising that we could purify clones with individual gene knockouts for over a quarter of all Vaccinia genes (Table 3.1). Indeed, cases of synthetic lethality have been described in which only the simultaneous knockout of two Vaccinia genes impairs viral replication [242]. To better understand viral gene interaction and find new cases of

synthetic lethality, a second transposon library could be built starting from a purified version of the first, showing synthetic lethality at the level of two genes.

As most interrupted genes in the clones we isolated had no *in vitro* effect on replication and no *in vivo* effect on virulence based on literature, we had decided to focus on recovered clones with large genomic deletions encompassing multiple genes. Being able to plaque purify and expand CopMD5p and CopMD3p suggested these viruses were not impaired in their ability to replicate in cancer cells. Since these viruses each had a different set of genes missing, I speculated combining these two sets of deletions may not yield a replication-competent virus. Interestingly that was not the case, as forcing recombination between CopMD5p and CopMD3p generated CopMD5p3p, a virus missing 32 genes. Even more surprisingly CopMD5p3p was not attenuated in its ability to replicate when compared to parental wild-type Copenhagen (Figure 3.6B and Figure 3.6C). Lack of attenuation *in vitro* suggests the deleted genes may have an important role *in vivo* in the presence of an immune system. Based on previous reports of synthetic lethality in Vaccinia, I speculate a transposon interruption library on CopMD5p3p would show a decreased ability to knock out genes in a virus which is already missing 10% of the genome. Nevertheless, such an experiment would suggest novel genetic interactions among deleted and remaining Vaccinia genes in CopMD5p3p.

CopMD5p3p is a tumor selective therapeutic capable of transgene expression

CopMD5p3p was shown to be more tumor-selective, more immunogenic and capable to express transgenes. Given a deletion larger than 20kb in CopMD5p3p, it is tempting to speculate that this space can now be filled up with multiple therapeutic payloads, including various cytokines previously inserted into other viruses and shown to be effective in CopMD5p3p (Figure 3.16). For instance, arming the virus with activating cytokines such as IL-12 and IL-2 can help overcome immune exhaustion in the tumor microenvironment [243]. However, this strategy alone may fail in poorly infiltrated cold tumors, in which case adding immune recruiting cytokines such as CXCL9, CXCL10 and CXCL11 may turn a tumor hot [244]. Depending on a tumor's microenvironment, such strategies may result in over-stimulation and immune shutdown via upregulation of checkpoint inhibitors CTLA4 and PD1/PDL1. Although antibodies targeting checkpoint inhibitors have shown clinical promise, they are both expensive to manufacture and toxic. Previous work has shown that Vaccinia is capable of encoding and producing functional antibodies, including one targeting PD1 [245]. Our *in vivo* luciferase signal shows CopMD5p3p to be tumor-selective (Figure 3.12B), suggesting that any transgene, including an antibody targeting checkpoint inhibitors would result in their localized expression in the tumor microenvironment, limiting off-target toxicity.

Most transgenes discussed in the previous paragraph are immune-stimulating, it is unsurprising that some have already been implicated in Vaccinia clearance [246]. Such a transgene expressed from Vaccinia may therefore benefit from the control of an inducible system [247], where transgene production is initiated upon successful spread in the tumor microenvironment. Furthermore, using a previously described Vaccinia T7 polymerase system driving transgene expression [248], an inducible T7 polymerase can

control expression of multiple transgenes under a T7 promoter. Aside for allowing for viral spread, controlling transgene expression has other benefits such as stopping expression in cases of observed toxicity.

Thesis Shortcomings and Pitfalls

An important aspect of an oncolytic virus is its ability to replicate and spread throughout a tumor. This will ensure better tumor control due to the direct lysis of cancer cells but also affect the amount and distribution of transgenes produced. When it comes to CopMD5p3p, an important question is if replication in the tumor can be boosted while maintaining the same degree of selectivity. In other words, have we deleted genes which don't contribute to off-target effect while helping virus replication in the tumor? The deletions in CopMD5p3p are clusters of genes localized in two genomic areas, leading to the possibility that some genes located within the deleted loci are needed for replication in an *in vivo* tumor. A labor-intensive way of investigating this avenue is the generation of CopMD5p3p viruses with deleted genes gradually added back in and tested *in vivo* for off-target replication. Perhaps a faster way would be to inject mouse tumors with mixtures of CopMD5p3p clones each with a different gene added back in. Targeted sequencing of the insertion locus in tumor DNA after viral inoculation would reveal whether any particular genes added back in can outcompete others. This virus can then separately be tested for off-target effects. A similar strategy can be applied to the transposon library where passaging it in tumors *in vivo* can reveal which Vaccinia genes causes loss of fitness when deleted. The caveat of such approaches remains genetic interactions, where the combined presence of a deleted gene in CopMD5p can synergize well with a deleted gene in CopMD3p to promote better replication and persistence in the tumor.

Lastly, it is important to consider an alternate avenue for creating the next best oncolytic Vaccinia virus. Our initial assays based on replication led us to select Copenhagen as the Vaccinia strain to move forward with, but is replication a key factor for a successful OV? Some studies involving replication-deficient or heat-inactivated Vaccinia have shown great efficacy in several hard to treat murine tumor models through activation of the interferon inducing cGAS/STING pathway [249]. I would argue that viral replication in the tumor microenvironment is absolutely crucial due to the need to produce transgenes. Activating the cGAS/STING pathway does not require heat-inactivated virus and can be achieved by encoding well-described STING agonists [250] in Vaccinia. However, the existence of different STING alleles in the human population can make it difficult to pick a universal STING agonist [251]. Importantly, cancer heterogeneity makes targeting a single molecular pathway an unlikely panacea.

Concluding statement

This thesis describes several strategies and assays that can be used in evaluating the oncolytic activity of a particular virus. Furthermore, it follows the rational design of a next generation Vaccinia OV. CopMD5p3p is a tumor selective, oncolytic and immunogenic virus able to encode and produce multiple therapeutic payloads in the tumor microenvironment. Overall, my thesis explored novel methods to generate Vaccinia oncolytic viruses showing a complex interplay between tumor selectivity and immunogenicity.

References

1. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
2. Toronto, O.C.C.S., *Canadian Cancer Society's Advisory Committee on Cancer Statistic.* Canadian Cancer Society, 2015.
3. Stetler-Stevenson, W.G., S. Aznavoorian, and L.A. Liotta, *Tumor cell interactions with the extracellular matrix during invasion and metastasis.* Annu Rev Cell Biol, 1993. **9**: p. 541-73.
4. Mikhail, S. and T. Bekaii-Saab, *Maintenance therapy for colorectal cancer: which regimen and which patients?* Drugs, 2015. **75**(16): p. 1833-42.
5. Bandla, A., et al., *Hypothermia for preventing chemotherapy-induced neuropathy - a pilot study on safety and tolerability in healthy controls.* Acta Oncol, 2016. **55**(4): p. 430-6.
6. Iyengar, N.M., et al., *A Pilot Study of Dose-Dense Paclitaxel With Trastuzumab and Lapatinib for Node-negative HER2-Overexpressed Breast Cancer.* Clin Breast Cancer, 2016. **16**(2): p. 87-94.
7. Dagogo-Jack, I. and A.T. Shaw, *Tumour heterogeneity and resistance to cancer therapies.* Nat Rev Clin Oncol, 2018. **15**(2): p. 81-94.
8. Bierman, H.R., et al., *Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia.* Cancer, 1953. **6**(3): p. 591-605.
9. Russell, S.J., K.W. Peng, and J.C. Bell, *Oncolytic virotherapy.* Nat Biotechnol, 2012. **30**(7): p. 658-70.
10. Hill, C. and R. Carlisle, *Achieving systemic delivery of oncolytic viruses.* Expert Opin Drug Deliv, 2019. **16**(6): p. 607-620.
11. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.
12. Schneider, W.M., M.D. Chevillotte, and C.M. Rice, *Interferon-stimulated genes: a complex web of host defenses.* Annu Rev Immunol, 2014. **32**: p. 513-45.
13. Katsoulidis, E., S. Kaur, and L.C. Platanias, *Deregulation of Interferon Signaling in Malignant Cells.* Pharmaceuticals (Basel), 2010. **3**(2): p. 406-418.
14. Critchley-Thorne, R.J., et al., *Impaired interferon signaling is a common immune defect in human cancer.* Proc Natl Acad Sci U S A, 2009. **106**(22): p. 9010-5.
15. Katlinskaya, Y.V., et al., *Suppression of Type I Interferon Signaling Overcomes Oncogene-Induced Senescence and Mediates Melanoma Development and Progression.* Cell Rep, 2016. **15**(1): p. 171-180.
16. Zhan, T., N. Rindtorff, and M. Boutros, *Wnt signaling in cancer.* Oncogene, 2017. **36**(11): p. 1461-1473.
17. Baril, M., et al., *Genome-wide RNAi screen reveals a new role of a WNT/CTNNB1 signaling pathway as negative regulator of virus-induced innate immune responses.* PLoS Pathog, 2013. **9**(6): p. e1003416.
18. Colombo, M., et al., *Cancer Cells Exploit Notch Signaling to Redefine a Supportive Cytokine Milieu.* Front Immunol, 2018. **9**: p. 1823.
19. Schaller, M.A., et al., *Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines.* J Exp Med, 2007. **204**(12): p. 2925-34.

20. Vaha-Koskela, M. and A. Hinkkanen, *Tumor Restrictions to Oncolytic Virus.* Biomedicines, 2014. **2**(2): p. 163-194.
21. Matveeva, O.V. and P.M. Chumakov, *Defects in interferon pathways as potential biomarkers of sensitivity to oncolytic viruses.* Rev Med Virol, 2018. **28**(6): p. e2008.
22. Stojdl, D.F., et al., *VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents.* Cancer Cell, 2003. **4**(4): p. 263-75.
23. Wu, L., et al., *rVSV(M Delta 51)-M3 is an effective and safe oncolytic virus for cancer therapy.* Hum Gene Ther, 2008. **19**(6): p. 635-47.
24. Brun, J., et al., *Identification of genetically modified Maraba virus as an oncolytic rhabdovirus.* Mol Ther, 2010. **18**(8): p. 1440-9.
25. Nikolic, J., et al., *Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein.* Nat Commun, 2018. **9**(1): p. 1029.
26. Musella, M., et al., *Type-I-interferons in infection and cancer: Unanticipated dynamics with therapeutic implications.* Oncoimmunology, 2017. **6**(5): p. e1314424.
27. Budhwani, M., R. Mazzieri, and R. Dolcetti, *Plasticity of Type I Interferon-Mediated Responses in Cancer Therapy: From Anti-tumor Immunity to Resistance.* Front Oncol, 2018. **8**: p. 322.
28. Smith, G.L., et al., *Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity.* J Gen Virol, 2013. **94**(Pt 11): p. 2367-92.
29. Haddad, D., *Genetically Engineered Vaccinia Viruses As Agents for Cancer Treatment, Imaging, and Transgene Delivery.* Front Oncol, 2017. **7**: p. 96.
30. Deng, L., et al., *Oncolytic efficacy of thymidine kinase-deleted vaccinia virus strain Guang9.* Oncotarget, 2017. **8**(25): p. 40533-40543.
31. Wong, H.H., N.R. Lemoine, and Y. Wang, *Oncolytic Viruses for Cancer Therapy: Overcoming the Obstacles.* Viruses, 2010. **2**(1): p. 78-106.
32. Martuza, R.L., et al., *Experimental therapy of human glioma by means of a genetically engineered virus mutant.* Science, 1991. **252**(5007): p. 854-6.
33. Rehman, H., et al., *Into the clinic: Talimogene laherparepvec (T-VEC), a first-in-class intratumoral oncolytic viral therapy.* J Immunother Cancer, 2016. **4**: p. 53.
34. Cripe, T.P., et al., *Phase 1 study of intratumoral Pexa-Vec (JX-594), an oncolytic and immunotherapeutic vaccinia virus, in pediatric cancer patients.* Mol Ther, 2015. **23**(3): p. 602-8.
35. Xiang, Y., et al., *Thymidine kinase 1 as a diagnostic tumor marker is of moderate value in cancer patients: A meta-analysis.* Biomed Rep, 2013. **1**(4): p. 629-637.
36. Kozin, S.V., et al., *Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation.* Cancer Res, 2010. **70**(14): p. 5679-85.
37. Veglia, F., M. Perego, and D. Gabrilovich, *Myeloid-derived suppressor cells coming of age.* Nat Immunol, 2018. **19**(2): p. 108-119.
38. Lin, E.Y., et al., *The macrophage growth factor CSF-1 in mammary gland development and tumor progression.* J Mammary Gland Biol Neoplasia, 2002. **7**(2): p. 147-62.
39. De Vlaeminck, Y., et al., *Cancer-Associated Myeloid Regulatory Cells.* Front Immunol, 2016. **7**: p. 113.
40. Munder, M., *Arginase: an emerging key player in the mammalian immune system.* Br J Pharmacol, 2009. **158**(3): p. 638-51.
41. Syed, V., *TGF-beta Signaling in Cancer.* J Cell Biochem, 2016. **117**(6): p. 1279-87.
42. Lee, G.R., *Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells.* Mediators Inflamm, 2017. **2017**: p. 5458178.

43. Gabrilovich, D.I., *Myeloid-Derived Suppressor Cells.* Cancer Immunol Res, 2017. **5**(1): p. 3-8.
44. Janghorban, M., et al., *Notch Signaling as a Regulator of the Tumor Immune Response: To Target or Not To Target?* Front Immunol, 2018. **9**: p. 1649.
45. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade.* Science, 1996. **271**(5256): p. 1734-6.
46. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.* Nature, 1992. **356**(6370): p. 607-9.
47. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation.* J Exp Med, 1995. **182**(2): p. 459-65.
48. Simpson, T.R., et al., *Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma.* J Exp Med, 2013. **210**(9): p. 1695-710.
49. Kvistborg, P., et al., *Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response.* Sci Transl Med, 2014. **6**(254): p. 254ra128.
50. Fife, B.T. and K.E. Pauken, *The role of the PD-1 pathway in autoimmunity and peripheral tolerance.* Ann N Y Acad Sci, 2011. **1217**: p. 45-59.
51. Iwai, Y., et al., *Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12293-7.
52. Miller, B.C., et al., *Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade.* Nat Immunol, 2019. **20**(3): p. 326-336.
53. Chae, Y.K., et al., *Current landscape and future of dual anti-CTLA4 and PD-1/PD-L1 blockade immunotherapy in cancer; lessons learned from clinical trials with melanoma and non-small cell lung cancer (NSCLC).* J Immunother Cancer, 2018. **6**(1): p. 39.
54. Marelli, G., et al., *Oncolytic Viral Therapy and the Immune System: A Double-Edged Sword Against Cancer.* Front Immunol, 2018. **9**: p. 866.
55. Saha, D., R.L. Martuza, and S.D. Rabkin, *Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade.* Cancer Cell, 2017. **32**(2): p. 253-267 e5.
56. Shim, K.G., et al., *Inhibitory Receptors Induced by VSV Viroimmunotherapy Are Not Necessarily Targets for Improving Treatment Efficacy.* Mol Ther, 2017. **25**(4): p. 962-975.
57. Liu, Z., et al., *Rational combination of oncolytic vaccinia virus and PD-L1 blockade works synergistically to enhance therapeutic efficacy.* Nat Commun, 2017. **8**: p. 14754.
58. Blondel, D., et al., *Resistance to Rhabdoviridae Infection and Subversion of Antiviral Responses.* Viruses, 2015. **7**(7): p. 3675-702.
59. Dai, P., et al., *Modified vaccinia virus Ankara triggers type I IFN production in murine conventional dendritic cells via a cGAS/STING-mediated cytosolic DNA-sensing pathway.* PLoS Pathog, 2014. **10**(4): p. e1003989.
60. Takasu, A., et al., *Immunogenic cell death by oncolytic herpes simplex virus type 1 in squamous cell carcinoma cells.* Cancer Gene Ther, 2016. **23**(4): p. 107-13.
61. Huang, C.Y., et al., *Cytosolic high-mobility group box protein 1 (HMGB1) and/or PD-1+ TILs in the tumor microenvironment may be contributing prognostic biomarkers for patients with locally advanced rectal cancer who have undergone neoadjuvant chemoradiotherapy.* Cancer Immunol Immunother, 2018. **67**(4): p. 551-562.

62. Bommareddy, P.K., et al., *Oncolytic virus immunotherapy induces immunogenic cell death and overcomes STING deficiency in melanoma.* Oncoimmunology, 2019. **8**(7): p. 1591875.
63. Serrano-Del Valle, A., et al., *Immunogenic Cell Death and Immunotherapy of Multiple Myeloma.* Front Cell Dev Biol, 2019. **7**: p. 50.
64. Chakravarty, P.K., et al., *Flt3L therapy following localized tumor irradiation generates long-term protective immune response in metastatic lung cancer: its implication in designing a vaccination strategy.* Oncology, 2006. **70**(4): p. 245-54.
65. Barnard, Z., et al., *Expression of FMS-like tyrosine kinase 3 ligand by oncolytic herpes simplex virus type I prolongs survival in mice bearing established syngeneic intracranial malignant glioma.* Neurosurgery, 2012. **71**(3): p. 741-8; discussion 748.
66. Riediger, C., et al., *Fms-like tyrosine kinase 3 receptor ligand (Flt3L)-based vaccination administered with an adenoviral vector prevents tumor growth of colorectal cancer in a BALB/c mouse model.* J Cancer Res Clin Oncol, 2013. **139**(12): p. 2097-110.
67. Zurkova, K., et al., *The expression of the soluble isoform of hFlt3 ligand by recombinant vaccinia virus enhances immunogenicity of the vector.* Oncol Rep, 2009. **21**(5): p. 1335-43.
68. Leveille, S., et al., *Vesicular stomatitis virus oncolytic treatment interferes with tumor-associated dendritic cell functions and abrogates tumor antigen presentation.* J Virol, 2011. **85**(23): p. 12160-9.
69. Lasek, W., R. Zagozdzon, and M. Jakobisiak, *Interleukin 12: still a promising candidate for tumor immunotherapy?* Cancer Immunol Immunother, 2014. **63**(5): p. 419-35.
70. Cheema, T.A., et al., *Multifaceted oncolytic virus therapy for glioblastoma in an immunocompetent cancer stem cell model.* Proc Natl Acad Sci U S A, 2013. **110**(29): p. 12006-11.
71. Veinalde, R., et al., *Oncolytic measles virus encoding interleukin-12 mediates potent antitumor effects through T cell activation.* Oncoimmunology, 2017. **6**(4): p. e1285992.
72. McFadden, G., *Poxvirus tropism.* Nat Rev Microbiol, 2005. **3**(3): p. 201-13.
73. Moss, B., *Poxvirus DNA replication.* Cold Spring Harb Perspect Biol, 2013. **5**(9).
74. Rice, A.D., et al., *An efficient method for generating poxvirus recombinants in the absence of selection.* Viruses, 2011. **3**(3): p. 217-32.
75. Fenner, F., *A successful eradication campaign. Global eradication of smallpox.* Rev Infect Dis, 1982. **4**(5): p. 916-30.
76. Kretzschmar, M., et al., *Frequency of adverse events after vaccination with different vaccinia strains.* PLoS Med, 2006. **3**(8): p. e272.
77. Breitbach, C.J., et al., *Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans.* Nature, 2011. **477**(7362): p. 99-102.
78. Garcia-Arriaza, J. and M. Esteban, *Enhancing poxvirus vectors vaccine immunogenicity.* Hum Vaccin Immunother, 2014. **10**(8): p. 2235-44.
79. Al Yaghchi, C., et al., *Vaccinia virus, a promising new therapeutic agent for pancreatic cancer.* Immunotherapy, 2015. **7**(12): p. 1249-58.
80. Volz, A. and G. Sutter, *Modified Vaccinia Virus Ankara: History, Value in Basic Research, and Current Perspectives for Vaccine Development.* Adv Virus Res, 2017. **97**: p. 187-243.
81. Stickl, H. and V. Hochstein-Mintzel, *[Intracutaneous smallpox vaccination with a weak pathogenic vaccinia virus ("MVA virus")].* Munch Med Wochenschr, 1971. **113**(35): p. 1149-53.
82. Mayr, A. and E. Munz, *[Changes in the vaccinia virus through continuing passages in chick embryo fibroblast cultures].* Zentralbl Bakteriol Orig, 1964. **195**(1): p. 24-35.

83. Shen, X., et al., *HIV-1 gp120 and Modified Vaccinia Virus Ankara (MVA) gp140 Boost Immunogens Increase Immunogenicity of a DNA/MVA HIV-1 Vaccine.* J Virol, 2017. **91**(24).
84. Brewitz, A., et al., *CD8(+) T Cells Orchestrate pDC-XCR1(+) Dendritic Cell Spatial and Functional Cooperativity to Optimize Priming.* Immunity, 2017. **46**(2): p. 205-219.
85. Bratke, K.A., A. McLysaght, and S. Rothenburg, *A survey of host range genes in poxvirus genomes.* Infect Genet Evol, 2013. **14**: p. 406-25.
86. Assarsson, E., et al., *Kinetic analysis of a complete poxvirus transcriptome reveals an immediate-early class of genes.* Proc Natl Acad Sci U S A, 2008. **105**(6): p. 2140-5.
87. Chung, C.S., et al., *Vaccinia virus proteome: identification of proteins in vaccinia virus intracellular mature virion particles.* J Virol, 2006. **80**(5): p. 2127-40.
88. Frei, A.P., et al., *Direct identification of ligand-receptor interactions on living cells and tissues.* Nat Biotechnol, 2012. **30**(10): p. 997-1001.
89. Mercer, J. and A. Helenius, *Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells.* Science, 2008. **320**(5875): p. 531-5.
90. Broyles, S.S., *Vaccinia virus transcription.* J Gen Virol, 2003. **84**(Pt 9): p. 2293-303.
91. Roberts, K.L. and G.L. Smith, *Vaccinia virus morphogenesis and dissemination.* Trends Microbiol, 2008. **16**(10): p. 472-9.
92. Jha, S., et al., *Trans-kingdom mimicry underlies ribosome customization by a poxvirus kinase.* Nature, 2017. **546**(7660): p. 651-655.
93. Chou, W., T. Ngo, and P.D. Gershon, *An overview of the vaccinia virus infectome: a survey of the proteins of the poxvirus-infected cell.* J Virol, 2012. **86**(3): p. 1487-99.
94. Moss, B., *Regulation of vaccinia virus transcription.* Annu Rev Biochem, 1990. **59**: p. 661-88.
95. Smith, G.L., A. Vanderplasschen, and M. Law, *The formation and function of extracellular enveloped vaccinia virus.* J Gen Virol, 2002. **83**(Pt 12): p. 2915-31.
96. Katz, E., et al., *Mutations in the vaccinia virus A33R and B5R envelope proteins that enhance release of extracellular virions and eliminate formation of actin-containing microvilli without preventing tyrosine phosphorylation of the A36R protein.* J Virol, 2003. **77**(22): p. 12266-75.
97. Vanderplasschen, A., et al., *Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7544-9.
98. Dobson, B.M. and D.C. Tscharke, *Redundancy complicates the definition of essential genes for vaccinia virus.* J Gen Virol, 2015. **96**(11): p. 3326-37.
99. Eaglesham, J.B., et al., *Viral and metazoan poxins are cGAMP-specific nucleases that restrict cGAS-STING signalling.* Nature, 2019. **566**(7743): p. 259-263.
100. Scutts, S.R., et al., *DNA-PK Is Targeted by Multiple Vaccinia Virus Proteins to Inhibit DNA Sensing.* Cell Rep, 2018. **25**(7): p. 1953-1965 e4.
101. Meng, X., et al., *Vaccinia virus K1L and C7L inhibit antiviral activities induced by type I interferons.* J Virol, 2009. **83**(20): p. 10627-36.
102. Unterholzner, L., et al., *Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7.* PLoS Pathog, 2011. **7**(9): p. e1002247.
103. Ferguson, B.J., et al., *Vaccinia virus protein N2 is a nuclear IRF3 inhibitor that promotes virulence.* J Gen Virol, 2013. **94**(Pt 9): p. 2070-81.

104. Colamonici, O.R., et al., *Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling.* J Biol Chem, 1995. **270**(27): p. 15974-8.
105. Mann, B.A., et al., *Vaccinia virus blocks Stat1-dependent and Stat1-independent gene expression induced by type I and type II interferons.* J Interferon Cytokine Res, 2008. **28**(6): p. 367-80.
106. Zhao, J., et al., *Recent advances on viral manipulation of NF-kappaB signaling pathway.* Curr Opin Virol, 2015. **15**: p. 103-11.
107. Lysakova-Devine, T., et al., *Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule.* J Immunol, 2010. **185**(7): p. 4261-71.
108. Maloney, G., M. Schroder, and A.G. Bowie, *Vaccinia virus protein A52R activates p38 mitogen-activated protein kinase and potentiates lipopolysaccharide-induced interleukin-10.* J Biol Chem, 2005. **280**(35): p. 30838-44.
109. Tang, Q., S. Chakraborty, and G. Xu, *Mechanism of vaccinia viral protein B14-mediated inhibition of IkappaB kinase beta activation.* J Biol Chem, 2018. **293**(26): p. 10344-10352.
110. Gedey, R., et al., *Poxviral regulation of the host NF-kappaB response: the vaccinia virus M2L protein inhibits induction of NF-kappaB activation via an ERK2 pathway in virus-infected human embryonic kidney cells.* J Virol, 2006. **80**(17): p. 8676-85.
111. Sumner, R.P., et al., *Vaccinia virus inhibits NF-kappaB-dependent gene expression downstream of p65 translocation.* J Virol, 2014. **88**(6): p. 3092-102.
112. Walsh, D., M.B. Mathews, and I. Mohr, *Tinkering with translation: protein synthesis in virus-infected cells.* Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a012351.
113. Liu, S.W., et al., *Poxvirus decapping enzymes enhance virulence by preventing the accumulation of dsRNA and the induction of innate antiviral responses.* Cell Host Microbe, 2015. **17**(3): p. 320-331.
114. Parrish, S. and B. Moss, *Characterization of a second vaccinia virus mRNA-decapping enzyme conserved in poxviruses.* J Virol, 2007. **81**(23): p. 12973-8.
115. Parrish, S., W. Resch, and B. Moss, *Vaccinia virus D10 protein has mRNA decapping activity, providing a mechanism for control of host and viral gene expression.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2139-44.
116. Yang, Z., et al., *Simultaneous high-resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing.* Proc Natl Acad Sci U S A, 2010. **107**(25): p. 11513-8.
117. Yang, Z., et al., *Pervasive initiation and 3'-end formation of poxvirus postreplicative RNAs.* J Biol Chem, 2012. **287**(37): p. 31050-60.
118. Benfield, C.T., et al., *Vaccinia virus protein K7 is a virulence factor that alters the acute immune response to infection.* J Gen Virol, 2013. **94**(Pt 7): p. 1647-57.
119. Pallett, M.A., et al., *Vaccinia Virus BBK E3 Ligase Adaptor A55 Targets Importin-Dependent NF-kappaB Activation and Inhibits CD8(+) T-Cell Memory.* J Virol, 2019. **93**(10).
120. Betakova, T., E.J. Wolffe, and B. Moss, *The vaccinia virus A14.5L gene encodes a hydrophobic 53-amino-acid virion membrane protein that enhances virulence in mice and is conserved among vertebrate poxviruses.* J Virol, 2000. **74**(9): p. 4085-92.
121. Qin, L., et al., *Evolution of and evolutionary relationships between extant vaccinia virus strains.* J Virol, 2015. **89**(3): p. 1809-24.

122. de Freitas, L.F.D., et al., *The Virulence of Different Vaccinia Virus Strains Is Directly Proportional to Their Ability To Downmodulate Specific Cell-Mediated Immune Compartments In Vivo.* J Virol, 2019. **93**(6).
123. Perkus, M.E., et al., *Vaccinia virus host range genes.* Virology, 1990. **179**(1): p. 276-86.
124. Hunter-Craig, I., et al., *Use of vaccinia virus in the treatment of metastatic malignant melanoma.* Br Med J, 1970. **2**(5708): p. 512-5.
125. Jefferson, A., V.E. Cadet, and A. Hielscher, *The mechanisms of genetically modified vaccinia viruses for the treatment of cancer.* Crit Rev Oncol Hematol, 2015. **95**(3): p. 407-16.
126. Parato, K.A., et al., *The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers.* Mol Ther, 2012. **20**(4): p. 749-58.
127. Potts, K.G., et al., *Deletion of F4L (ribonucleotide reductase) in vaccinia virus produces a selective oncolytic virus and promotes anti-tumor immunity with superior safety in bladder cancer models.* EMBO Mol Med, 2017. **9**(5): p. 638-654.
128. McCart, J.A., et al., *Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes.* Cancer Res, 2001. **61**(24): p. 8751-7.
129. Downs-Canner, S., et al., *Phase 1 Study of Intravenous Oncolytic Poxvirus (vvDD) in Patients With Advanced Solid Cancers.* Mol Ther, 2016. **24**(8): p. 1492-501.
130. Andrade, A.A., et al., *The vaccinia virus-stimulated mitogen-activated protein kinase (MAPK) pathway is required for virus multiplication.* Biochem J, 2004. **381**(Pt 2): p. 437-46.
131. Seshacharyulu, P., et al., *Targeting the EGFR signaling pathway in cancer therapy.* Expert Opin Ther Targets, 2012. **16**(1): p. 15-31.
132. Beerli, C., et al., *Vaccinia virus hijacks EGFR signalling to enhance virus spread through rapid and directed infected cell motility.* Nat Microbiol, 2019. **4**(2): p. 216-225.
133. Lun, X.Q., et al., *Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide.* Clin Cancer Res, 2009. **15**(8): p. 2777-88.
134. Kedia, S., G. Garcia, and M. Dhar, *Stage IV EGFR Mutation-Negative and ALK Mutation-Negative Lung Adenocarcinoma: Long-Term Survival is Possible.* Cureus, 2015. **7**(12): p. e419.
135. Burgess, H.M., et al., *Targeting Poxvirus Decapping Enzymes and mRNA Decay to Generate an Effective Oncolytic Virus.* Mol Ther Oncolytics, 2018. **8**: p. 71-81.
136. Liu, S.W., et al., *The D10 decapping enzyme of vaccinia virus contributes to decay of cellular and viral mRNAs and to virulence in mice.* J Virol, 2014. **88**(1): p. 202-11.
137. Backes, S., et al., *Viral host-range factor C7 or K1 is essential for modified vaccinia virus Ankara late gene expression in human and murine cells, irrespective of their capacity to inhibit protein kinase R-mediated phosphorylation of eukaryotic translation initiation factor 2alpha.* J Gen Virol, 2010. **91**(Pt 2): p. 470-82.
138. Kuruppu, D. and K.K. Tanabe, *Viral oncolysis by herpes simplex virus and other viruses.* Cancer Biol Ther, 2005. **4**(5): p. 524-31.
139. Thorne, S.H., *Immunotherapeutic potential of oncolytic vaccinia virus.* Front Oncol, 2014. **4**: p. 155.
140. Sampath, P. and S.H. Thorne, *Arming viruses in multi-mechanistic oncolytic viral therapy: current research and future developments, with emphasis on poxviruses.* Oncolytic Virother, 2014. **3**: p. 1-9.

141. Mejias-Perez, E., L. Carreno-Fuentes, and M. Esteban, *Development of a Safe and Effective Vaccinia Virus Oncolytic Vector WR-Delta4 with a Set of Gene Deletions on Several Viral Pathways.* Mol Ther Oncolytics, 2018. **8**: p. 27-40.
142. Yan, W.L., et al., *Recent progress in GM-CSF-based cancer immunotherapy.* Immunotherapy, 2017. **9**(4): p. 347-360.
143. Chon, H.J., et al., *Tumor Microenvironment Remodeling by Intratumoral Oncolytic Vaccinia Virus Enhances the Efficacy of Immune-Checkpoint Blockade.* Clin Cancer Res, 2019. **25**(5): p. 1612-1623.
144. Kowalsky, S.J., et al., *Superagonist IL-15-Armed Oncolytic Virus Elicits Potent Antitumor Immunity and Therapy That Are Enhanced with PD-1 Blockade.* Mol Ther, 2018. **26**(10): p. 2476-2486.
145. Panelli, M.C., et al., *Forecasting the cytokine storm following systemic interleukin (IL)-2 administration.* J Transl Med, 2004. **2**(1): p. 17.
146. Liu, Z., et al., *Modifying the cancer-immune set point using vaccinia virus expressing re-designed interleukin-2.* Nat Commun, 2018. **9**(1): p. 4682.
147. Liu, Z., et al., *CXCL11-Armed oncolytic poxvirus elicits potent antitumor immunity and shows enhanced therapeutic efficacy.* Oncoimmunology, 2016. **5**(3): p. e1091554.
148. Chan, W.M. and G. McFadden, *Oncolytic Poxviruses.* Annu Rev Virol, 2014. **1**(1): p. 119-141.
149. Wilkinson, M.J., et al., *Oncolytic vaccinia virus combined with radiotherapy induces apoptotic cell death in sarcoma cells by down-regulating the inhibitors of apoptosis.* Oncotarget, 2016. **7**(49): p. 81208-81222.
150. Binz, E., et al., *Chemovirotherapy of Pancreatic Adenocarcinoma by Combining Oncolytic Vaccinia Virus GLV-1h68 with nab-Paclitaxel Plus Gemcitabine.* Mol Ther Oncolytics, 2017. **6**: p. 10-21.
151. Jiang, Y., Y. Li, and B. Zhu, *T-cell exhaustion in the tumor microenvironment.* Cell Death Dis, 2015. **6**: p. e1792.
152. Azoury, S.C., D.M. Straughan, and V. Shukla, *Immune Checkpoint Inhibitors for Cancer Therapy: Clinical Efficacy and Safety.* Curr Cancer Drug Targets, 2015. **15**(6): p. 452-62.
153. Postow, M.A., et al., *Nivolumab and ipilimumab versus ipilimumab in untreated melanoma.* N Engl J Med, 2015. **372**(21): p. 2006-17.
154. Ottaviano, M., S. De Placido, and P.A. Ascierto, *Recent success and limitations of immune checkpoint inhibitors for cancer: a lesson from melanoma.* Virchows Arch, 2019. **474**(4): p. 421-432.
155. Rojas, J.J., et al., *Defining Effective Combinations of Immune Checkpoint Blockade and Oncolytic Virotherapy.* Clin Cancer Res, 2015. **21**(24): p. 5543-51.
156. Jonas, B.A., *Combination of an oncolytic virus with PD-L1 blockade keeps cancer in check.* Sci Transl Med, 2017. **9**(386).
157. Fend, L., et al., *Immune Checkpoint Blockade, Immunogenic Chemotherapy or IFN-alpha Blockade Boost the Local and Abscopal Effects of Oncolytic Virotherapy.* Cancer Res, 2017. **77**(15): p. 4146-4157.
158. Perdiguero, B. and M. Esteban, *The interferon system and vaccinia virus evasion mechanisms.* J Interferon Cytokine Res, 2009. **29**(9): p. 581-98.
159. Rice, A.D., et al., *Roles of vaccinia virus genes E3L and K3L and host genes PKR and RNase L during intratracheal infection of C57BL/6 mice.* J Virol, 2011. **85**(1): p. 550-67.
160. Wasilenko, S.T., et al., *The vaccinia virus F1L protein interacts with the proapoptotic protein Bak and inhibits Bak activation.* J Virol, 2005. **79**(22): p. 14031-43.
161. Baxby, D., *The origins of vaccinia virus.* J Infect Dis, 1977. **136**(3): p. 453-5.

162. McIntosh, A.A. and G.L. Smith, *Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus.* J Virol, 1996. **70**(1): p. 272-81.
163. Turner, G.S., *Respiratory infection of mice with vaccinia virus.* J Gen Virol, 1967. **1**(3): p. 399-402.
164. Pelin, A., et al., *Deletion of Apoptosis Inhibitor F1L in Vaccinia Virus Increases Safety and Oncolysis for Cancer Therapy.* Mol Ther Oncolytics, 2019. **14**: p. 246-252.
165. Rintoul, J.L., et al., *A selectable and excisable marker system for the rapid creation of recombinant poxviruses.* PLoS One, 2011. **6**(9): p. e24643.
166. Smith, E.R. and E.A. Chiocca, *Oncolytic viruses as novel anticancer agents: turning one scourge against another.* Expert Opin Investig Drugs, 2000. **9**(2): p. 311-27.
167. Postigo, A., et al., *Vaccinia-induced epidermal growth factor receptor-MEK signalling and the anti-apoptotic protein F1L synergize to suppress cell death during infection.* Cell Microbiol, 2009. **11**(8): p. 1208-18.
168. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
169. Tzahar, E., et al., *Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network.* EMBO J, 1998. **17**(20): p. 5948-63.
170. Elkins, K.L., et al., *In vivo delivery of interleukin-4 by a recombinant vaccinia virus prevents tumor development in mice.* Hum Gene Ther, 1994. **5**(7): p. 809-20.
171. Martin, N.T. and J.C. Bell, *Oncolytic Virus Combination Therapy: Killing One Bird with Two Stones.* Mol Ther, 2018. **26**(6): p. 1414-1422.
172. Parviainen, S., et al., *CD40 ligand and tdTomato-armed vaccinia virus for induction of antitumor immune response and tumor imaging.* Gene Ther, 2014. **21**(2): p. 195-204.
173. Zhang, Y.Q., et al., *Enhancing the therapeutic effect against ovarian cancer through a combination of viral oncolysis and antigen-specific immunotherapy.* Mol Ther, 2010. **18**(4): p. 692-9.
174. Kim, J.H., et al., *Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF.* Mol Ther, 2006. **14**(3): p. 361-70.
175. Pin, R.H., M. Reinblatt, and Y. Fong, *Employing tumor hypoxia to enhance oncolytic viral therapy in breast cancer.* Surgery, 2004. **136**(2): p. 199-204.
176. Postigo, A., et al., *Interaction of F1L with the BH3 domain of Bak is responsible for inhibiting vaccinia-induced apoptosis.* Cell Death Differ, 2006. **13**(10): p. 1651-62.
177. Zhai, D., et al., *Vaccinia virus protein F1L is a caspase-9 inhibitor.* J Biol Chem, 2010. **285**(8): p. 5569-80.
178. Gerlic, M., et al., *Vaccinia virus F1L protein promotes virulence by inhibiting inflammasome activation.* Proc Natl Acad Sci U S A, 2013. **110**(19): p. 7808-13.
179. Postigo, A. and M. Way, *The vaccinia virus-encoded Bcl-2 homologues do not act as direct Bax inhibitors.* J Virol, 2012. **86**(1): p. 203-13.
180. Rainov, N.G. and H. Ren, *Oncolytic viruses for treatment of malignant brain tumours.* Acta Neurochir Suppl, 2003. **88**: p. 113-23.
181. Yoo, S.Y., S.Y. Lee, and N.C. Yoo, *Cytokine expression and cancer detection.* Med Sci Monit, 2009. **15**(3): p. RA49-56.
182. Perdiguero, B., et al., *Deletion of the viral anti-apoptotic gene F1L in the HIV/AIDS vaccine candidate MVA-C enhances immune responses against HIV-1 antigens.* PLoS One, 2012. **7**(10): p. e48524.
183. Eltom, S., et al., *TLR4 activation induces IL-1beta release via an IPAF dependent but caspase 1/11/8 independent pathway in the lung.* Respir Res, 2014. **15**: p. 87.
184. Taylor, J.M. and M. Barry, *Near death experiences: poxvirus regulation of apoptotic death.* Virology, 2006. **344**(1): p. 139-50.

185. Guo, Z.S., et al., *The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2.* Cancer Res, 2005. **65**(21): p. 9991-8.
186. Nichols, D.B., W. De Martini, and J. Cottrell, *Poxviruses Utilize Multiple Strategies to Inhibit Apoptosis.* Viruses, 2017. **9**(8).
187. Lathe, R., J.L. Vilotte, and A.J. Clark, *Plasmid and bacteriophage vectors for excision of intact inserts.* Gene, 1987. **57**(2-3): p. 193-201.
188. Foloppe, J., et al., *Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus.* Gene Ther, 2008. **15**(20): p. 1361-71.
189. Barquist, L., C.J. Boinett, and A.K. Cain, *Approaches to querying bacterial genomes with transposon-insertion sequencing.* RNA Biol, 2013. **10**(7): p. 1161-9.
190. Blasco, R., J.R. Sisler, and B. Moss, *Dissociation of progeny vaccinia virus from the cell membrane is regulated by a viral envelope glycoprotein: effect of a point mutation in the lectin homology domain of the A34R gene.* J Virol, 1993. **67**(6): p. 3319-25.
191. Antoine, G., et al., *The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses.* Virology, 1998. **244**(2): p. 365-96.
192. Pires de Miranda, M., et al., *The vaccinia virus kelch-like protein C2L affects calcium-independent adhesion to the extracellular matrix and inflammation in a murine intradermal model.* J Gen Virol, 2003. **84**(Pt 9): p. 2459-71.
193. Maluquer de Motes, C., et al., *Inhibition of apoptosis and NF-kappaB activation by vaccinia protein N1 occur via distinct binding surfaces and make different contributions to virulence.* PLoS Pathog, 2011. **7**(12): p. e1002430.
194. Ryerson, M.R., et al., *Vaccinia Virus Encodes a Novel Inhibitor of Apoptosis That Associates with the Apoptosome.* J Virol, 2017. **91**(23).
195. Bravo Cruz, A.G. and J.L. Shisler, *Vaccinia virus K1 ankyrin repeat protein inhibits NF-kappaB activation by preventing RelA acetylation.* J Gen Virol, 2016. **97**(10): p. 2691-2702.
196. Zhou, J., et al., *The vaccinia virus K2L gene encodes a serine protease inhibitor which inhibits cell-cell fusion.* Virology, 1992. **189**(2): p. 678-86.
197. Carroll, K., et al., *Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase.* J Biol Chem, 1993. **268**(17): p. 12837-42.
198. Eckert, D., et al., *Vaccinia virus nicking-joining enzyme is encoded by K4L (VACWR035).* J Virol, 2005. **79**(24): p. 15084-90.
199. Prichard, M.N., et al., *Vaccinia virus lacking the deoxyuridine triphosphatase gene (F2L) replicates well in vitro and in vivo, but is hypersensitive to the antiviral drug (N)-methanocarbathymidine.* Virol J, 2008. **5**: p. 39.
200. Froggatt, G.C., G.L. Smith, and P.M. Beard, *Vaccinia virus gene F3L encodes an intracellular protein that affects the innate immune response.* J Gen Virol, 2007. **88**(Pt 7): p. 1917-21.
201. Spriggs, M.K., et al., *Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein.* Cell, 1992. **71**(1): p. 145-52.
202. Liu, B., et al., *Identification of Poxvirus Genome Uncoating and DNA Replication Factors with Mutually Redundant Roles.* J Virol, 2018. **92**(7).
203. Alcami, A., J.A. Symons, and G.L. Smith, *The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN.* J Virol, 2000. **74**(23): p. 11230-9.
204. Shchelkunov, S.N., *Orthopoxvirus genes that mediate disease virulence and host tropism.* Adv Virol, 2012. **2012**: p. 524743.

205. Alcami, A., et al., *Vaccinia virus strains Lister, USSR and Evans express soluble and cell-surface tumour necrosis factor receptors.* J Gen Virol, 1999. **80 (Pt 4)**: p. 949-59.
206. Zhang, L., et al., *Analysis of vaccinia virus-host protein-protein interactions: validations of yeast two-hybrid screenings.* J Proteome Res, 2009. **8**(9): p. 4311-8.
207. Turner, P.C. and R.W. Moyer, *An orthopoxvirus serpinlike gene controls the ability of infected cells to fuse.* J Virol, 1992. **66**(4): p. 2076-85.
208. Belongia, E.A. and A.L. Naleway, *Smallpox vaccine: the good, the bad, and the ugly.* Clin Med Res, 2003. **1**(2): p. 87-92.
209. Engelmayer, J., et al., *Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion.* J Immunol, 1999. **163**(12): p. 6762-8.
210. Di Pilato, M., et al., *Distinct Roles of Vaccinia Virus NF-kappaB Inhibitor Proteins A52, B15, and K7 in the Immune Response.* J Virol, 2017. **91**(13).
211. Di Pilato, M., et al., *NFkappaB activation by modified vaccinia virus as a novel strategy to enhance neutrophil migration and HIV-specific T-cell responses.* Proc Natl Acad Sci U S A, 2015. **112**(11): p. E1333-42.
212. Patel, M.R., *Immunotherapy for thoracic oncology gone viral.* Immunotherapy, 2018. **10**(5): p. 383-390.
213. Sharon, E., et al., *Immune checkpoint inhibitors in clinical trials.* Chin J Cancer, 2014. **33**(9): p. 434-44.
214. Giri, A., S.S. Walia, and A. Gajra, *Clinical Trials Investigating Immune Checkpoint Inhibitors in Non-Small-Cell Lung Cancer.* Rev Recent Clin Trials, 2016. **11**(4): p. 297-305.
215. Remy-Ziller, C., et al., *Sequential administration of MVA-based vaccines and PD-1/PD-L1-blocking antibodies confers measurable benefits on tumor growth and survival: Preclinical studies with MVA-betaGal and MVA-MUC1 (TG4010) in a murine tumor model.* Hum Vaccin Immunother, 2018. **14**(1): p. 140-145.
216. Beavis, P.A., et al., *Dual PD-1 and CTLA-4 Checkpoint Blockade Promotes Antitumor Immune Responses through CD4(+)Foxp3(-) Cell-Mediated Modulation of CD103(+) Dendritic Cells.* Cancer Immunol Res, 2018. **6**(9): p. 1069-1081.
217. Lu, Y.C. and P.F. Robbins, *Cancer immunotherapy targeting neoantigens.* Semin Immunol, 2016. **28**(1): p. 22-7.
218. McGray, A.J.R., et al., *Oncolytic Maraba virus armed with tumor antigen boosts vaccine priming and reveals diverse therapeutic response patterns when combined with checkpoint blockade in ovarian cancer.* J Immunother Cancer, 2019. **7**(1): p. 189.
219. Pol, J.G., et al., *Maraba virus as a potent oncolytic vaccine vector.* Mol Ther, 2014. **22**(2): p. 420-429.
220. Chakrabarti, S., J.R. Sisler, and B. Moss, *Compact, synthetic, vaccinia virus early/late promoter for protein expression.* Biotechniques, 1997. **23**(6): p. 1094-7.
221. Baur, K., et al., *Immediate-early expression of a recombinant antigen by modified vaccinia virus ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses.* J Virol, 2010. **84**(17): p. 8743-52.
222. Yang, Z., et al., *Deciphering poxvirus gene expression by RNA sequencing and ribosome profiling*. J Virol, 2015. **89**(13): p. 6874-86.
223. Breitbach, C.J., et al., *A Phase 2, Open-Label, Randomized Study of Pexa-Vec (JX-594) Administered by Intratumoral Injection in Patients with Unresectable Primary Hepatocellular Carcinoma.* Methods Mol Biol, 2015. **1317**: p. 343-57.
224. Fend, L., et al., *Oncolytic virotherapy with an armed vaccinia virus in an orthotopic model of renal carcinoma is associated with modification of the tumor microenvironment.* Oncoimmunology, 2016. **5**(2): p. e1080414.

225. Buller, R.M., et al., *Deletion of the vaccinia virus growth factor gene reduces virus virulence.* J Virol, 1988. **62**(3): p. 866-74.
226. Zhao, Y., E.B. Butler, and M. Tan, *Targeting cellular metabolism to improve cancer therapeutics.* Cell Death Dis, 2013. **4**: p. e532.
227. McLysaght, A., P.F. Baldi, and B.S. Gaut, *Extensive gene gain associated with adaptive evolution of poxviruses.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15655-60.
228. Massague, J., *TGFbeta in Cancer.* Cell, 2008. **134**(2): p. 215-30.
229. Qin, L. and D.H. Evans, *Genome scale patterns of recombination between coinfecting vaccinia viruses.* J Virol, 2014. **88**(10): p. 5277-86.
230. Samson, A., et al., *Intravenous delivery of oncolytic reovirus to brain tumor patients immunologically primes for subsequent checkpoint blockade.* Sci Transl Med, 2018. **10**(422).
231. Kearse, M., et al., *Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data.* Bioinformatics, 2012. **28**(12): p. 1647-9.
232. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-20.
233. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* J Comput Biol, 2012. **19**(5): p. 455-77.
234. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
235. Katoh, K., et al., *MAFFT version 5: improvement in accuracy of multiple sequence alignment.* Nucleic Acids Res, 2005. **33**(2): p. 511-8.
236. Quinlan, A.R., *BEDTools: The Swiss-Army Tool for Genome Feature Analysis.* Curr Protoc Bioinformatics, 2014. **47**: p. 11 12 1-34.
237. Kim, D., B. Langmead, and S.L. Salzberg, *HISAT: a fast spliced aligner with low memory requirements.* Nat Methods, 2015. **12**(4): p. 357-60.
238. Pertea, M., et al., *StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.* Nat Biotechnol, 2015. **33**(3): p. 290-5.
239. Eglen, S.J., *A quick guide to teaching R programming to computational biology students.* PLoS Comput Biol, 2009. **5**(8): p. e1000482.
240. Oliveira, G.P., et al., *Poxvirus Host Range Genes and Virus-Host Spectrum: A Critical Review.* Viruses, 2017. **9**(11).
241. Williamson, J.D., et al., *Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route.* J Gen Virol, 1990. **71 (Pt 11)**: p. 2761-7.
242. Sivan, G., et al., *Human Host Range Restriction of the Vaccinia Virus C7/K1 Double Deletion Mutant Is Mediated by an Atypical Mode of Translation Inhibition.* J Virol, 2018. **92**(23).
243. Kaufman, H.L., et al., *Insertion of interleukin-2 (IL-2) and interleukin-12 (IL-12) genes into vaccinia virus results in effective anti-tumor responses without toxicity.* Vaccine, 2002. **20**(13-14): p. 1862-9.
244. Tokunaga, R., et al., *CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy.* Cancer Treat Rev, 2018. **63**: p. 40-47.
245. Kleinpeter, P., et al., *Vectorization in an oncolytic vaccinia virus of an antibody, a Fab and a scFv against programmed cell death -1 (PD-1) allows their intratumoral delivery and an improved tumor-growth inhibition.* Oncoimmunology, 2016. **5**(10): p. e1220467.

246. Gherardi, M.M., J.C. Ramirez, and M. Esteban, *IL-12 and IL-18 act in synergy to clear vaccinia virus infection: involvement of innate and adaptive components of the immune system.* J Gen Virol, 2003. **84**(Pt 8): p. 1961-72.
247. Mortimer, C.L., B. Dugdale, and J.L. Dale, *Updates in inducible transgene expression using viral vectors: from transient to stable expression.* Curr Opin Biotechnol, 2015. **32**: p. 85-92.
248. Elroy-Stein, O. and B. Moss, *Gene expression using the vaccinia virus/T7 RNA polymerase hybrid system.* Curr Protoc Mol Biol, 2001. **Chapter 16**: p. Unit16 19.
249. Dai, P., et al., *Intratumoral delivery of inactivated modified vaccinia virus Ankara (iMVA) induces systemic antitumor immunity via STING and Batf3-dependent dendritic cells.* Sci Immunol, 2017. **2**(11).
250. Rivera Vargas, T., I. Benoit-Lizon, and L. Apetoh, *Rationale for stimulator of interferon genes-targeted cancer immunotherapy.* Eur J Cancer, 2017. **75**: p. 86-97.
251. Yi, G., et al., *Single nucleotide polymorphisms of human STING can affect innate immune response to cyclic dinucleotides.* PLoS One, 2013. **8**(10): p. e77846.

Contribution of Collaborators

Chapters 2 and 3 contain a Preface section detailing contribution of various lab members to our projects. This section will focus on contributions of collaborators from a different institute.

Chapter 2

Dr. Johann Foloppe and Dr. Philippe Erbs have performed *in vivo* and *in vitro* experiments using the F1L virus (Figure 2.3). They have also contributed to manuscript writing and response to reviewer concerns.

Dr. Michael Way and Dr. Antonio Postigo have shared data on a different strain of Vaccinia deleted for F1L. They have also contributed in drafting the manuscript.

Chapter 3

Dr. David Evans has sent our lab recombinant Tian Tan viruses from which we were able to purify wild-type Tian Tan, thank you.

Dr. Victoria A. Jennings performed flow analysis on human PBMCs infected with CopMD5p3p (Figure 3.13A). Dr. Alan Melcher provided advice and guidance.

Dr. Tyson Graber and Dr. Hoàng Dũng performed infections and polysome isolation for ribosome profiling experiments (Figure 3.16).

Appendices

A1. Supplemental information for Chapter 2

Supplemental Figure

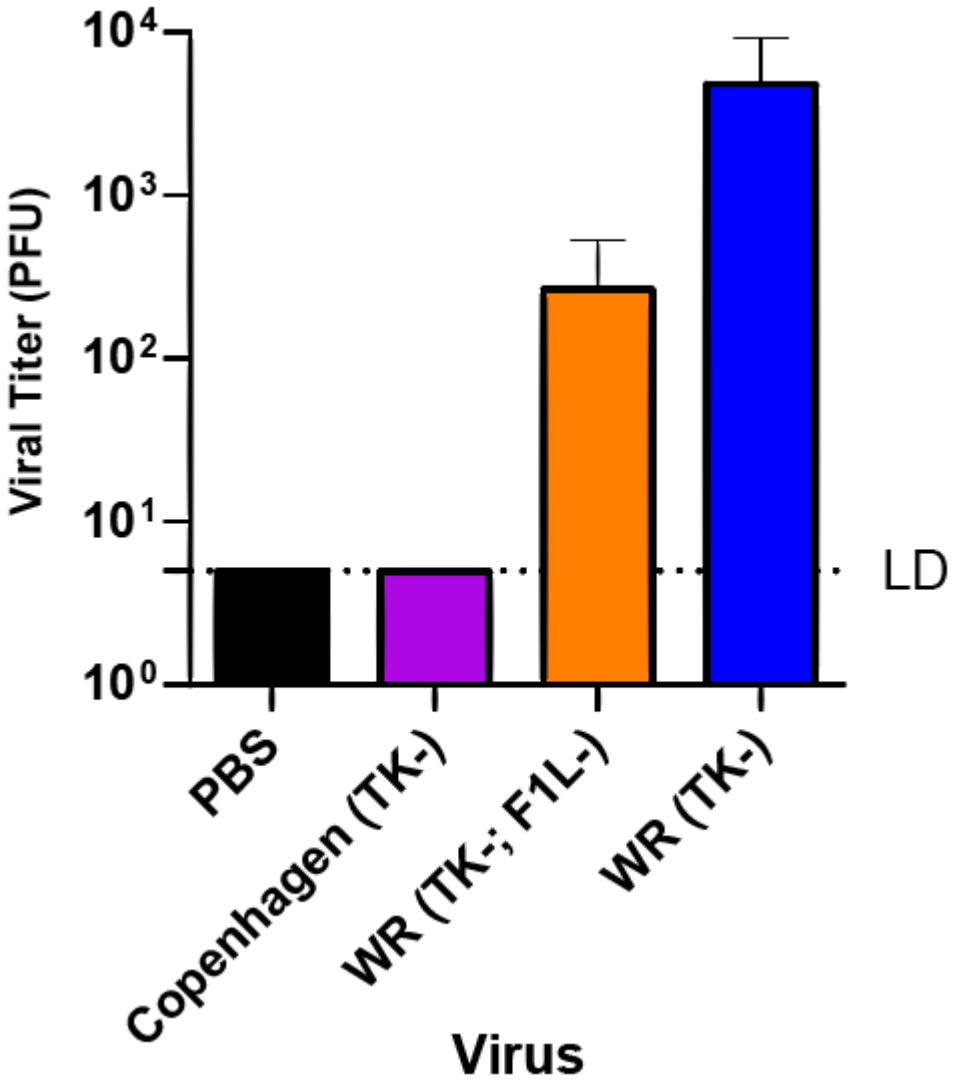


Figure S2.1: F1L deletion decreases neurovirulence.

Viral brain titer 3 days post infection of nude CD-1 mice treated with one dose 1e7 pfu intra-venously with either Copenhagen ΔTK, WR ΔTK or WR ΔTK/F1L virus.

A2. Supplemental information for Chapter 3

Supplemental Tables

Table S3.1: List of poxvirus genomes and their respective GenBank Accession numbers used for phylogenetic analysis performed in Figure 3.1

Taxon	GenBank Accession
Vaccinia Brazil	KF179385
Vaccinia Cantagalo	KT013210
Vaccinia Copenhagen	M35027
Vaccinia CVA	AM501482
Vaccinia IHDW	KJ125439
Vaccinia IOC	KT184690
Vaccinia Lister	DQ121394
Vaccinia Mulfords1902	MF477237
Vaccinia MVA	U94848
Vaccinia Tashkent	KM044309
Vaccinia TianTan	AF095689
Vaccinia WAU86881	KF866253
Vaccinia WR	NC_006998
Vaccinia Wyeth	JN654976
Akhmetapox	MH607141
BeAn58058	NC_032111
Bovinepapularstomatitis	NC_005337
Buffalopox	MG599038
Camelpox	AF438165
Canarypox	NC_005309
CotiaSPAn232	NC_016924
Cowpox	AF482758
DeerpoxW84883	NC_006966
Ectromelia	AF012825
Eptesipox	NC_035460
Flamingopox	NC_036582
Fowlpox	AF198100
Goatpox	AY077835
Horsepox	DQ792504
Lumpyskindisease	AF325528
Molluscumcontagiosum	NC_001731
Monkeypox	AF380138

Murmanskpox	NC_035468
Myxoma	AF170726
Nilecrocodilepox	NC_008030
NY014pox	NC_035469
Orf	NC_005336
Parapoxreddeer	NC_025963
Penguinpox	KJ859677
Pigeonpox	NC_024447
Pseudocowpox	NC_013804
Pteropox	NC_030656
Rabbitfibroma	AF170722
Rabbitpox	AY484669
Raccoonpox	NC_027213
Salmongillpox	NC_027707
Sealparapox	NC_035188
Seaotterpox	MH427217
Shearwaterpox	KX857216
SheeppoxA	AY077833
Skunkpox	NC_031038
Squirrelpox	NC_022563
Swinepox	AF410153
Tanapox	NC_009888
Taterapox	NC_008291
Turkeypox	NC_028238
Variola	NC_001611
Volepox	NC_031033
WesternKangaroopox	MF467280
Yabalikedisease	AJ293568
Yabamonkeytumor	NC_005179
Yokapox	NC_015960
Adoxophyeshonmaientomopox	NC_021247
Amsactamooreientomopox	AF250284
Anomalacupreaentomopox	NC_023426
Choristoneurabiennisentomopox	NC_021248
Choristoneurarosaceanaentomopox	NC_021249
Melanoplussanguinipesentomopox	AF063866
Mythimnaseparataentomopox	NC_021246

Supplemental Figure

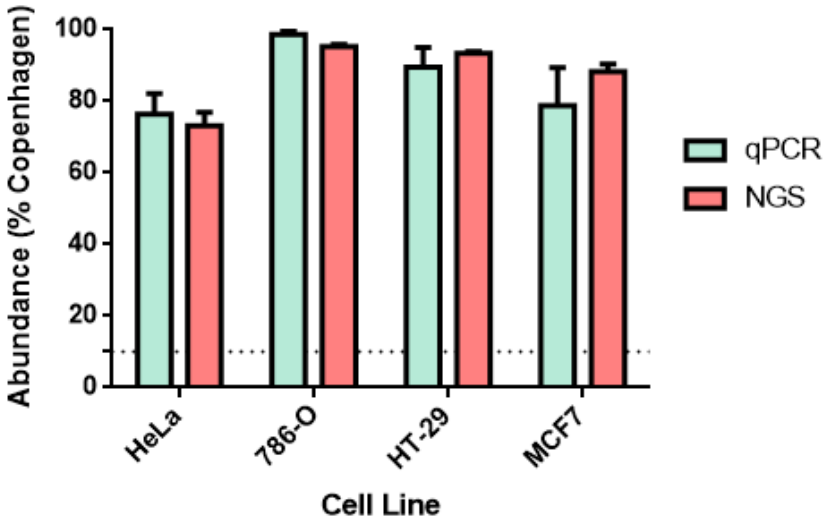


Figure S3.1: Relative fitness of five Vaccinia wild-type strains after passaging three times in various cancer models.

Validation of Copenhagen abundance using qPCR technique on Copenhagen specific genomic locus on data shown in Figure 3.3.

A3. Enhanced susceptibility of cancer cells to oncolytic rhabdo-virotherapy by expression of Nodamura virus protein B2 as a suppressor of RNA interference

Preface

Work described in this chapter has been previously published as an article in the Journal for ImmunoTherapy of Cancer. The published paper is titled "Enhanced susceptibility of cancer cells to oncolytic rhabdo-virotherapy by expression of Nodamura virus protein B2 as a suppressor of RNA interference" and has the following authors: Donald Bastin, Amelia S. Aitken, Adrian Pelin, Larissa A. Pikor, Mathieu J. F. Crupi, Michael S. Huh, Marie-Claude Bourgeois-Daigneault, John C. Bell and Carolina S. Ilkow.

As I joined the Bell lab work on this project was underway, supervised by Dr. Ilkow and Dr. Bell. My contribution to this work revolved primarily around bioinformatic analysis, namely RNA-seq analysis (Figure 1a, Figure 2c) and Microarray analysis (Figure 3a, Figure 3b). Because my contribution was rather focused on certain aspects of the project, I have not included this paper as a standalone chapter in my thesis.

Abstract

Antiviral responses are barriers that must be overcome for efficacy of oncolytic virotherapy. In mammalian cells, antiviral responses involve the interferon pathway, a protein-signaling cascade that alerts the immune system and limits virus propagation. Tumour-specific defects in interferon signaling enhance viral infection and responses to oncolytic virotherapy, but many human cancers are still refractory to oncolytic viruses. Given that invertebrates, fungi and plants rely on RNA interference pathways for antiviral protection, we investigated the potential involvement of this alternative antiviral mechanism in cancer cells. Here, we detected viral genome-derived small RNAs, indicative of RNAi-mediated antiviral responses, in human cancer cells. As viruses may encode suppressors of the RNA interference pathways, we engineered an oncolytic vesicular stomatitis virus variant to encode the Nodamura virus protein B2, a known inhibitor of RNAi-mediated immune responses. B2-expressing oncolytic virus showed enhanced viral replication and cytotoxicity, impaired viral genome cleavage and altered microRNA processing in cancer cells. Our data establish the improved therapeutic potential of our novel virus which targets the RNAi-mediated antiviral defense of cancer cells.

Introduction

Oncolytic viruses (OVs) possess an intrinsic or engineered ability to selectively target, replicate in and kill cancer cells [1]. These promising cancer therapeutics exploit cellular defects that promote tumour growth [2], destroy tumour-associated vasculature [3], induce anti-tumour immunity [1] and synergize with other treatments [4]. OVs must overcome barriers triggered by viral infection, including those mounted by cancer cells and components of the tumour microenvironment [5]. The type I interferon (IFN) pathway is a well-characterized mammalian signaling cascade triggered upon viral attack to protect the surrounding cells and alert the immune system to contain infection [6]. The production of type I IFNs promotes an antiviral and anti-proliferative state in addition to inducing innate and adaptive immunity [2]. This antiviral response represents a major barrier to virus replication and spread in healthy tissues and is necessary to the safety of OV therapy [7]. Interestingly, the genetic alterations promoting tumourigenesis are associated with enhanced cancer cell susceptibility to viral infection [2]. Many pathways activated in response to infection that inhibit cell growth, activate apoptosis, and alert the immune system, are incompatible with malignant growth and are often defective in cancer cells [2]. Since these defects are common in cancer cells, they facilitate the targeted killing of cancer cells by certain OVs. Despite having IFN pathway defects, many cancers are still quite resistant to OV therapy [7]. For example, the vesicular stomatitis virus (VSV) is an OV platform with promising potential for clinical translation [8]. A VSV variant with an improved therapeutic index (VSVΔ51) is impaired in its capacity to block the IFN response and infect normal tissues [7]. The degree of susceptibility to VSVΔ51 killing varies among human cancers [7], due to the

IFN status of the cancer cells and the potential involvement of other antiviral mechanisms within resistant tumours.

An alternative antiviral strategy relies on RNA interference (RNAi) [9], in order to combat infection in plants, fungi and invertebrates. This system is similar to the microRNA (miRNA) processing pathway used for post-transcriptional regulation in most eukaryotes. Viral double-stranded RNA generated during replication and transcription is bound and cleaved by the host cytoplasmic enzyme Dicer to form 22–23 nucleotides long RNA fragments [10]. These short RNA fragments are loaded into the RNA-induced silencing complex (RISC) where a single strand is selected to target homologous viral RNA and therefore prevent viral genome replication and translation [10]. To counteract this RNAi-mediated antiviral response, many plant and insect viruses have evolved viral suppressors of RNAi (VSRs) [11]. One such virus is the Nodamura virus, which primarily infects insects but is also highly virulent to certain mammals like suckling mice and hamsters [11–13]. Nodamura virus encodes a VSR known as B2, which binds double-stranded RNA and inhibits processing by Dicer which prevents the production of antiviral siRNAs [14–16].

RNAi- and protein-mediated immunity were long thought to be non-overlapping mechanisms, with insects and invertebrates using one strategy and mammals using the other. Interestingly, recent discoveries suggest that these mechanisms might not be mutually exclusive. In fact, antiviral RNAi has been shown to function in mammalian embryonic or undifferentiated cells [17]. Considering the discovery of mammalian antiviral RNAi in embryonic stem cells and the genetic similarities between cancer cells and embryonic stem cells [18–22]; we hypothesized a role for antiviral RNAi in cancer

cells. To investigate antiviral RNAi and its effects on OV therapy, we engineered a recombinant VSVΔ51 to express the Nodamura virus B2 protein. Herein, we characterize this novel OV and demonstrate the interplay between the B2-expressing virus and RNA processing pathways in cancer. Our results show enhanced cancer-specific killing by our virus as well as improved viral replication in vivo. Together, our data strongly suggest the involvement of the RNAi pathway in the antiviral defense of cancer cells.

Results

Evidence of a functional antiviral RNAi mechanism detected in VSVΔ51 infected cancer cells

To investigate the potential involvement of antiviral RNAi mechanisms in cancer cells, we infected human cancer cell lines with a VSV variant impaired in its ability to block IFN response (VSVΔ51) and performed small-RNA deep sequencing. We showed that virus-derived small RNAs (vsRNAs) have a length bias towards 22-mers in several cell lines (Figure 1 and Additional file 1: Figure S1A), consistent with the size of Dicer cleavage products. Importantly, this enrichment for 22-mers is present in positive strand vsRNAs, suggesting the occurrence of double stranded RNA cleavage. This is also characteristic of Dicer products, and likely acts during the synthesis of positive strands in VSV genome replication.

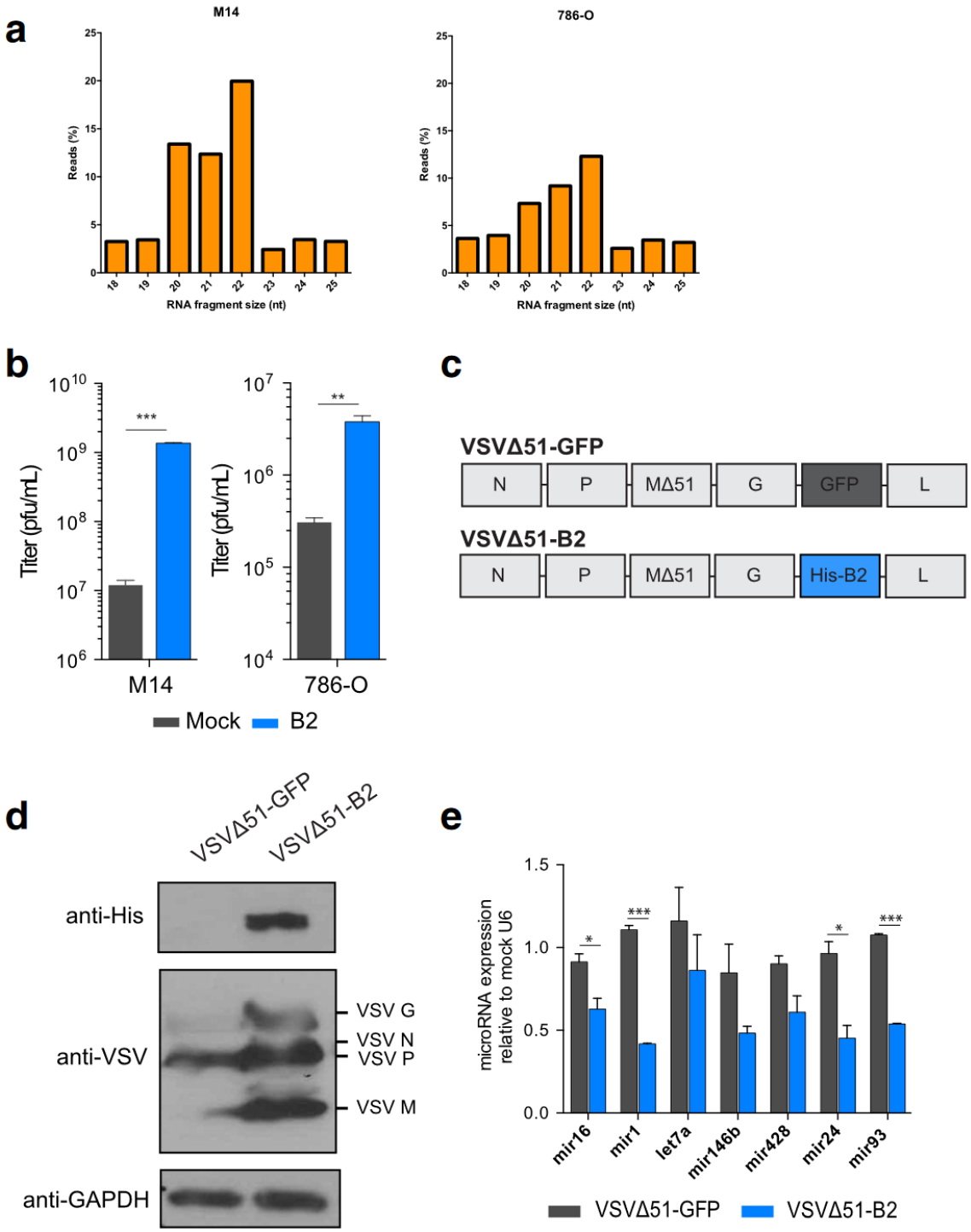

a
M14
786-O
Reads (%)
RNA fragment size (nt)
b
Titer (pfu/mL)
M14
786-O
Mock
B2
c
VSVΔ51-GFP
N
P
MΔ51
G
GFP
L
VSVΔ51-B2
His-B2
d
VSVΔ51-GFP
VSVΔ51-B2
anti-His
anti-VSV
VSV G
VSV N
VSV P
VSV M
anti-GAPDH
e
microRNA expression relative to mock U6
mir16
mir1
let7a
mir146b
mir428
mir24
mir93
VSVΔ51-GFP
VSVΔ51-B2

Figure 1: B2 enhances VSVΔ51 replication and alters miRNA levels in cancer cell lines.

a) M14 or 786-O cells were infected with VSVΔ51 virus and small-RNA deep sequencing was performed. Virus-derived small RNAs have a length bias towards 22-mers. The enrichment for 22-mers is indicated for positive strand vsRNAs.
b) Virus concentrations of supernatants collected from M14 or 786-O cells expressing fluorescently-tagged B2 or empty vector and infected with VSVΔ51 at an MOI of 0.1 for 24 h. NS: $P > 0.1$; *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test. Only significantly different pairs are indicated.
c) Schematic representation of the VSVΔ51-B2 and VSVΔ51-GFP viral backbones.
d) Western blot analysis of Vero cells infected at an MOI of 1 with VSVΔ51 or VSVΔ51-B2 for 24 h. The membranes were probed for VSV proteins, His-tagged B2 and GAPDH.
e) MiRNA levels from 786-O cells infected with VSVΔ51-GFP or VSVΔ51-B2 for 18 h as determined by qPCR. The results were normalized to mock uninfected levels as explained in the material and methods section. NS: $P > 0.1$, *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test. Only significantly different pairs are indicated on the figure

B2 protein enhances VSVΔ51 replication in virus-resistant cancer cells

We reasoned that if antiviral RNAi is triggered in mammalian cancer cells upon VSVΔ51 virus infection, expression of an RNAi viral suppressor should significantly enhance virus growth and cytotoxicity. To this end, we investigated the effects of the VSR protein B2 on VSVΔ51 replication in cancer cells, and characterized two human cancer cell lines (melanoma M14 cells and renal carcinoma 786-O cells) transfected with fluorescently-tagged B2 or empty vector (mock control). M14 and 786-O cell lines were selected as models for further characterization as they are both resistant to VSV infection and have functional type I IFN pathways. Upon drug-selection and sorting of positive cells, we confirmed ectopic expression of fluorescently-tagged constructs, as shown in fluorescence microscopy images (Additional file 1: Figure S1B). Viral outputs from B2-expressing M14 and 786-O cells infected with VSVΔ51 were significantly higher relative to mock controls (Figure 1B), suggesting that B2 may enhance viral production.

VSVΔ51-mediated expression of B2 enhances cytotoxicity in cancer cell lines

As ectopic expression of the B2 protein enhanced VSVΔ51 titers in both M14 and 786-O cell lines, we engineered a VSVΔ51 virus variant encoding His-tagged B2 to assess effects of virus-mediated B2 expression. B2, or GFP as a control, was cloned between the G and L genes of the VSVΔ51 backbone (Figure 1C), using a strategy previously shown to support expression of transgenes without impairing virus replication [7, 23]. We infected Vero cells with VSVΔ51-B2 and confirmed transgene expression by immunoblotting for His-tagged B2. As predicted, expression of B2 enhanced expression of VSV viral proteins (Figure 1D).

While the mechanism of action of VSVΔ51-encoded B2 on mammalian cancer cells remains to be elucidated, previous studies have shown that B2 blocks processing of small RNAs by Dicer [24, 25]. Given that B2 enhanced VSVΔ51 production by mammalian cancer cells, we investigated whether miRNA levels were affected by VSVΔ51-B2 using quantitative PCR (qPCR) for various miRNAs from infected 786-O cells. For the majority of miRNAs tested, including miR-1, miR-16, miR-24, and miR-93, the miRNA expression levels measured in VSVΔ51-B2 infected samples were significantly lower compared to VSVΔ51-GFP samples (Figure 1E), suggesting inhibition of small RNA processing by B2.

To determine if VSVΔ51-B2 could kill cancer cells more efficiently than VSVΔ51-GFP, we screened a panel of 38 different human cancer cell lines. The cells were infected at a multiplicity of infection (MOI) of 1 and cell viability was assessed. B2-expressing virus showed enhanced killing in the majority of cancer cell lines tested, including M14 and 786-O cells (Figure 2A). In our screen, we also included an additional virus variant encoding vaccinia Copenhagen virus VP55, a different VSR [26], which similarly enhanced virus-mediated cell killing in our studies (Figure 2A).

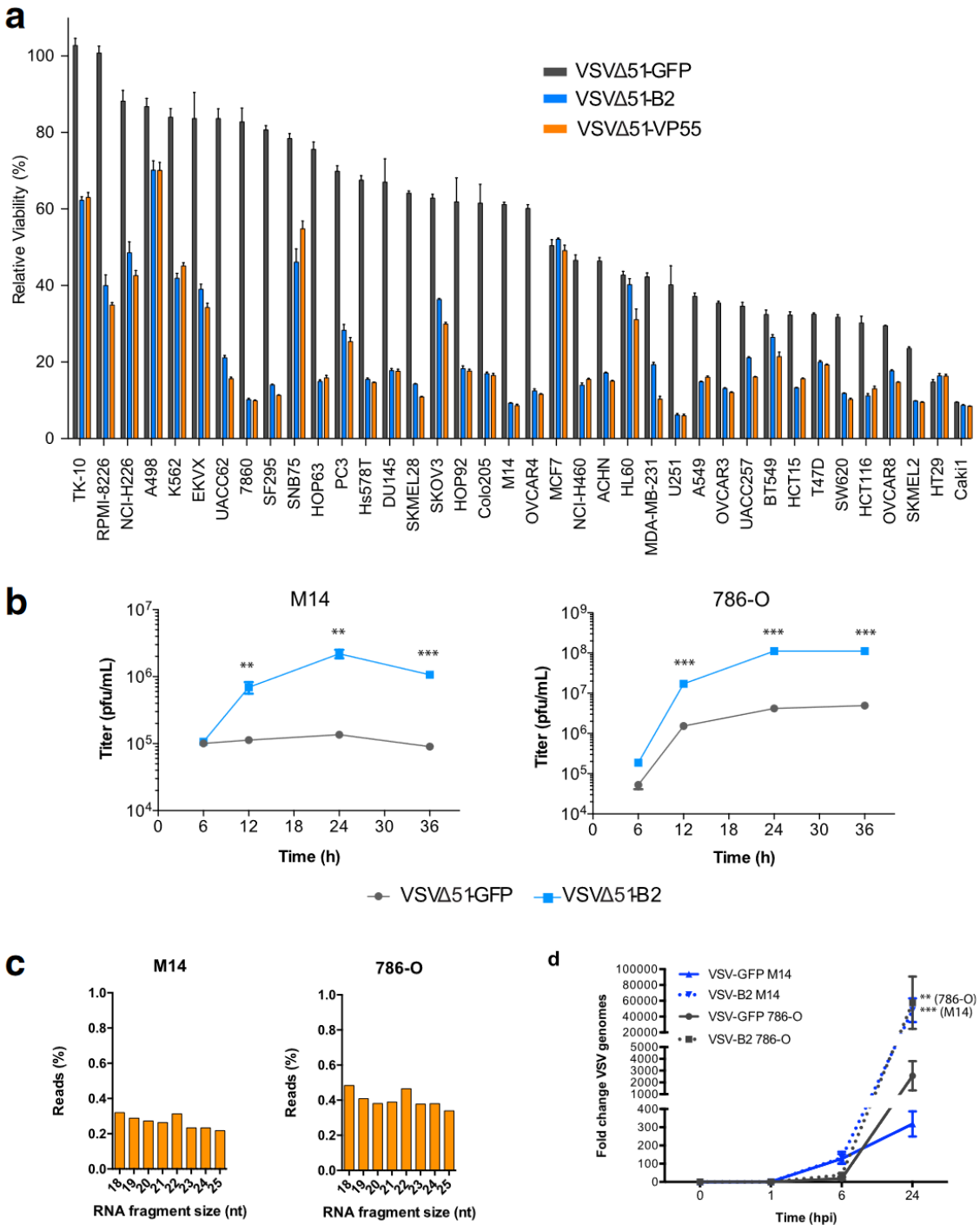

a
Relative Viability (%)
VSVΔ51-GFP
VSVΔ51-B2
VSVΔ51-VP55
TK-10
RPMI-8226
NCI-H226
A498
K562
EKVX
UACC62
7860
SF295
SNB75
HOP63
PC3
Hs578T
DU145
SKMEL28
SKOV3
HOP92
Colo205
M14
OVCAR4
MCF7
NCI-H460
ACHN
HL60
MDA-MB-231
U251
A549
OVCAR3
UACC257
BT549
HCT15
T47D
SW620
HCT116
OVCAR8
SKMEL2
HT29
Caki1
b
M14
786-O
Titer (pfu/mL)
Time (h)
VSVΔ51-GFP
VSVΔ51-B2
c
M14
786-O
Reads (%)
RNA fragment size (nt)
d
VSV-GFP M14
VSV-B2 M14
VSV-GFP 786-O
VSV-B2 786-O
** (786-O)
*** (M14)
Fold change VSV genomes
Time (hpi)

Figure 2: VSVΔ51-B2 alters cytotoxicity and viral genome cleavage.

a) Relative metabolic activity of 38 human cancer cell lines infected with VSVΔ51-GFP or VSVΔ51-B2 or additionally VSVΔ51-VP55 for 48 h at an MOI of 1. The results are expressed as a percentage of the signal obtained compared to mock treatment.
b) Time-course of viral titers from 786-O and M14 cell lines infected with VSVΔ51-GFP or VSVΔ51-B2 at an MOI of 3. NS: $P > 0.1$, *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test. Only significantly different pairs are indicated on the fig.
c) We performed small-RNA deep-sequencing using M14 or 786-O cells infected with VSVΔ51-B2 at an MOI of 0.1 for 18 h. B2 expression in VSVΔ51 virus abrogates genomic cleavage as 22-mer vsRNAs are no longer prominent. VSVΔ51-B2 derived vsRNAs display a bias towards positive strand reads in M14 and 786-O cells.
d) The indicated human cancer cell lines were infected with VSVΔ51-GFP or VSVΔ51-B2 (MOI = 0.1). At the indicated time points, the expression level of virus genomes for each sample was quantified and normalized to GAPDH. Levels of VSV genomes are expressed relative to the level observed in the VSVΔ51-GFP 1 h-post-infection samples, which were arbitrarily set to 1. Error bars indicate ±SD among triplicates. NS: $P > 0.1$, *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test

To determine whether VSVΔ51-B2 could affect virus production, we assessed viral replication at multiple time points in M14 and 786-O cells, and found that VSVΔ51-B2 significantly enhanced replication over time, relative to VSVΔ51-GFP (Figure 2B). Further, we infected GM38 fibroblasts with VSVΔ51-B2 or VSVΔ51-GFP to investigate if B2 expression affects virus replication in healthy cells, and found that VSVΔ51-B2 infection did not significantly increase viral cytotoxicity at an MOI of 1 (Additional file 1: Figure S2A).

VSVΔ51-B2 prevents VSV genome cleavage in cancer cells

To determine whether B2 protects VSV from genome cleavage, we performed small-RNA deep-sequencing on VSVΔ51-B2 infected cancer cells similar to the experiment in Figure 1. We showed that B2 expression in VSVΔ51 virus abrogates genomic cleavage as 22-mer vsRNAs are no longer prominent in different cell lines (Figure 2C and Additional file 1: Figure. S2B). Interestingly, VSVΔ51-B2 derived vsRNAs display a bias towards positive strand reads compared to VSVΔ51 vsRNAs in cancer cells (Figure 2C and Additional file 1: Figure S2B). Since the viral positive strand consists of viral mRNAs and positive sense genome copies, a larger bias for positive sense vRNAs suggests more efficient mRNA transcription in our VSVΔ51-B2 virus. In addition, we infected M14 and 786-O cells with VSVΔ51-GFP or VSVΔ51-B2 and showed that the expression level of virus genomes for each sample was enhanced in response to VSVΔ51-B2, relative to VSVΔ51-GFP (Figure 2D). Taken together, these data suggest that VSVΔ51-B2 inhibits direct cleavage of the viral genome and host RNA processing pathways.

VSVΔ51-B2 and the type I IFN response

To characterize the effect of B2 expression on mammalian cancer cells at a transcriptome level, we performed a microarray analysis on samples from M14 cells infected with either VSVΔ51-GFP or VSVΔ51-B2. Our results show that at a low MOI, VSVΔ51-B2 virus induced the expression of a variety of immune related genes, which were not affected by VSVΔ51 infection (Figure 3A). Through GO-term analysis, we detected an upregulation of genes by at least four-fold in response to VSVΔ51-B2, but not VSVΔ51-GFP. We also showed enrichment of cytokine and cytokine activity predominantly associated with an IFN response (Figure 3B). Interestingly, at high MOI, most immune genes upregulated by VSVΔ51-B2 at low MOI remain unchanged with few visible differences between the viruses (Figure 3A).

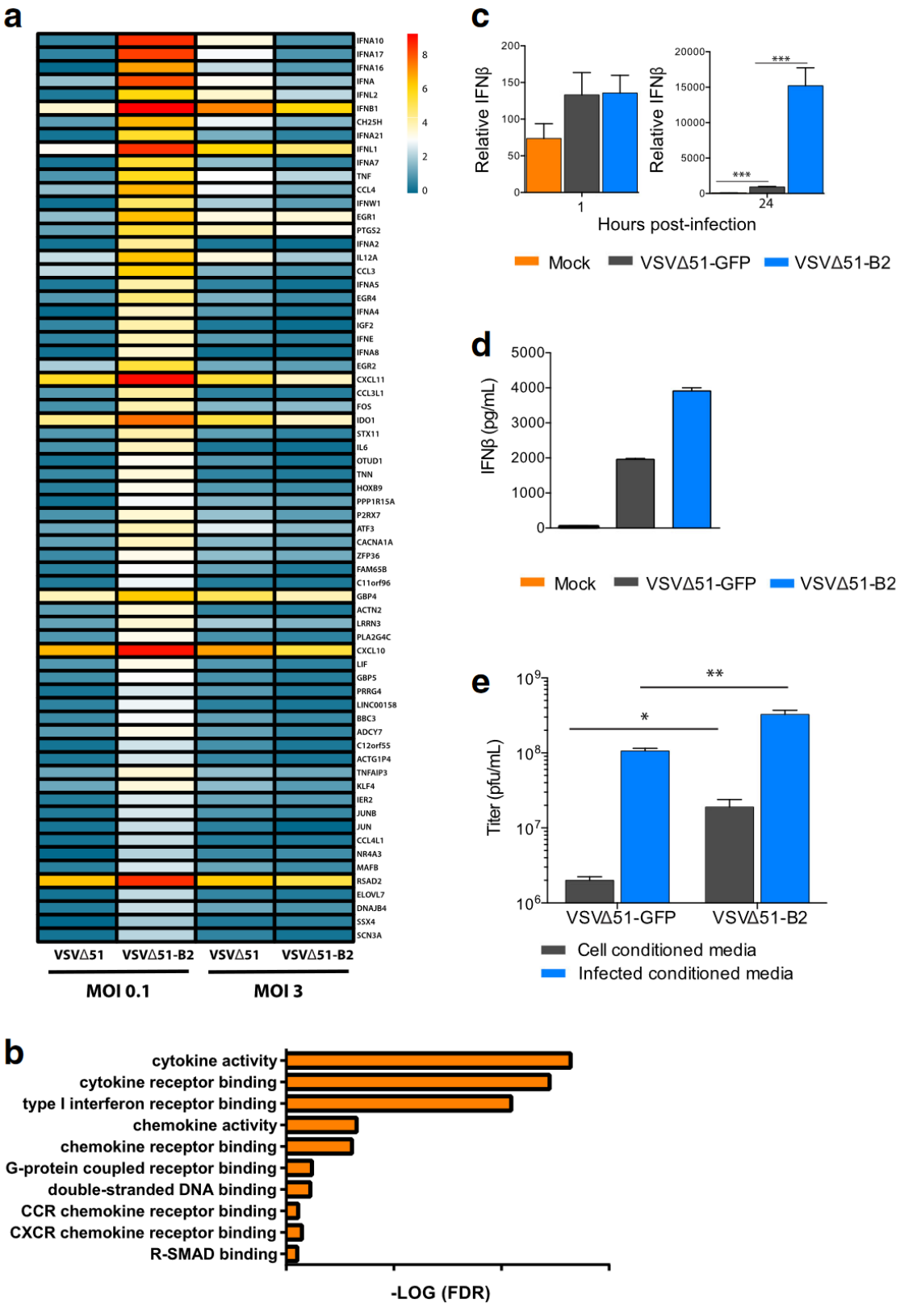

a
IFNA10
IFNA17
IFNA16
IFNA
IFNL2
IFNB1
CH25H
IFNA21
IFNL1
IFNA7
TNF
CCL4
IFNW1
EGR1
PTGS2
IFNA2
IL12A
CCL3
IFNA5
EGR4
IFNA4
IGF2
IFNE
IFNA8
EGR2
CXCL11
CCL3L1
FOS
IDO1
STX11
IL6
OTUD1
TNN
HOXB9
PPP1R15A
P2RX7
ATF3
CACNA1A
ZFP36
FAM65B
C11orf96
GBP4
ACTN2
LRRN3
PLA2G4C
CXCL10
LIF
GBP5
PRRG4
LINC00158
BBC3
ADCY7
C12orf55
ACTG1P4
TNFAIP3
KLF4
IER2
JUNB
JUN
CCL4L1
NR4A3
MAFB
RSAD2
ELOVL7
DNAJB4
SSX4
SCN3A
8
6
4
2
0
VSVΔ51
VSVΔ51-B2
VSVΔ51
VSVΔ51-B2
MOI 0.1
MOI 3
c
Relative IFNβ
200
150
100
50
0
1
Relative IFNβ
20000
15000
10000
5000
0
***
***
24
Hours post-infection
Mock
VSVΔ51-GFP
VSVΔ51-B2
d
IFNβ (pg/mL)
5000
4000
3000
2000
1000
0
Mock
VSVΔ51-GFP
VSVΔ51-B2
e
Titer (pfu/mL)
10^9
10^8
10^7
10^6
**
*
VSVΔ51-GFP
VSVΔ51-B2
Cell conditioned media
Infected conditioned media
b
cytokine activity
cytokine receptor binding
type I interferon receptor binding
chemokine activity
chemokine receptor binding
G-protein coupled receptor binding
double-stranded DNA binding
CCR chemokine receptor binding
CXCR chemokine receptor binding
R-SMAD binding
-LOG (FDR)

Figure 3: VSVΔ51-B2 modulates IFN response and cytokine production.

a) Microarray analysis of M14 cells infected with VSVΔ51-GFP or VSVΔ51-B2 at low and high MOI as indicated.
b) Enrichment of cytokine and cytokine activity in the microarray, associated with an IFN response.
c) qPCR analysis of IFN-β expression of 786-O cells infected for various times. IFN-β levels were normalized to GAPDH levels within each sample.
d) ELISA for IFN-β from supernatants of 786-O cells infected with VSVΔ51-GFP or VSVΔ51-B2 at an MOI of 0.1 for 24 h.
e) Virus outputs of VSVΔ51-GFP and VSVΔ51-B2 obtained from 786-O cells pre-treated with vaccinia Copenhagen virus conditioned-media. Virus-cleared supernatants from HeLa cells that were infected with vaccinia Copenhagen virus at an MOI of 1 for 48 h or left uninfected were transferred onto 786-O cells prior to infection with VSVΔ51-GFP or VSVΔ51-B2 for 48 h. NS: $P > 0.1$, *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test. Only significantly different pairs are indicated on the figure

Given that the IFN response is an important antiviral mechanism in mammalian cells, we investigated the potential impact of B2 on IFN responsiveness by qPCR after infection of 786-O cells. Consistent with our microarray results, we observed a significant increase in IFN-ß levels 24 h post-infection with VSVΔ51-B2 relative to the control virus (Figure 3C). We also showed that VSVΔ51-B2 enhances IFN-ß secretion in 786-O cells, relative to VSVΔ51-GFP, by ELISA (Figure 3D). Lastly, we investigated if the production of VSVΔ51-B2 could be further enhanced by blocking the IFN pathway. We have previously shown that B19R, a soluble type I IFN scavenger expressed by vaccinia Copenhagen virus, enhances VSVΔ51 production [26]. In order to block the antiviral effect of the IFN that would be produced in response to VSVΔ51 infection, we generated conditioned-media from vaccinia virus-infected HeLa cells and pre-treated 786-O cells with B19R-containing media. We found that viral titers were significantly higher for VSVΔ51-B2 with both control media and vaccinia Copenhagen virus conditioned-media compared to VSVΔ51-GFP (Figure 3E); however, the absolute increase in virus titers after exposing the cells to vaccinia Copenhagen virus-conditioned media was similar for both VSVΔ51-GFP and VSVΔ51-B2. Although induction of antiviral RNAi has been shown in mature mammalian cells [27, 28], our data suggest that the RNAi-based pathway is an IFN-independent antiviral mechanism in cancer cells as B2 expression alone enhances viral titers, which can be further enhanced by blocking the IFN response.

Virus-mediated B2 expression enhances replication and cytokine production *in vivo*

To establish an in vivo mouse model, we first screened RENCA mouse renal carcinoma cells in vitro to determine whether VSVΔ51-B2 enhanced cytotoxicity. We showed that,

as observed using human cell lines, RENCA cells were more efficiently killed by the B2-expressing virus (Additional file 1: Figure S2C). In vivo, we subsequently tested the RENCA cell line which is syngeneic to Balb/c mice. In addition, we used the human M14 melanoma cell line as a xenograft model in nude mice. For both models, VSVΔ51-B2 titers from subcutaneous tumours harvested 24 h post-intratumoural viral injection were significantly higher compared to VSVΔ51-GFP (Figure 4A). Consistent with our microarray analysis, the IFN-γ, TNF-α and MCP-1 concentrations from the serum of the RENCA tumour-bearing Balb/c mice were significantly increased for the VSVΔ51-B2 treated mice compared to VSVΔ51-GFP treated mice (Figure 4B). In contrast, Il-6 concentrations did not significantly increase (Figure 4B). Biodistribution analyses following intravenous administration revealed unchanged amounts of virus obtained from the different organs for VSVΔ51 and VSVΔ51-B2 (Additional file 1: Figure S2D-E).

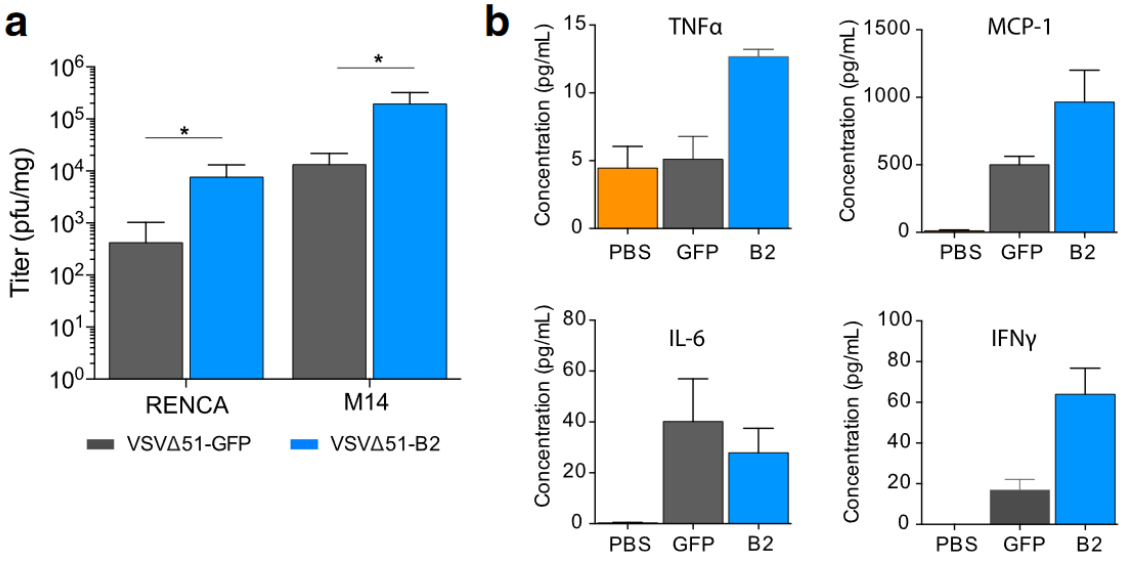

a
Titer (pfu/mg)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
RENCA
M14
VSVΔ51-GFP
VSVΔ51-B2
b
TNFα
Concentration (pg/mL)
15
10
5
0
PBS
GFP
B2
MCP-1
1500
1000
500
0
IL-6
80
60
40
20
IFNγ
100

Figure 4: VSVΔ51-B2 enhances replication and cytokine levels within in vivo tumour models.

a) Viral titers obtained 24 hpi from subcutaneous M14 or RENCA tumours. Virus was administered intratumourally at a dose of 1E9 pfu of VSVΔ51-GFP or VSVΔ51-B2. NS: $P > 0.1$, $*P < 0.1$, $**P < 0.01$, $***P < 0.001$, using Student's t-test. Only significantly different pairs are indicated on the fig.
b) Serum levels of TNF-α, MCP-1, IL-6 and IFN-γ from RENCA tumour-bearing C57BL/6 mice. Virus was administered intratumourally at a dose of 1E9 pfu of VSVΔ51-GFP or VSVΔ51-B2 and serum collected 24 hpi. NS: $P > 0.1$, $*P < 0.1$, $**P < 0.01$, $***P < 0.001$, using Student's t-test. Only significantly different pairs are indicated on the figure.

Discussion

In this study, we demonstrate that B2 expression is sufficient to enhance VSVΔ51 replication and cytotoxicity (Figure 1 and Figure 2) in mammalian cancer cells. Our screen of human cancer cells demonstrated enhanced efficacy of VSVΔ51-B2 in killing the majority of cancer cell lines tested. The enhanced cytotoxic ability of VSVΔ51-B2 suggests that RNAi may be an important factor hindering virus replication in resistant cancer cells. It is important to note that the cell lines in which there was no difference in cytotoxicity were the most sensitive to viral infection (Figure 2), suggesting that the lack of improvement could potentially be attributed to the already maximal virus production by these cells. An antiviral RNAi system may still function in these cells but may only be apparent in certain conditions, such as within the tumour microenvironment where many factors come together and create additional barriers to infection.

Interestingly, virus variants encoding B2 and VP55, two VSRs that impair the RNAi response by different mechanisms, show the same improvement in killing ability for all cell lines tested (Figure 2). The mechanism of B2 involves binding of small RNA fragments which could either prevent their processing by Dicer or loading into RISC. On the other hand, VP55 polyadenylates miRNAs which targets them for degradation [29]. Given that both VSRs improve VSVΔ51-mediated killing to the same extent, this suggests that inhibition of the RNAi pathway improves virus replication regardless of the mechanism through which inhibition is achieved. VP55 does not polyadenylate all small RNAs and key features such as the presence of a 2'O methyl group protect a subset of small RNAs from degradation [29]. Notably, vsRNAs have been previously shown to be 2'O methylated, which protects them from degradation [30]. This may be advantageous for cell lines in which direct viral genome cleavage occurs as ideally,

cleaved genome fragments can provide additional protection through targeting homologous viral genomes and transcripts.

To begin exploring the mechanism of B2 on VSVΔ51 replication, we investigated the potential impact of B2 on the IFN response. A number of cellular proteins such as Toll-like receptor 3, retinoic acid inducible gene I, 2′-5′ oligoadenylate synthetase and protein kinase R recognize dsRNA and trigger a potent antiviral immune response [31]. Therefore, B2 may sequester the dsRNA substrates of these antiviral factors or interact with these proteins to prevent the sensing of dsRNA. As such, we investigated the effects of B2 on the IFN response, which is downstream of these pathways. We demonstrate that B2 does not suppress but actually significantly increases IFN-ß production (Figure 3) compared to control virus, which is likely a result of enhanced replication. More specifically, at low MOI we detect an upregulation of immune genes with VSVΔ51-B2 due to the ability of the VSVΔ51-B2 virus to replicate in M14 cells and establish a successful infection which triggers a more robust IFN response (Figure 3). However, at high MOI we do not see upregulation of immune genes by either VSVΔ51-B2 or VSVΔ51-GFP virus (Figure 3). Higher MOI is often used to overcome resistance to infectivity and often leads to faster cell death, suggesting there was insufficient time to mount a type I IFN response as most, if not all, cells were infected in the first round of replication. Of note, low MOI levels are more comparable to in vivo systems, in which VSVΔ51-B2 virus may be more immunogenic. This prediction is in line with higher cytokine (IFN-γ, TNF-α and MCP-1) levels found in the serum of tumour bearing VSVΔ51-B2 treated immune-competent mice (Figure 4).

Additionally, our vaccinia Copenhagen virus-conditioned media transfer experiments demonstrated that the blocking of type I IFN did not further enhance VSVΔ51-B2 replication compared to VSVΔ51-GFP. This is an indirect way to neutralize IFN-1 [26] and further suggested that IFN and B2 have different mechanisms of action (Figure 3). Consistent with this notion, a similar induction of IFN-stimulated genes was observed in wildtype and RNAi-defective mouse embryonic fibroblasts [27]. In addition, suppression of RNAi by Nodamura virus protein B2 protein does not change the expression levels of IFN-stimulated genes in infected mice [17]. Importantly, our data do not eliminate the possibility of IFN-stimulated miRNAs limiting VSVΔ51 efficacy. Despite stimulating a type I IFN response, VSVΔ51-B2 has enhanced replication, suggesting that B2 expression is sufficient to overcome the antiviral response it stimulates.

One study has shown production of abundant influenza viral siRNAs in IFN-competent A549 cancer cells [27], but the existence of antiviral RNAi in cancer cells remains largely unexplored. We were able to detect viral genome fragments (vsRNAs) following infection with VSVΔ51 in a number of cell lines (Figure 1 and Additional file 1: Figure S1), suggesting that viral genome cleavage is occurring. This may be facilitated by RNAi machinery being repurposed during viral infection. Interestingly, VSVΔ51-B2 infection resulted in a decrease in the percentage of vsRNAs (Figure 2 and Additional file 1: Figure S2), suggesting the efficient prevention of this antiviral mechanism. A number of recent reports support the notion of antiviral genome cleavage in mammalian cells. It has been demonstrated that infection with RNA viruses can trigger the production of viral siRNAs, presumably as a result of direct virus genome

cleavage [17, 32]. The fact that many mammalian viruses encode VSRs, further supports the concept of a mammalian RNAi system. For example, Influenza A encodes the NS1 protein [33, 34], Ebola encodes VP35 [35, 36], HIV-1 encodes Tat [37, 38] and vaccinia Copenhagen virus encodes VP55 [29]. These proteins all have VSR-like functions, which suggests an evolutionary advantage of blocking antiviral RNAi.

It is possible that both direct inhibition of viral genome cleavage and inhibition of cellular antiviral miRNA production are co-occurring within certain cancer cell lines, as we observe changes in both VSV-specific and total RNA read length distributions (Additional file 1: Figure S1 and S2 and data not shown). The potential interplay between viruses and infected host cell miRNAs is a concept that is supported by several studies. For example, miR-29 has been reported to bind the 3' UTR of HIV mRNA which inhibits its translation and results in sequestering the mRNA into processing bodies [39]. IFN-ß itself induces transcription of a number of miRNAs in hepatocytes that are complimentary to the hepatitis C virus genomic RNA and inhibit viral replication [40]. In fact, activation of the IFN pathway has been shown to lead to the upregulation of a number of miRNAs including miR-1 [40–42], miR-129 [43], miR-146 [42] and miR-155 [42, 44, 45], some of which likely function to control infection. Perhaps most relevant to our study, 2 miRNAs (miR-24 and miR-93) have been previously shown to directly target the VSV genome and limit VSV replication [46]. Our results show that VSVΔ51-B2 infection leads to the downregulation of both of these miRNAs, providing a potential explanation for the increased virus production using the B2 virus.

Overall, we demonstrate a novel role of the RNAi pathway as an intrinsic antiviral mechanism in cancer cells and how RNAi inhibition may be used to improve OV replication. Mechanistically, inhibition of direct viral genome cleavage and/or modulation of miRNA processing contribute to enhancing VSVΔ51 infection in a cell line specific manner. This work provides insight into the basic biology of viral defense mechanisms in cancer and promises to improve current OV therapies by tailoring viruses to overcome alternative antiviral mechanisms.

Materials and methods

Cell lines and culture

All cell lines were purchased from the American Type Culture Collections (Manassas, VA). Mammalian cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning cellgro, Manassas, VA) or RPMI-1640 (K562, OVCAR3, OVCAR4, OVCAR8) (Corning cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma life science, St-Louis, MO) and maintained at 37°C with 5% CO2. Drosophila melanogaster Schneider line 2 (S2) cells were cultured in SF900II serum-free medium (Invitrogen) at 25 °C under atmospheric pressures.

DNA constructs and viral constructs

The pcDNA3.1-puro B2 (Nodamura gene) plasmid used for generating the B2-expressing stable cell lines was made available by the Christopher Sullivan lab (Addgene plasmid # 17228). The pEGFP-N1 plasmid (Catalog # 6085–1) was purchased from Clontech (Moutain View, CA).

The B2 gene was amplified by PCR of the Nodamura virus genome. The primers were designed to include the XhoI and NheI restriction sites as well as to insert a 6× histidine tag at the 5′ end of the B2 sequence. The digested PCR fragment was cloned into the XhoI and NheI digested VSVΔ51 backbone, as previously described. The primer pairs for inserting B2 into the VSV backbone are listed in Additional file 2: Table S1.

VP55 was PCR amplified from the Copenhagen strain of vaccinia virus and subcloned into pcDNA3.1 with an N-terminal Flag epitope. Flag-VP55 was subsequently PCR amplified and cloned into VSVΔ51M using the same strategy.

Transfection and selection of cell lines

M14 and 786-O cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Briefly, cells were plated in 6 well format 1 day prior to transfection. Plasmid and Lipofectamine reagent were incubated for 20 min and then added to plated cells in OptiMEM (Thermoscientific, Waltham, MA). 24 h post-transfection, medium was replaced by DMEM with 10% FBS and cultured for 48 h. Cells were then subjected to drug selection by the addition of Geneticin (800 μg/mL) (Thermo Fisher, Carlsbad, CA). Cells were expanded and GFP- or YFP-positive cells were sorted twice by FACS (MoFlo Astrios).

Virus quantification

Viral titers were obtained by plaque assays. Serial dilutions of the samples were prepared in serum-free DMEM. The dilutions were then transferred to monolayers of Vero cells and incubated at 37 °C for 1 h. After the incubation, cells were overlaid with 0.5% agarose in DMEM supplemented with 10% FBS. Plates were incubated for 24 h at 37 °C with 5% CO2 and plaques were counted.

Virus rescue and purification

Virus rescues were performed as previously described. Vero cells were infected with T7 polymerase-expressing vaccinia Copenhagen virus at an MOI of 3. Following a 2 h incubation, media was removed and cells were transfected with T7-driven plasmids

encoding VSV N, P, and L genes as well as the VSVΔ51-B2 backbone. Supernatants collected 48 h post-transfection were passed through a 0.22 μm filter (MillexGP, Carrigtwohill, Ireland) to remove vaccinia Copenhagen virus.

For expansion and purification of the viral preparations, Vero cells were infected at an MOI of 0.001 and culture supernatants were collected 24 h post-infection. Supernatants were then filtered through a 0.2 μm bottle top filter (Millipore, Etobicoke, Canada) and centrifuged at 30,100 g for 90 min. The supernatant was discarded and pelleted virus was resuspended in Dulbecco's phosphate-buffered saline (Corning cellgro, Manassas, VA). Purified virus was kept at − 80°C.

Deep sequencing of vsRNAs

Total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer instructions. Library preparation for Illumina sequencing was performed (TCAG DNA Sequencing Facility, Toronto, ON). Briefly, RNA was enriched for 15–25 nt sizes before strand-specific, small-RNA library preparation and 50 bp single end read sequencing. Adapter trimming was done with Trimmomatic [47] following default parameters. Before read mapping, VSVΔ51 genome was constructed from the VSV reference genome (NC_001560), manually edited to delete the 51st methionine amino acid in the M gene. Reads were mapped to VSVΔ51 genome using bbmap.sh script from the BBMap toolkit (http://sourceforge.net/projects/bbmap) with a minimum alignment identity of 100%. SAMtools was used to separate positive sense mapping from bbmap produced sam files [48]. Lastly, positive sense reads were analyzed for size distribution using the reformat.sh script from the BBMap toolkit.

Western blotting

Cell pellets were lysed on ice for 30 min using complete protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada) supplemented lysis buffer (1% NP40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4). Lysates were centrifuged for 10 min at 16,000 g and cleared supernatants were mixed with dithiothreitol-supplemented loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-Mercaptoethanol, 0.02% bromophenol blue). The samples were migrated on Bio-Rad Mini Protean 4–15% TGX Protein Gels (Bio-Rad, Mississauga, ON) and transferred onto PVDF membranes (GE Healthcare, Buckinghanshire, UK) prior to blocking with 5% skim milk powder (Oxoid Ltd., Basingstoke, UK) in Tris-buffered saline (TBS)with 0.1% Tween-20. The membranes were proofed using specific rabbit antibodies for 6× His tag (Abcam, Cambridge, UK), VSV (polyclonal anti-VSV serum for hyperimmune rabbits) [49]. Rabbit anti-GAPDH (Abcam, Cambridge, UK) and rat anti-tubulin (Novus Biologicals, Oakville, ON) antibodies were used as loading controls. Membranes were then probed with a horse radish peroxidase-coupled goat anti-rabbit secondary antibody (Millipore, Etobicoke, Canada) or goat anti-rat secondary antibody (Life Technologies, Carlsbad, CA) and the signal was revealed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK). The gels were analyzed using FluorChem FC2 (Alpha Innotech, San Leandro, CA).

Cell viability assay

Relative metabolic activity of cells was used as a readout of cell viability and was determined using alamarBlue reagent (Bio-Rad, Mississauga, Ontario, Canada). The assays were performed according to manufacturer instructions. Briefly, cells were plated

in 96-well plates and infected with the different viruses 24 h later. 48 h after virus infection, alamarBlue was added to each well to a final concentration of 1:10. The samples were incubated 1 to 5 h and the fluorescence readings (excitation and emission wavelengths of 530 nm and 590 nm, respectively) were taken using a Fluoroskan Ascent FL (Thermo Labsystems, Beverly, MA).

Quantitative PCR

For miRNA qPCRs, RNA was extracted from infected cell pellets using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer instructions. The RNA concentration and purity was assessed using a NanoDrop ND-1000 spectrophotometer (Thermoscientific, Waltham, MA) prior to reverse transcription using Quanta miRNA cDNA synthesis kit (Gaithersburg, MD).

For all other qPCRs, RNA was extracted using RNAeasy RNA extraction kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer's instructions. The RNA concentration and purity was assessed using a NanoDrop ND-1000 spectrophotometer (Thermoscientific, Waltham, MA) prior to reverse transcription using RevertAid H Minus First Strand cDNA Synthesis kit (Thermoscientific, Waltham, MA).

qRT -PCR was performed on the non-pooled triplicate samples. Following conversion to cDNA by Superscript RT II (Invitrogen, Carlsbad, CA), qRT-PCR was carried out using Sybergreen (Invitrogen) according to the manufacturer's instructions. Analyses were performed on a Rotor-Gene RG-3000A machine (Corbett Research, Mortlake, AU) according to the manufacture instructions. The primer pairs specific for

various gene products used in our experiments are listed in Additional file 2: Table S1. qRT-PCR measurements were normalized to the U6 or GAPDH house-keeping genes for miRNA or RNA transcripts, respectively, using the Pfaffl method [50].

Microarray

Monolayers of M14 cells were treated at an MOI of 0.1 or 3 for 24 h with either VSVΔ51 or VSVΔ51 encoding B2 gene virus Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer instructions. Experimental triplicate total RNA samples were processed by The Center for Applied Genomics at The Hospital for Sick Children for microarray analysis on a Human Prime View chip. Raw files were analyzed using the Transcriptome Analysis Console v3.0 (Affymetrix) software. Normalized transcript expression values further processed with R. Heatmaps were produced using the R package "pheatmap" v1.0.8. GO Term Enrichment analysis (http://CRAN.R-project.org/package=pheatmap) was performed using the online EnrichR tool [51]. Genes selected for enrichment analysis are the subset of genes upregulated by the expression of the B2 gene in VSVΔ51 by at least 4-fold.

ELISA

The concentration of IFN-β was determined using the human IFN- β ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Supernatant transfer experiments

Vaccinia stocks were propagated in U-2 OS cells and cell-associated virus was collected by repeat [3] freeze–thaw cycles. Purification of viral stocks was done by centrifugation at 20,700 g through a 36% sucrose cushion (in 1 mM Tris) before resuspension in 1 mM Tris, pH 9.

To generate infected cell conditioned media, U-2 OS cells were either mock treated or infected with VVdd-mCherry at a multiplicity of 10 PFU/cell for 24 h, harvested and then pelleted by centrifugation. Supernatants were collected and passed through a 0.22 μm filter to eliminate cell-free vaccinia virions. To test for factors enhancing VSV infectivity, tumour cell monolayers were pre-treated for 2 h with conditioned U-2 OS supernatant. Tumour cells were then infected with VSV in the presence of conditioned medium.

In vivo experiments and tumour models

6–8 weeks old female Balb/c or nude mice (Charles River Laboratories, Wilmington, MA) were used. For the Balb/c mice 5 × 105 RENCA tumour cells were implanted subcutaneously 21 days prior to treatment. For the nude mice 1 × 108 M14 tumour cells were implanted subcutaneously 14 days prior to treatment. A single intratumoural injection of 1E8 PFU of VSVΔ51-GFP or VSVΔ51-B2 was performed. Tumours were harvested 24 h post-treatment, weighed and homogenized in PBS using a Powergen 125 tissue homogenizer (Fischer Scientific, Hampton, New Hampshire). Serial dilutions of tumour homogenates were tittered to obtain intratumoural titer data. For biodistribution experiments, naïve Balb/c mice were treated with a single intravenous injection of 1E8 PFU of VSVΔ51-GFP or VSVΔ51-B2. Mice were sacrificed 24 h or 48 h post-treatment and organs were harvested, frozen and homogenized in PBS as above. All experiments

were approved by the University of Ottawa animal care and veterinary services (ME-2258).

Serum was obtained upon centrifugation of blood samples collected using lithium-heparin coated capillary tubes (Sarstedt, Newton) at 16000 g for 5 min. The concentrations of IFNγ, TNFα, MCP-1 and IL-6 were measured using a mouse inflammation cytometric bead array kit (BD Biosciences, San Jose, CA) according to manufacturer instructions. The results were acquired on a LSR Fortessa flow cytometer and analyzed using the FCAP array software (BD Biosciences). Acknowledgements

experiments indicate the genes deleted in CopMD5p3p attenuate viral replication in normal

Acknowledgements

We would like to thank the exceptional technical support of Catia Cemeus, Christiano Tanese de Souza and Julia Petryk, as well as members of the Bell, Auer, Atkins, Ilkow and Diallo laboratories for feedback on this project.

Funding

This work was funded by grants from the Terry Fox Research Foundation and the Canadian Institutes of Health Research to J.C.B. and C.S.I. J.C.B. is also supported by the Ontario Institute for Cancer Research and the Ottawa Regional Cancer Foundation. A.A. is funded by the Canadian Institutes of Health Research Frederick Banting and Charles Best Master's Award. A.P. is funded by the Joseph and Wolf Lebovic Cancer Genomics and Immunity Fellowship and Ontario Graduate Scholarship. L.P. and M.C.B.D. are funded by the Canadian Institutes of Health Research post-doctoral Fellowship.

Availability of data and materials

Deep-sequencing datasets of VSV genome reads generated during the current study are available in the Sequence Read Archive (SRA) repository (Accession number: SRP132124). Microarray data are deposited in the NCBI Gene Expression Omnibus (Accession number: GSE115138).

Authors' contributions

Conceptualization: DB, ASA, AP, LAP, MJFC, MSH, JCB, CSI; Methodology: DB, ASA, AP, LAP, MJFC, MSH, JCB, CSI; Validation: DB, ASA, AP, LAP, MJFC, MSH, JCB, CSI; Formal analysis: DB, ASA, AP, LAP, MJFC, MSH, JCB, CSI.; Investigation: DB, ASA, AP, LAP, MJFC, MSH, JCB, CSI; Resources: DB, MSH; Data curation: DB, ASA, AP, LAP, MCBD; Writing - review & editing: ASA, AP, MJFC, CSI; Visualization: DB, ASA, AP, MJFC, CSI; Supervision: JCB, CSI; Project administration: CSI; Funding acquisition: JCB, CSI. All authors read and approved the final manuscript.

References

1. Lichty BD, Breitbach CJ, Stojdl DF, Bell JC. Going viral with cancer immunotherapy. Nat Rev Cancer. 2014;14(8):559-567.

2. Ilkow CS, Swift SL, Bell JC, Diallo JS. From scourge to cure: tumour-selective viral pathogenesis as a new strategy against cancer. PLoS Pathog. 2014;10(1):e1003836.

3. Arulanandam R, Batenchuk C, Angarita FA, Ottolino-Perry K, Cousineau S, Mottashed A, Burgess E, Falls TJ, De Silva N, Tsang J, Howe GA, Bourgeois-Daigneault MC, Conrad DP, Daneshmand M, Breitbach CJ, et al. VEGF-mediated induction of PRD1-BF1/Blimp1 expression sensitizes tumor vasculature to oncolytic virus infection. Cancer Cell. 2015;28(2):210-224.

4. Bourgeois-Daigneault MC, Roy DG, Aitken AS, El Sayes N, Martin NT, Varette O, Falls T, St-Germain LE, Pelin A, Lichty BD, Stojdl DF, Ungerechts G, Diallo JS, Bell JC. Neoadjuvant oncolytic virotherapy before surgery sensitizes triple-negative breast cancer to immune checkpoint therapy. Sci Transl Med. 2018;10(422)

5. Ilkow CS, Marguerie M, Batenchuk C, Mayer J, Ben Neriah D, Cousineau S, Falls T, Jennings VA, Boileau M, Bellamy D, Bastin D, de Souza CT, Alkayyal A, Zhang J, Le Boeuf F, et al. Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. Nat Med. 2015;21(5):530-536.

6. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014;14(1):36-49.

7. Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S, Marius R, Reynard J, Poliquin L, Atkins H, Brown EG, Durbin RK, Durbin JE, Hiscott J, Bell JC. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell. 2003;4(4):263-275.

8. Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N, Bell JC. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med. 2000;6(7):821-825.

9. Wilson RC, Doudna JA. Molecular mechanisms of RNA interference. Annu Rev Biophys. 2013;42:217-239.

10. Yan N, Chen ZJ. Intrinsic antiviral immunity. Nat Immunol. 2012;13(3):214-222.

11. Jiang L, Wei C, Li Y. Viral suppression of RNA silencing. Sci China Life Sci. 2012;55(2):109-118.

12. Bailey L, Newman JF, Porterfield JS. The multiplication of Nodamura virus in insect and mammalian cell cultures. J Gen Virol. 1975;26(1):15-20.

13. Bailey L, Scott HA. The pathogenicity of Nodamura virus for insects. Nature. 1973;241(5391):545.

14. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834-838.

15. Sullivan CS, Ganem D. A virus-encoded inhibitor that blocks RNA interference in mammalian cells. J Virol. 2005;79(12):7371-7379.

16. Aliyari R, Wu Q, Li HW, Wang XH, Li F, Green LD, Han CS, Li WX, Ding SW. Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in Drosophila. Cell Host Microbe. 2008;4(4):387-397.

17. Li Y, Lu J, Han Y, Fan X, Ding SW. RNA interference functions as an antiviral immunity mechanism in mammals. Science. 2013;342(6155):231-234.

18. Boumahdi S, Driessens G, Lapouge G, Rorive S, Nassar D, Le Mercier M, Delatte B, Caauwe A, Lenglez S, Nkusi E, Brohee S, Salmon I, Dubois C, del Marmol V, Fuks F, et al. SOX2 controls tumour initiation and cancer stem-cell functions in squamous-cell carcinoma. Nature. 2014;511(7508):246-250.

19. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet. 2008;40(5):499-507.

20. Somervaille TC, Matheny CJ, Spencer GJ, Iwasaki M, Rinn JL, Witten DM, Chang HY, Shurtleff SA, Downing JR, Cleary ML. Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. Cell Stem Cell. 2009;4(2):129-140.

21. Schoenhals M, Kassambara A, De Vos J, Hose D, Moreaux J, Klein B. Embryonic stem cell markers expression in cancers. Biochem Biophys Res Commun. 2009;383(2):157-162.

22. Mizuno H, Spike BT, Wahl GM, Levine AJ. Inactivation of p53 in breast cancers correlates with stem cell transcriptional signatures. Proc Natl Acad Sci U S A. 2010;107(52):22745-22750.

23. Bourgeois-Daigneault MC, Roy DG, Falls T, Twumasi-Boateng K, St-Germain LE, Marguerie M, Garcia V, Selman M, Jennings VA, Pettigrew J, Amos S, Diallo JS, Nelson B, Bell JC. Oncolytic vesicular stomatitis virus expressing interferon-gamma has enhanced therapeutic activity. Mol Ther Oncolytics. 2016;3:16001.

24. Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR. Dual modes of RNA-silencing suppression by flock house virus protein B2. Nat Struct Mol Biol. 2005;12(11):952-957.

25. Johnson KL, Price BD, Eckerle LD, Ball LA. Nodamura virus nonstructural protein B2 can enhance viral RNA accumulation in both mammalian and insect cells. J Virol. 2004;78(12):6698-6704.

26. Le Boeuf F, Diallo JS, McCart JA, Thorne S, Falls T, Stanford M, Kanji F, Auer R, Brown CW, Lichty BD, Parato K, Atkins H, Kirn D, Bell JC. Synergistic interaction between oncolytic viruses augments tumor killing. Mol Ther. 2010;18(5):888-895.

27. Li Y, Basavappa M, Lu J, Dong S, Cronkite DA, Prior JT, Reinecker HC, Hertzog P, Han Y, Li WX, Cheloufi S, Karginov FV, Ding SW, Jeffrey KL. Induction and suppression of antiviral RNA interference by influenza a virus in mammalian cells. Nat Microbiol. 2016;2:16250.

28. Qiu Y, Xu Y, Zhang Y, Zhou H, Deng YQ, Li XF, Miao M, Zhang Q, Zhong B, Hu Y, Zhang FC, Wu L, Qin CF, Zhou X. Human virus-derived small RNAs can confer antiviral immunity in mammals. Immunity. 2017;46(6):992-1004.

29. Backes S, Shapiro JS, Sabin LR, Pham AM, Reyes I, Moss B, Cherry S, tenOever BR. Degradation of host microRNAs by poxvirus poly(a) polymerase reveals terminal RNA methylation as a protective antiviral mechanism. Cell Host Microbe. 2012;12(2):200-210.

30. Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Zust R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, et al. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature. 2010;468(7322):452-456.

31. Gantier MP, Williams BR. The response of mammalian cells to double-stranded RNA. Cytokine Growth Factor Rev. 2007;18(5-6):363-371.

32. Maillard PV, Ciaudo C, Marchais A, Li Y, Jay F, Ding SW, Voinnet O. Antiviral RNA interference in mammalian cells. Science. 2013;342(6155):235-238.

33. Lu Y, Wambach M, Katze MG, Krug RM. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. Virology. 1995;214(1):222-228.

34. Bucher E, Hemmes H, de Haan P, Goldbach R, Prins M. The influenza a virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. J Gen Virol. 2004;85(Pt 4):983-991.

35. Cardenas WB, Loo YM, Gale M, Jr, Hartman AL, Kimberlin CR, Martinez-Sobrido L, Saphire EO, Basler CF. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. J Virol. 2006;80(11):5168-5178.

36. Haasnoot J, de Vries W, Geutjes EJ, Prins M, de Haan P, Berkhout B. The Ebola virus VP35 protein is a suppressor of RNA silencing. PLoS Pathog. 2007;3(6):e86.

37. Bennasser Y, Le SY, Benkirane M, Jeang KT. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity. 2005;22(5):607-619.

38. Bennasser Y, Jeang KT. HIV-1 tat interaction with dicer: requirement for RNA. Retrovirology. 2006;3:95.

39. Nathans R, Chu CY, Serquina AK, Lu CC, Cao H, Rana TM. Cellular microRNA and P bodies modulate host-HIV-1 interactions. Mol Cell. 2009;34(6):696-709.

40. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature. 2007;449(7164):919-922.

41. Scagnolari C, Zingariello P, Vecchiet J, Selvaggi C, Racciatti D, Taliani G, Riva E, Pizzigallo E, Antonelli G. Differential expression of interferon-induced microRNAs in patients with chronic hepatitis C virus infection treated with pegylated interferon alpha. Virol J. 2010;7:311.

42. Bruni R, Marcantonio C, Tritarelli E, Tataseo P, Stellacci E, Costantino A, Villano U, Battistini A, Ciccaglione AR. An integrated approach identifies IFN-regulated microRNAs and targeted mRNAs modulated by different HCV replicon clones. BMC Genomics. 2011;12:485.

43. Zhang J, Li S, Yan Q, Chen X, Yang Y, Liu X, Wan X. Interferon-beta induced microRNA-129-5p down-regulates HPV-18 E6 and E7 viral gene expression by targeting SP1 in cervical cancer cells. PLoS One. 2013;8(12):e81366.

44. Zhang J, Zhao H, Chen J, Xia B, Jin Y, Wei W, Shen J, Huang Y. Interferon-beta-induced miR-155 inhibits osteoclast differentiation by targeting SOCS1 and MITF. FEBS Lett. 2012;586(19):3255-3262.

45. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007;104(5):1604-1609.

46. Otsuka M, Jing Q, Georgel P, New L, Chen J, Mols J, Kang YJ, Jiang Z, Du X, Cook R, Das SC, Pattnaik AK, Beutler B, Han J. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity. 2007;27(1):123-134.

47. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-2120.

48. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. Genome project data processing S. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25(16):2078-2079.

49. Roy DG, Power AT, Bourgeois-Daigneault MC, Falls T, Ferreira L, Stern A, Tanese de Souza C, McCart JA, Stojdl DF, Lichty BD, Atkins H, Auer RC, Bell JC, Le Boeuf F.

Programmable insect cell carriers for systemic delivery of integrated cancer biotherapy. J Control Release. 2015;220(Pt A):210-221.

50. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45.

51. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, Koplev S, Jenkins SL, Jagodnik KM, Lachmann A, McDermott MG, Monteiro CD, Gundersen GW, Ma'ayan A. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44(W1):W90-W97.

Rights and Permissions

The following details the specific rights and permissions needed for the reproduction of published material in this thesis.

Chapter 2

Rights and permission for reprint and use in this thesis of this published article were obtained from American Society of Gene & Cell Therapy (ASGCT) via an email communication. No changes to the original content of the article were made.

Appendix A3

Authors: Donald Bastin, Amelia S. Aitken, Adrian Pelin, Larissa A. Pikor, Mathieu J. F. Crupi, Michael S. Huh, Marie-Claude Bourgeois-Daigneault, John C. Bell and Carolina S. Ilkow.

Title: Enhanced susceptibility of cancer cells to oncolytic rhabdo-virotherapy by expression of Nodamura virus protein B2 as a suppressor of RNA interference.

Publication Source: Journal for immunotherapy of cancer 6 (1), 62

Curriculum Vitae

Adrian Pelin

PhD candidate in Biochemistry
Specialization in Human and Molecular Genetics
Bioengineering Vaccinia Viruses for increased Oncolytic Potential
Department of Biochemistry, Faculty of Medicine, University of Ottawa.

EDUCATION

2019 (expected)	**Doctor of Philosophy,** Biochemistry, Specialization in Human and Molecular Genetics Advisor: Dr. John Bell
Spring 2015	**Masters of Science,** Biology, Specialization in Bioinformatics**,** University of Ottawa Advisor: Dr. Nicolas Corradi
Spring 2013	**Bachelor of Science,** Specialization in Biochemistry**,** University of Ottawa

WORK EXPERIENCE

Graduate PhD Student May 2015 – present
Bell Lab - Ottawa Hospital Research Institute

- Inventor on three provisional patents describing modified Vaccinia viruses serving as oncolytic agents expressing several therapeutic transgenes.
- Discovered, bioengineered and characterized a novel Vaccinia virus which has been licensed to Turnstone Biologics for pre-clinical studies and clinical trials.
- Involved in pre-clinical study designs as well as drafting an FDA pre-IND briefing document.
- Co-author on 6 publications.

Graduate MSc Student September 2013 - May 2015
Corradi Lab - University of Ottawa

- Masters project on Population Genomics of *Nosema ceranae*; Analysis of 9 geographically distinct isolates infecting the European Honeybee.
- Phylogenetic reconstruction and analysis of Microsporidian Transposons and recent Horizontal Gene Transfers.
- Species description and phylogentic analysis of *Agmasoma penaei*, microsporidian infecting white shrimp.
- First author on 4 and co-author on 10 publications.

COOP/Summer Student: Bioinformatics May 2011 - August 2013
Corradi Lab - University of Ottawa

- Worked on the genomics of:
  - Arbuscular Mycorrhizal Fungus *Gigaspora margarita* mitochondria (Plant symbiont).

- First sequenced Cryptomycota member *Rozella allomycis*.

- Linked different contigs using bioinformatic software Consed and Geneious.
- Inferred phylogenetic relationships of basal fungal taxa using Bayesian and Maximum Likelihood analysis.
- Practiced traditional molecular methods including DNA/RNA extraction, PCR and Molecular cloning.

COOP Student - PCB Program January 2011 - August 2013
Environment Canada

- Provided support over the phone to regulatees (corporations subject to Canadian regulations) in their on-line submission of annual reports.
- Helped plan the development of a new database. Verified recent data submissions with data from older databases.
- Conducted presentations during meetings with regulatees, compliance promotion officers and enforcement officers. Prepared guides and fact sheets to be posted on the EC Website.

ACCOMPLISHMENTS

Publications

1. Adrian Pelin, Johann Foloppe, Julia Petryk, Marianne Hussein, Florian Gossart, Victoria Jennings, Lawton Stubbert, Maddie Foster, Chris Storbeck, Antonio Postigo, Elena Scut, Brian Laight, Michael Way, Philippe Erbs, Fabrice Le Boeuf and John C. Bell. **2019**. Targeting apoptosis inhibitor F1L in a Vaccinia virus increases safety and oncolysis for intravenous brain cancer therapy. **In press.** ***Molecular Therapy Oncolytics.***

2. Chadi Zakaria, Polen Sean, Huy-Dung Hoang, Louis-Phillipe Leroux, Margaret Watson, Samuel Tekeste Workenhe, Jaclyn Hearnden, Dana Pearl, Vinh Tai Truong, Nathaniel Robichaud, Akiko Yanagiya, Soroush Tahmasebi, Seyed Mehdi Jafarnejad, Jian-Jun Jia, Adrian Pelin, Jean-Simon Diallo, Fabrice Le Boeuf, John Cameron Bell, Karen Louise Mossman, Tyson Ernst Graber, Maritza Jaramillo, Nahum Sonenberg, Tommy Alain. **2018**. Active-site mTOR inhibitors augment HSV1-dICP0 infection in cancer cells via dysregulated eIF4E/4E-BP axis. ***PLoS Pathogens.*** 14 (8)

3. María C Rosales Gerpe, Jacob P van Vloten, Lisa A Santry, Jondavid de Jong, Robert C Mould, Adrian Pelin, John C Bell, Byram W Bridle, Sarah K Wootton (2018). Use of Precision-Cut Lung Slices as an Ex Vivo Tool for Evaluating Viruses and Viral Vectors for Gene and Oncolytic Therapy. ***Molecular Therapy-Methods & Clinical Development.*** 10, 245-256

4. Adrian Pelin, Jiahu Wang, John Bell, Fabrice Le Boeuf. **2018**. The importance of imaging strategies for pre-clinical and clinical in vivo distribution of oncolytic viruses. ***Oncolytic virotherapy***. 7, 25

5. Donald Bastin, Amelia S. Aitken, Adrian Pelin, Larissa A. Pikor, Mathieu J. F. Crupi, Michael S. Huh, Marie-Claude Bourgeois-Daigneault, John C. Bell and Carolina S. Ilkow. **2018**. Enhanced susceptibility of cancer cells to oncolytic rhabdo-virotherapy by expression of Nodamura virus protein B2 as a suppressor of RNA interference. ***Journal for ImmunoTherapy of Cancer.*** 6 (1), 62

6. Marie-Claude Bourgeois-Daigneault, Dominic Guy Roy, Amelia S. Aitken, Nader El Sayes, Nikolas Tim Martin, Oliver Varette, Theresa Falls, Lauren Elizabeth St-Germain, Adrian Pelin, Brian Dennis Lichty, David Francis Stojdl, Guy Ungerechts, Jean-Simon Diallo, John Cameron Bell. **2018**. Neoadjuvant oncolytic virotherapy before surgery sensitizes triple-negative breast cancer to immune checkpoint therapy. ***Science translational medicine.*** 10 (422)

7. Steven R Ahrendt, C Alisha Quandt, Doina Ciobanu, Alicia Clum, Asaf Salamov, Bill Andreopoulos, Jan-Fang Cheng, Tanja Woyke, Adrian Pelin, Bernard Henrissat, Nicole K Reynolds, Gerald L Benny, Matthew E Smith, Timothy Y James, Igor V Grigoriev. **2018**. Leveraging single-cell genomics to expand the fungal tree of life. ***Nature microbiology.*** 3 (12), 1417

8. Emmanuelle Morin, Shingo Miyauchi, Hélène San Clemente, Eric CH Chen, Adrian Pelin, Ivan de la Providencia, Steve Ndikumana, Denis Beaudet, Mathieu Hainaut, Elodie Drula, Alan Kuo, Nianwu Tang, Sébastien Roy, Julie Viala, Bernard Henrissat, Igor V Grigoriev, Nicolas Corradi, Christophe Roux, Francis M Martin. **2018**. Comparative genomics of *Rhizophagus irregularis*, *R. cerebriforme*, *R. diaphanus* and *Gigaspora rosea* highlights specific genetic features in Glomeromycotina. **Accepted** - ***New Phytologist.***

9. Melissa J Peters, Guntima Suwannapong, Adrian Pelin, Nicolas Corradi. **2018**. Genetic and Genome Analyses Reveal Genetically Distinct Populations of the Bee Pathogen *Nosema ceranae* from Thailand. ***Microbial ecology.*** 1-13

10. Eric CH Chen, Stephanie Mathieu, Anne Hoffrichter, Kinga Sedzielewska-Toro, Max Peart, Adrian Pelin, Steve Ndikumana, Jeanne Ropars, Steven Dreissig, Jorg Fuchs, Andreas Brachmann, Nicolas Corradi. **2018**. Single nucleus sequencing reveals evidence of inter-nucleus recombination in arbuscular mycorrhizal fungi. ***eLife.*** Vol. 7

11. Dillon F Da Fonte, Christopher J Martyniuk, Lei Xing, Adrian Pelin, Nicolas Corradi, Wei Hu, Vance L Trudeau. **2017**. Secretoneurin A regulates neurogenic and inflammatory transcriptional networks in goldfish (Carassius auratus) radial glia. ***Scientific Reports.*** 7 (1), 14930

12. Kevin L. Schauer, Christophe M. R. LeMoine, Adrian Pelin, Nicolas Corradi, Wesley Warren, Martin Grosell. **2016**. A proteinaceous organic

matrix regulates carbonate mineral production in the marine teleost intestine. ***Scientific Reports.*** 6, 34494

13. Steve Ndikumana, Adrian Pelin, Alex Williot, Justin L Sanders, Michael Kent, Nicolas Corradi. **2016**. Genome Analysis of Pseudoloma neurophilia: a Microsporidian Parasite of Zebrafish (Danio rerio). ***Journal of Eukaryotic Microbiology.*** 64 (1), 18-30

14. Marie-Claude Bourgeois-Daigneault; Lauren Elizabeth St-Germain; Dominic Guy Roy; Adrian Pelin; Amelia Sadie Aitken; Rozanne Arulanandam; Theresa Falls; Vanessa Garcia; Jean-Simon Diallo; John Cameron Bell. **2016**. Combination of Paclitaxel and MG1 oncolytic virus as a successful strategy for breast cancer treatment. ***Breast Cancer Research.*** 18 (1), 83

15. Ropars J., Sędzielewska K., Noel J., Pelin A., Charron P., Farinelli L., Marton T., Krüger M., Fuchs J., Brachmann A. and Corradi N. **2016**. Evidence for a sexual origin of heterokaryosis in a supposedly ancient asexual fungus. ***Nature - Microbiology.*** 1 (6), 16033

16. Pelin A., Moteshareie H., Sak B., Selman M., Naor A., Eyahpaise M-È., Farinelli L., Golshani A., Kvac M. & Corradi N. **2015**. The genome of an *Encephalitozoon cuniculi* type III strain reveals insights into the genetic diversity and mode of reproduction of a ubiquitous vertebrate pathogen. ***Heredity.*** 116 (5), 458

17. Pelin, A., Selman, M., Aris-Brosou, S., Farinelli, L., & Corradi, N. **2015**. Genome analyses suggest the presence of polyploidy and recent human-driven expansions in eight global populations of the honeybee pathogen *Nosema ceranae*. ***Environmental microbiology***. 17 (11), 4443-4458

18. Sokolova Y., Pelin A., Hawke J.and Corradi N. **2014.** Morphology and phylogeny of *Agmasoma penae* (Microsporidia) from white Atlantic shrimp *Litopenaeus setiferus*. ***International Journal of Parasitology*.** 45 (1), 1-16

19. Parisot N.*, Pelin A.*, Gasc C., Polonais V., Belkorchia A., Panek J.,El Alaoui H., Biron D.G., Brasset E., Vaury C., Peyret P., Corradi N.$^{+}$, Peyretaillade E.$^{+}$ and E. Lerat $^{+}$. **2014**. Microsporidian genomes harbour a diverse array of transposable elements that demonstrate an ancestry of horizontal exchange with metazoan. ***Genome Biology and Evolution***. 6 (9): 2289-2300. *Contributed equally. $^{+}$ Co-Corresponding authors.

20. James T.Y., Pelin A., Bonen L., Ahrendt S., Sain D., Corradi N. and J.E. Stajich. **2013**. Shared signatures of parasitism and phylogenomics unite the Cryptomycota with the Microsporidia. ***Current Biology***. 23 (16), 1548–1553

21. Pelin A.*, Pombert J.F.*, Salvioli A., Bonen L., Bonfante P. and Corradi N. **2012.**The mitochondrial genome of the arbuscular mycorrhizal fungus

Gigaspora margarita reveals two unsuspected trans-splicing events of group I introns. ***New Phytologist.*** 194 (3) 836-845. *Contributed equally;

Patents

1. **Modified Poxvirus Vectors,** US #62/614,349, filling date January 5th, 2018
2. **Modified Poxvirus Vectors,** US #62/614,350, filling date January 5th, 2018
3. **Modified Poxvirus Vectors,** US #62/614,745, filling date July 3rd, 2018

Awards

1. **The Joseph and Wolf Lebovic Cancer Genomics and Immunity Fellowship -75000$**

Institute for Medical Research Israel-Canada funding for Graduate Studies 2017 and 2018.

2. **Ontario Graduate Scholarship - 45,000$**

University of Ottawa, 2016/5 - 2019/4

3. **BioCanRx HQP Travel Award – 3,000$**

Summit for Cancer Immunotherapy, 2016, 2017 and 2018

4. **CBW Registration Award – 950$**

Canadian Bioinformatic Workshops, 2016/5

5. **Travel Awards - Institute Community Support - 2,750$**

Canadian Institutes of Health Research, 2016/4, 2017/10 and 2018/11

6. **Admission Scholarship - 36,000$**

University of Ottawa, 2015/9 - 2019/9

7. **Dean's scholarships - 1,500$**

University of Ottawa, 2015/6

Mentorship

1. **Jessie Duong**, Graduate Summer Student, University of Ottawa

Project: Charachterization of major deletions in Vaccinia virus

2. **Mariam Maltseva**, Undergraduate Summer Student, University of Ottawa

Project: Assessing cytotoxicity of chimeric viruses

3. **Justin Cowen**, Undergraduate Summer Student, University of Queens Ottawa

Project: Creating new recombinant Vaccinia viruses expressing immune stimulating transgenes

4. **Brian Laight,** Undergraduate Summer Student, University of Ottawa

Project: Fitness analysis of various Vaccinia strains

5. **Haodong Zhou**, Undergraduate Student, Summer Volunteer, University of Ottawa

Project: Assembly and annotation of microsporidian species.

6. **Marie-Eve Eyahpaise**, Undergraduate Student, Honours Project, University of Ottawa

Project: Evolution of *Encephalitozoon cuniculi* parasites through genome sequencing.

7. **Aaron Naor**, Undergraduate Student, Summer Volunteer, UBC

Project: Variant discovery in *Encephalitozoon cuniculi* spores.

8. **Laura Richards**, Undergraduate Student, UROP, University of Ottawa

Project: In vitro recombination mediated by PCR template switching

Conferences Attended

1. **AACR Tumor Immunology and Immunotherapy**

Miami Beach, US, 2018
Oral Presentation: Utilizing novel oncolytic vaccinia virus for selective expression of immunotherapeutic payloads in metastatic tumors

2. **Summit for Cancer Immunotherapy**
Banff, Canada, 2018
Oral Presentation: Poxvirus genome editing in the development of a safe and multi-purpose immunotherapeutic platform

3. **Canadian Society of Virologists**
Halifax, Canada, 2018
Poster Presentation: Redundant genes in Vaccinia virus

4. **Ottawa Hospital Department of Medicine Research Day**
Ottawa, Canada, 2018
Oral Presentation: Engineering a novel oncolytic Vaccinia

5. **11th International Oncolytic Virus Meeting**
London, UK, 2018
Poster Presentation: Large genomic deletions of a novel oncolytic Vaccinia Virus increase safety, efficacy and anti-tumour immunogenicity

6. **Summit for Cancer Immunotherapy**
Gatineau, Canada, 2017
Oral Presentation: Large genomic deletions of a novel oncolytic Vaccinia Virus (OncoVac) increase safety, efficacy and anti-tumour immunogenicity

7. **Keystone Symposia. Cancer Immunology and Immunotherapy**
Whistler, Canada, 2017
Poster Presentation: Large genomic deletions of a novel oncolytic Vaccinia Virus increase anti-tumour immunogenicity

8. **10th International Oncolytic Virus Meeting**
Vancouver, Canada, 2016
Poster Presentation: Bioengineering an optimal oncolytic Vaccinia virus variant for cancer therapy.

9. **Summit for Cancer Immunotherapy**
Halifax, Canada, 2016
Poster Presentation: Bioengineering an optimal oncolytic Vaccinia virus variant with important deletions of multiple immunosuppressive genes for efficient viro-immunotherapy.

10. **XXI International Poxvirus, Asfarvirus and Iridovirus Conference**
France, 2016
Oral Presentation: Bioengineering an optimal oncolytic Vaccinia virus variant for cancer therapy.

11. **9th International Conference on Oncolytic Virus Therapeutics**
Boston, MA 2015

12. **Gordon Research Conference**, Cellular & Molecular Fungal Biology
Holderness, NH, USA, 2014
Poster presentation: Genome wide analysis of inter and intra isolate variation suggests a recent and rapid clonal expansion of the western honey bee pathogen *Nosema ceranae* globally

13. **Canadian Institute for Advanced Research**, Integrated Microbial Biodiversity
Whistler, BC, Canada, 2013
Poster presentation: The mitochondrial genome of *Rozella allomycis*: on the edge of getting lost forever?

Professional Workshops Attended

1. **Canadian Bioinformatics Workshops** – Toronto, Canada, 2018
Novel methods in RNA-seq Analysis
2. **Canadian Center for Computational Genomics (C3G)**– Montreal, Canada, 2017
Genomic virology workshop
3. **Canadian Bioinformatics Workshops** – Toronto, Canada, 2016
Cancer Genomics
4. **Canadian Bioinformatics Workshops** – Toronto, Canada, 2015
Informatics on High-Throughput Sequencing Data
5. **Canadian Bioinformatics Workshops** – Montreal, Canada, 2014
Informatics for RNA-seq Analysis

SKILLS

Informatics

Bioinformatics

- Expertise in Genome Assembly and analysis using a variety of methods.
- Proficiency in RNA-Seq analysis, including differential transcript expression as well as transcript prediction.
- Worked on fungal, human and viral DNA-Seq and RNA-Seq experiments.
- Experience in handling microarray data.

Linux

- Experienced in Linux Systems for over 10 years. Operating systems include: Fedora, CentOS and Ubuntu.
- Installation of various programs including software for Bioinformatic Analysis.
- System administration of servers and resource allocation

Programs and Software

- Used and extensive array of bioinformatical programs such as: Geneious, Consed, PhyML, Phylobayes, samtools, FreeBayes, etc.
- Proficient in using R, a program for basic statistics, regression analysis, tables and graphics.
- Have designed multiple scripts in Tcl, perl and R for file manipulation.

Wet lab work

Molecular

- DNA and RNA Purification.
- PCR and qPCR analysis.
- Cloning into and from various vectors. Worked with bacterial and mammalian expression vectors.

Tissue Culture

- Experience in sterile culturing both normal cells from patient donors and cancer cells (worked with NCI-60).
- Propagation of cells in both FBS and NCS.
- Worked with both suspension and adherent cells from multiple organisms.
- Have performed clonal isolation.

Virology

- Worked with Vaccinia (and other poxviruses) virus, Rhabdovirus (VSV and Maraba), Measles virus, Adenovirus and HSV.
- Can expand and quantify (plaque assay and qPCR).
- Experience with engineering of recombinant viruses expressing transgenes. Created more than 20 new Poxviruses and 2 new Rhabdovirus expressing transgenes (Anti-CTLA4 Ab, OX40L, CD40L, IL12, etc.).
- Have induced genomic deletions and recombination in various poxviruses.

In vivo work

- Worked with BalbC (849 mice), C57BL/6 (398 mice) and Nude CD1 (188 mice). Several experiments with DBA/2 and NON-SCID mice.
- Experience with seeding tumours SubQ and intra-peritoneally several models (CT26, 4T1, B16, MC38, EMT6, HT-29, MiaPaca2 and HeLa). Validated transgene activity with qPCR, Western Blotting as well as other immune assays.
- Have treated mice with oncolytic virus intra-tumourally or intra-peritoneally.
- Have performed mouse dissections and bio-distribution of viruses in 12 organs.
- Some experience with saph bleeds.

Immunology

- Performed cytokine arrays and RNA-seq on human and murine PBMCs.
- Basic experience with flow-cytometry. Performed a 5-colour experiment to test CD8 T cell antigen (OVA, DCT) recognition after heterologous prime/boost using various oncolytic viruses.
- Performed several experiments using ELISPOT and ELISA techniques.
- Have computationally identified tumour neo-epitopes and tested spleen activation with synthetic peptides.

Social

Hobbies and Interests

- Languages: Fluent in English, French, Romanian and Russian.
- Traveled extensively within Europe and North America; lived in three countries: Canada, United States and Moldova.

Team Work

- VP-Finance, Biology Graduate Student Association, University of Ottawa
- Enjoyed playing team sports. Played water polo for 4 years. Teams: CAMO Quebec, DDO Montreal and Mavericks Toronto.