

# **ENHANCING ONCOLYTIC VACCINIA VIROTHERAPY USING FUNCTIONAL GENOMIC SCREENING**

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Thesis submitted to the University of Ottawa  
in partial fulfillment of the requirements for the  
Doctorate of Philosophy in Biochemistry

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## ABSTRACT

The treatment of cancer patients with oncolytic vaccinia virotherapy has yielded encouraging results in early phase clinical trials, but there are yet unexplored opportunities for improving this therapy that could lead to even better outcomes for patients. To this end, we harnessed the power of functional genomic screening to identify both host and vaccinia virus (VV) genes that could be manipulated to enhance the efficacy of VV. We hypothesized that one approach to enhancing the efficacy of VV was to increase its ability to spread in tumours by manipulating host genes. We conducted human genome-wide RNAi screens in three tumour cell lines and identified 237 genes that upon silencing enhanced spread in two out of the three cells lines tested and 23 across all three cell lines. Muscle RAS oncogene homolog (MRAS) emerged as a promising candidate following *in vitro* validation studies. To test the impact of inhibiting this gene *in vivo*, we engineered an oncolytic VV encoding a dominant negative of MRAS. The treatment of tumour-bearing mice with this virus significantly delayed tumour progression, prolonged survival and resulted in durable responses. As a parallel approach to enhancing the therapy, we also sought to discover alternative means of conferring VV tumour specificity that would lead to a larger therapeutic index than that of the current clinical candidates that rely on deletions of thymidine kinase and/or vaccinia growth factor. We conducted RNAi VV genome-wide screens in two tumour cell lines to identify VV genes that could be deleted without diminishing its potency in tumour cells but would be concomitantly attenuating in normal cells. We identified 35 VV gene candidates and engineered deletion mutants of 13 of these. We found a VV with the DNA ligase gene deleted to be as attenuating as the current clinical

candidate vvDD in normal cells, but more potent in multiple tumour cell lines and provided a therapeutic advantage *in vivo*. This work serves as a valuable resource for designing the next generation of oncolytic VVs and showcases functional genomic screening as a powerful tool for identifying genes that can be manipulated to potentially improve clinical outcomes.

## **ACKNOWLEDGEMENTS**

I would first like to express my appreciation to Dr. David Stojdl for giving me the tremendous opportunity to work in his lab first as a co-op student and later as a graduate student. When I was first offered a job to work in his lab as a co-op student, I was very torn between a number of job opportunities. I ended up choosing to work in the Stojdl lab primarily because I thought it would be the most challenging but, at the same time, the most intellectually stimulating and fulfilling, and would likely give me the greatest opportunity to work on the greatest number of things. It has been all this and more.

I have so many other people to thank. I have had the great fortune to work and interact with so many incredible people both in the Stojdl lab and outside. I do not think it is possible to thank everyone who has contributed to this work because there are so many who have contributed even just by giving a tip, sharing a protocol, providing an aliquot of reagent, or taking the time out of their busy schedules to share their knowledge and expertise, and offer advice.

I would like to specifically mention by name those who have directly contributed to the work described in this thesis. First, I would like to thank Dr. Stephen Baird who assisted with many of the high-throughput screening aspects including taking calls from me on the weekend with my asking for help on fixing equipment issues. A big thanks goes to Charles Lefebvre, the King of Cloning among many things, who helped seed cells for all of the high throughput screens, provided expert advice for my cloning work and was an all-around knowledgeable resource for all things lab related with his wealth of experience. I learned from the best! Many thanks to Katelynn Rowe for being



a great encourager, for being someone to discuss ideas with and for her initial help with the mouse work including teaching me how to implant tumours, perform various injections and harvest tissues. Many thanks also to Melanie Labelle and Dr. Stephanie Swift, who pitched in when extra help was needed, in particular helping with the mouse work. An extra special thanks goes to Dr. Justyna Kmeicik for being a great mentor and for all her support, encouragement and input, especially as I was writing up, and for also helping me with the mouse work. I would like to very much thank her, Dr. Stephanie Pontier and Dr. Sebastien Delpet for taking the time to read over all or parts of this thesis and providing apt and constructive feedback. I am also thankful to all of the Stojdl lab members who may not have contributed directly to this work but with whom I had the opportunity to work alongside and learn from.

I also cannot help but mention the people I was fortunate to work alongside at CHEO, principally members of the Alain, Cowan, and Kumar labs. I would be remiss in not specifically thanking Lynn Kyte. You could not find a better lab manager. I cannot count all the times she tracked down reagents for me and tried to find the best deals on supplies and reagents. Also, thanks to Keith Wilson for taking so much pride in his work in keeping the TC rooms and labs stocked and humming.

My greatest thanks go to my friends and family, especially my parents, Frank and Jean, and my four siblings Juanita, Ryan, Rodney and Byron and their spouses Steve, Chantal, Jessie and Kim. They more than anyone know how hard-fought this journey has been, especially given my health challenges. I could not have made it without their love, encouragement, and support. Ultimately, I cannot help but thank God for giving me the health and strength to make it to the end of this journey.

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## LIST OF ABBREVIATIONS

|                   |                                                          |
|-------------------|----------------------------------------------------------|
| 5-FC              | 5-fluorocytosine                                         |
| 5-FU              | 5-fluorouracil                                           |
| AGO2              | Argonaute 2                                              |
| ANOVA             | Analysis of variance                                     |
| ARAF              | A-Raf proto-oncogene, serine/threonine kinase            |
| ARF6              | ADP ribosylation factor 6                                |
| ATCC              | American Type Culture Collection                         |
| BAF               | Barrier-to-autointegration factor                        |
| BCL2              | B-cell CLL/lymphoma-2                                    |
| BH                | Board of Health                                          |
| BRAF              | B-Raf proto-oncogene, serine/threonine kinase            |
| c-ABL             | Abelson tyrosine-protein kinase 1                        |
| CDC               | Complement-dependent cytotoxicity                        |
| CDC42             | Cell division cycle 42                                   |
| <i>C. elegans</i> | <i>Caenorhabditis elegans</i>                            |
| CEV               | Cell-associated enveloped virion                         |
| CMC               | Carboxymethyl cellulose                                  |
| CPE               | Cytopathic effects                                       |
| CRAF              | C-Raf proto-oncogene, serine/threonine kinase            |
| CRISPR            | Clustered regularly interspaced short palindromic repeat |
| c-SRC             | SRC proto-oncogene, non-receptor tyrosine kinase         |
| CTLA-4            | Cytotoxic T-lymphocyte-associated protein 4              |

|         |                                                                      |
|---------|----------------------------------------------------------------------|
| CVA     | Choriolallantois vaccinia Ankara                                     |
| DAI     | DNA-dependent activator of IFN regulatory factors                    |
| DAVID   | The Database for Annotation, Visualization, and Integrated Discovery |
| DENV    | Dengue virus                                                         |
| DGCR8   | DiGeorge syndrome critical region 8                                  |
| DMEM    | Dulbecco's Modified Eagle Medium                                     |
| dNDP    | Deoxyribonucleoside diphosphate                                      |
| dNTP    | Deoxynucleoside triphosphate                                         |
| dsRNA   | Double-stranded RNA                                                  |
| dT      | Deoxythymidine                                                       |
| dTMP    | deoxythymidine monophosphate                                         |
| EEV     | Extracellular enveloped virion                                       |
| EFC     | Entry fusion complex                                                 |
| EGF     | Epidermal growth factor                                              |
| eGFP    | Enhanced green fluorescent protein                                   |
| EGFR    | Epidermal growth factor receptor                                     |
| ELISPOT | Enzyme-Linked ImmunoSpot                                             |
| EMA     | European Medicines Agency                                            |
| ER      | Estrogen receptor                                                    |
| EV      | Extracellular enveloped virion                                       |
| FACS    | Fluorescent activated cell sorting                                   |
| FBS     | Fetal bovine serum                                                   |
| FDA     | U.S. Food and Drug Administration                                    |

|        |                                                  |
|--------|--------------------------------------------------|
| FEN1   | Flap endonuclease 1                              |
| FGF-2  | Fibroblast growth factor 2                       |
| FLUC   | Firefly luciferase                               |
| GDP    | Guanosine diphosphate                            |
| GEF    | Guanine-nucleotide-exchange factor               |
| GFP    | Green fluorescent protein                        |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| GPRT   | Xanthine-guanine phosphoribosyltransferase       |
| GRB2   | Growth factor receptor bound protein 2           |
| GTP    | Guanosine triphosphate                           |
| HBSS   | Hanks' Balanced Salt Solution                    |
| HCC    | Hepatocellular carcinoma                         |
| HDI    | Histone deacetylase inhibitor                    |
| HDMEC  | Human dermal microvascular endothelial cells     |
| HIV-1  | Human immunodeficiency virus type 1              |
| Hpi    | Hours post-infection                             |
| HRAS   | HRAS proto-oncogene, GTPase                      |
| HSF1   | Heat shock factor 1                              |
| HSV-1  | Herpes simplex virus type 1                      |
| HUVEC  | Human umbilical vein endothelial cells           |
| IFITM  | Interferon-inducible transmembrane protein       |
| IFN    | Interferon                                       |
| IHD    | International Health Department (VV strain)      |

|               |                                                                                      |
|---------------|--------------------------------------------------------------------------------------|
| IHD-J         | International Health Department-Japan (VV strain)                                    |
| IL            | Interleukin                                                                          |
| IRE1 $\alpha$ | Inositol-requiring enzyme 1 alpha                                                    |
| IRF3          | Interferon regulator factor 3                                                        |
| IT            | Intratumoural                                                                        |
| IVIS          | <i>In Vivo</i> Imaging System                                                        |
| KRAS          | KRAS proto-oncogene, GTPase                                                          |
| LIVP          | Lister-Institute for Viral Preparations                                              |
| MDSC          | Myeloid-derived suppressor cell                                                      |
| miRNA         | microRNA                                                                             |
| MMP-9         | Metalloproteinase 9                                                                  |
| MOI           | Multiplicity of infection                                                            |
| MPXV          | Monkeypox                                                                            |
| MRAS          | Muscle RAS oncogene homolog                                                          |
| MV            | Mature virion                                                                        |
| MVA           | Modified Vaccinia Ankara                                                             |
| NCK           | NCK adaptor protein 1                                                                |
| NF $\kappa$ B | Nuclear factor kappa B                                                               |
| NIS           | Sodium iodide symporter                                                              |
| NLRP1         | Nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein 1 |
| NRAS          | NRAS proto-oncogene, GTPase                                                          |
| NT            | Non-targeting                                                                        |



|                  |                                         |
|------------------|-----------------------------------------|
| NUP62            | Nucleoporin protein 62                  |
| NUP98            | Nucleoporin protein 98                  |
| NUP153           | Nucleoporin protein 153                 |
| N-WASP           | Neural Wiskott-Aldrich syndrome protein |
| NYCBH            | New York City Board of Health           |
| OAS              | 2'-5'-oligoadenylate synthetase         |
| ORF              | Open reading frame                      |
| OV               | Oncolytic virus                         |
| PACT             | Protein activator of protein kinase     |
| PAK1             | p21 activated kinase 1                  |
| PBS              | Phosphate-buffered saline               |
| PCR              | Polymerase chain reaction               |
| PD-1             | Programmed cell death protein 1         |
| PD-L1            | Programmed death ligand 1               |
| Pexa-Vec         | Pexastimogene devacirepvec, JX-594      |
| PFU              | Plaque-forming unit                     |
| PGE <sub>2</sub> | Prostaglandin E2                        |
| PI3K             | Phosphatidylinositol 3-kinase           |
| piRNA            | PIWI-interacting                        |
| PKC              | Protein kinase C                        |
| PKR              | Protein kinase R                        |
| Pre-miRNA        | Precursor microRNA                      |
| Pri-miRNA        | Primary microRNA                        |

|        |                                                    |
|--------|----------------------------------------------------|
| PTGS   | Post-transcriptional gene silencing                |
| qPCR   | Quantitative polymerase chain reaction             |
| RALGDS | Ral guanine nucleotide dissociation stimulator     |
| RFC3   | Replication factor C subunit 3                     |
| RFP    | Red fluorescent protein                            |
| RIN1   | Ras interaction protein 1                          |
| RISC   | RNA-induced silencing complex                      |
| RNAi   | RNA interference                                   |
| rNDP   | ribonucleoside diphosphate                         |
| RNR    | Ribonucleotide reductase                           |
| RPO    | RNA polymerase                                     |
| RRM1   | Ribonucleotide reductase catalytic subunit M1      |
| RRM2   | Ribonucleotide reductase regulatory subunit M2     |
| SCR    | Short consensus repeat                             |
| SEM    | Standard error of the mean                         |
| SHIP-2 | SH2 domain-containing inositol 5-phosphatase 2     |
| shRNA  | Short hairpin RNA                                  |
| siRNA  | Small interfering RNA                              |
| SPI-1  | Serine protease inhibitor 1                        |
| SPI-2  | Serine protease inhibitor 2                        |
| SRSF2  | Serine/arginine-rich splicing factor 2             |
| STAT1  | Signal transducer and activator of transcription 1 |
| TAA    | Tumour-associated antigens                         |

|                   |                                                                                              |
|-------------------|----------------------------------------------------------------------------------------------|
| TK (-)            | Thymidine kinase (negative)                                                                  |
| TLR               | Toll-like receptor                                                                           |
| TOP2              | Topoisomerase II                                                                             |
| TRAF2             | Tumour necrosis factor receptor (TNFR)-associated factor 2                                   |
| TRBP              | Transactivating RNA-binding protein                                                          |
| T-Vec             | Talimogene laherparepvec                                                                     |
| UDG               | Uracil-DNA glycosylase                                                                       |
| UTR               | Untranslated region                                                                          |
| VARV              | Variola virus                                                                                |
| VEGF              | Vascular endothelial growth factor                                                           |
| VGF               | Vaccinia growth factor                                                                       |
| VITF              | Vaccinia intermediate transcription factor                                                   |
| VLTF              | Vaccinia late transcription factor                                                           |
| VV                | Vaccinia virus                                                                               |
| vvDD              | Vaccinia virus double deleted ( $\Delta$ TK and $\Delta$ VGF)                                |
| vvDD-eGFP         | vvDD expressing enhanced GFP                                                                 |
| vvDD-FLUC         | vvDD expressing FLUC                                                                         |
| vvDD-hMRAS (S27N) | vvDD expressing human MRAS dominant negative mutant (S27N) and firefly luciferase            |
| vvDD-mMRAS (S27N) | vvDD expressing mouse MRAS dominant negative mutant (S27N) and firefly luciferase            |
| vvDD-MRAS (S27N)  | vvDD expressing MRAS dominant negative mutant (S27N) (mouse or human) and firefly luciferase |
| vvDD-R2RLUC       | vvDD with R2RLUC cassette expressing mCherry (RFP) and <i>Renilla</i> luciferase             |

|            |                                                                       |
|------------|-----------------------------------------------------------------------|
| VV-eGFP    | TK- Western Reserve strain of VV expressing enhanced GFP              |
| vSP        | VV deletion mutant ( $\Delta$ SPI-1 and $\Delta$ SPI-2)               |
| vvWT       | rWT-F13L+ ( <i>i.e.</i> wild-type VV with LacZ insertion)             |
| vvWT-RFP   | Wild-type VV (rWT-F13L+) with dsRed inserted into a non-coding region |
| WHO        | World Health Organization                                             |
| WNV        | West Nile virus                                                       |
| WR         | Western Reserve                                                       |
| WR A5-eGFP | WR strain of VV with A5 protein tagged with enhanced GFP tagged       |
| YFP        | Yellow fluorescent protein                                            |

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## **CHAPTER 1: Introduction**

### **1.1 Cancer**

#### **1.1.1 The Burden of Cancer**

Cancer is the second leading cause of death worldwide.<sup>1</sup> In 2012, the latest year for which statistics are available, 8.2 million people died of cancer, 14.1 million people were newly diagnosed with cancer, and 32.6 million people were living with cancer within 5 years of the initial diagnosis.<sup>2</sup> The majority of new cancer cases (*i.e.* 57%) and deaths (*i.e.* 65%) were in less developed regions.<sup>2</sup>

In Canada, cancer is the leading cause of death accounting for 30% of all deaths.<sup>3</sup> In 2017, it was estimated that 80 800 Canadians would die from cancer (*i.e.* 221 Canadians per day).<sup>3</sup> It is also predicted that 1 in 2 Canadians will develop cancer at some point in their lives and that 1 out of 4 Canadians will die from cancer.<sup>3</sup>

Not only does cancer exact a significant physical and social toll, the economic burden is also high and increasing. It is difficult to determine the exact cost of cancer including both direct healthcare costs and indirect costs such as lost productivity due to illness or premature death. Globally, in 2010, the economic cost of cancer was estimated to be US\$ 1.16 trillion annually.<sup>1</sup> In Canada, in 2008, the Public Health Agency of Canada estimated the cost to be \$4.4 billion annually.<sup>4</sup>

#### **1.1.2 The Biology of Cancer**

In reality, cancer is not a single disease, but a constellation of diseases characterized by the uncontrolled proliferation of aberrant cells that can invade surrounding tissues and metastasize to distant sites of the body. Despite the

heterogeneity of cancer, in 2000, Hanahan and Weinberg published a seminal review where they provided an organizing framework for understanding the complex biology of cancer.<sup>5</sup> They proposed six hallmarks shared by most types of human cancers; these include self-sustained cell growth signalling, insensitivity to growth suppressors, evasion of cell death, cell immortality, promotion of angiogenesis, and invasion and metastasis.<sup>5</sup> In 2011, they revisited and elaborated on the original hallmarks and described two additional emerging hallmarks, namely metabolic rewiring and immune evasion.<sup>6</sup> Underpinning these hallmarks are genome instability, which results in the generation of random mutations that promote the acquisition of these hallmarks; and inflammation, which leads to the recruitment of bioactive molecules such as proangiogenic and survival factors as well as the releasing of reactive oxygen species that are mutagenic. The contribution of seemingly normal cells that exist as part of the tumour microenvironment also play a role in the acquisition of these features and are the subject of their most recent review.

### **1.1.3 The Treatment of Cancer**

Due to the complex nature of cancer, a cure remains elusive. The standard therapies for cancer include surgery, radiation therapy and chemotherapy. In the last couple of decades, targeted therapies such as small-molecule drugs and monoclonal antibodies have come online. However, perhaps one of the most promising therapies to make a foray into the landscape of cancer medicine in recent years is immunotherapy. It was heralded in *Science* as the “Breakthrough of the Year for 2013.”<sup>7</sup> Immunotherapy employs biotherapeutics to direct the host immune system to target and destroy



tumour cells. Some of these include checkpoint inhibitors, adoptive cellular therapy, cancer vaccines and oncolytic viruses (OVs).<sup>8</sup>

OVs are viruses that selectively target and destroy cancer growths by directly infecting, replicating within and killing tumour cells and by engaging the immune system to direct a response against the tumour. They have attributes that make them distinct from and provide advantages over current therapeutic modalities including their high safety profile as they are relatively non-pathogenic and replicate in a tumour-selective manner with the capacity for built-in safety features, and their low probability of generating resistance as they use multiple means of cytotoxicity including both virus- and immune-mediated mechanisms.<sup>9-12</sup> They also have the potential for working synergistically with other immunotherapies. While remarkable outcomes have been observed and patients have benefited from novel immunotherapies, there is still much to be learned. Efforts are ongoing to further hone these therapies including oncolytic virotherapy.

## **1.2 Oncolytic Virotherapy**

### **1.2.1 A Brief History and Current Progress**

One of the first reports of a virus having antitumour effects dates back to 1904 when a patient diagnosed with leukemia is described to have experienced remission following an apparent infection with influenza.<sup>13</sup> While there were other reports in the early 1900s of natural viral infections concurring with tumour regressions, it was not until the mid-1900s with the development of tissue culture techniques and rodent cancer models that studies began in earnest.<sup>14,15</sup> Dr. Alice Moore pioneered some of the first OV studies in mice. In 1949, she reported tumour regression in mice infected with

Russian Far East encephalitis virus.<sup>16</sup> One of the first human studies was also reported in 1949 when twenty-two patients with Hodgkin's disease were treated with hepatitis virus (likely Hepatitis B virus); seven patients had improvement in a clinical aspect of the disease including tumour regression in four cases, fourteen out of the twenty-two developed hepatitis, and there was one recorded death.<sup>14,17</sup> While there were other case studies and clinical trials conducted in the latter half of the 20<sup>th</sup> century with wild-type virus and often crude virus preparations, it was not until the advent of recombinant DNA technologies in the 1990s and with it the ability to engineer tumour selective viruses that the modern era of OV research dawned.<sup>11,14,15,18</sup> In 1991, Martuza *et al.* reported the first study with a genetically engineered tumour-specific virus, a herpes simplex virus type-1 (HSV-1) lacking the thymidine kinase (TK) gene.<sup>19</sup> They were able to demonstrate efficacy in mice implanted with human glioma cells. In 1996, ONYX-015, a genetically modified adenovirus, became the first virus to enter a phase I clinical trial.<sup>15,20,21</sup> H101, another adenovirus-based virus almost identical to ONYX-015, became the first OV approved for cancer treatment by a regulatory agency;<sup>22</sup> China's State Food And Drug Administration approved it for the treatment of head and neck cancer in 2005. In late 2015, Imlygic (talimogene laherparepvec or T-Vec), an HSV-1 virus engineered to replicate selectively in tumour cells and to express granulocyte-macrophage colony-stimulating factor (GM-CSF), was the first OV approved by the U.S. Food and Drug Administration (FDA) and by the European Medicines Agency (EMA); it was approved for the treatment of melanoma.

While Imlygic is currently the only fully FDA-approved OV, Reolysin (pelareorep), a proprietary isolate of the human wild-type reovirus, has received

Orphan Drug Designation by the FDA for the treatment of ovarian cancer as well as Fast Track designation for the treatment of metastatic breast cancer. In April 2017, Oncolytics Biotech reported positive overall survival data for a phase II trial in metastatic breast cancer patients treated with Reolysin concurrently receiving paclitaxel. Patients had an overall median survival of 10.4 months on the control arm receiving only paclitaxel versus 20.9 months on the test arm receiving both Reolysin and paclitaxel.<sup>23</sup> Pexa-Vec (pexastimogene devacirepvec), which has also received Orphan Drug Designation for the treatment of patients with hepatocellular carcinoma (HCC), is currently undergoing a phase III trial for patients with advanced HCC (described in further detail below). In addition to adenovirus, HSV-1, reovirus and VV, there are a host of other OV platforms currently being tested or have been tested in early phase clinical trials including coxsackie virus, measles virus, Newcastle disease virus, parvovirus, poliovirus, Seneca Valley virus, vesicular stomatitis virus (VSV) and Maraba virus.<sup>11,18,24,25</sup>

OVs are not only being tested on their own, but also in combination with other therapeutic modalities including chemotherapy, radiation, small molecules and, in particular, immune checkpoint inhibitors, a type of immunotherapy. Immune checkpoint inhibitors consist of antibodies that act to relieve T-cell exhaustion by blocking ligands or receptors [*e.g.* programmed cell death protein 1 (PD-1), programmed death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4)] within the tumour microenvironment involved in immune inhibitory pathways.<sup>18</sup> T-Vec, Cavatak (a coxsackie-based OV) and Reolysin are currently being tested clinically in combination with checkpoint inhibitors<sup>18</sup> with others on the horizon

including one with a Maraba-based OV that is currently recruiting. In a recent study with triple-negative breast cancer mouse models, Bourgeois-Daigneault *et al.* demonstrated that neoadjuvant treatment with a Maraba-based OV prior to surgery sensitized mice to immune checkpoint therapy as this induced inflammation, immune cell infiltration and upregulation of PD-L1.<sup>26</sup>

### **1.2.2 Mechanisms of Action**

OVs have two key mechanisms of action: direct killing of infected tumour cells and indirect killing of infected and uninfected tumour cells resulting from the induction of antitumour immunity. Antitumour immunity is generated as a result of local inflammation and the release of tumour and viral antigens at the site of infection. The nature and type of cancer, the viral vector and its interaction with the tumour microenvironment and the host immune system (*e.g.* the presence of neutralizing antibodies and/or virus-reactive T cells generated during previous exposure) dictates the relative contribution of each mechanism.<sup>18,27</sup>

OVs are not limited to employing these two mechanisms of action and are rightly referred to as multi-mechanistic anti-cancer agents. Some OVs such as VV and VSV have been shown to intrinsically induce tumour vascular disruption while others, such as adenovirus, measles virus and HSV-1, have been engineered with this additional capability.<sup>28</sup> OVs, with their capacity to encode transgenes, can engage additional mechanisms or potentiate existing ones. For example, a number of OVs including adenovirus,<sup>29</sup> HSV-1,<sup>30</sup> and measles virus<sup>31</sup> have been engineered to encode the prodrug converting enzyme, cytosine deaminase, which converts non-toxic 5-fluorocytosine (5-FC) to highly cytotoxic 5-fluorouracil (5-FU) to enhance tumour cell

killing *in situ*. Another recent strategy has been to engineer viruses with tumour-associated antigens (TAAs)<sup>32-35</sup> in order maximize virus replication using a vaccination-based strategy where the host is first vaccinated or “primed” with a TAA and subsequently treated or “boosted” with an OV expressing the same TAA. The secondary immune response directed against the TAA can serve to overcome the primary immune response directed against the virus proteins maximizing virus replication.<sup>34,36</sup>

### **1.2.3 Tumour Selectivity**

OVs are described as viruses that selectively target and kill tumour cells. OVs may be inherently oncotropic (*e.g.* reovirus) or may have been genetically altered to enhance their tumour specificity. The first genetic alteration to make a virus tumour-specific was the deletion of TK in HSV-1<sup>19</sup> exploiting the difference in the expression of TK in cancer cells versus normal cells. There are a number of the distinctive features common to many tumours that make them optimal niches for virus replication or that can be exploited to enhance tumour selectivity such as the enhanced expression of cell surface receptors that serve as virus-host receptors, the deregulation of the antiviral response, increased nucleotide and protein synthesis, enhanced cell proliferation, and resistance to apoptosis.<sup>37</sup>

## **1.3 Oncolytic Vaccinia Virotherapy**

### **1.3.1 A Brief History and Current Progress**

It was in the early 1920s that VV was first discovered to be naturally oncolytic in Levatidi’s laboratory.<sup>38,39</sup> In the late 1960s, Cassell and Garret conducted studies in mice with malignant ascites.<sup>40</sup> The first human trials occurred in the 1970s in patients with melanoma with promising results.<sup>41,42</sup> As with other oncolytic virotherapy

research, interest waned due to short-lived success and unacceptable side effects.<sup>43</sup> It was not until the 1990s and the advent of recombinant DNA technology that research intensified.

Currently there are three oncolytic VV platforms that have been tested clinically: Pexa-Vec (Wyeth strain), vvDD (Western Reserve strain) and GL-ONC1 (Lister strain). Pexa-Vec is the most advanced clinical candidate with a phase III trial currently underway. Pexa-Vec is a TK-deficient VV expressing GM-CSF. It was constructed in Dr. Edmund Lattime's lab at the Thomas Jefferson University in Pennsylvania. His group was the first to show that it was safe in melanoma patients in 1999.<sup>44</sup> Based on the success of this study, further clinical trials have been conducted using Pexa-Vec for the treatment of HCC (both adult and paediatric populations), colorectal cancer, neuroblastoma and Ewing sarcoma, among others.<sup>45</sup> A landmark phase II dose-ranging trial of Pexa-Vec in HCC patients resulted in a significantly better overall median survival for patients in the high dose treatment group: 14.1 months compared to 6.7 months for the low dose treatment group (hazard ratio 0.39;  $p=0.020$ ).<sup>46</sup> vvDD and GL-ONC1 are only in early-stage clinical development. A couple of phase I studies have been completed with vvDD where it was shown to be safe in patients with solid tumours.<sup>47,48</sup> Additionally, a phase I study was recently completed with GL-ONC1 in patients with advanced head and neck cancer where it was also found to be safe.<sup>49</sup>

### **1.3.2 Mechanisms of Action**

Oncolytic VV has been shown to target and destroy tumours by three primary mechanisms of action: (1) direct infection and lysis (*i.e.* oncolysis), (2) induction of antitumour immunity, and (3) induction of tumour vasculature collapse (Reviewed

in<sup>50</sup>). Preclinical *in vitro* and *in vivo* studies have shown VV to selectively infect, replicate within and lyse tumour cells.<sup>51-53</sup> Importantly, evidence of amplification within tumours following intravenous and intratumoural injection has also been demonstrated in human trials with all three clinical trial candidates.<sup>47-49,54,55</sup>

The capacity of oncolytic VV to induce antitumour immunity has been demonstrated in mice as evidenced by tumour rejection following re-challenge in mice having exhibited complete tumour responses.<sup>52</sup> The antitumour immune response following VV treatment has also been observed in cancer patients. A study by Kim *et al.* designed to examine functional cancer immune responses in cancer patients with differing types of solid tumours showed that treatment with Pexa-Vec gave rise to polyclonal antibody-mediated complement-dependent cytotoxicity (CDC) against multiple tumour cell antigens.<sup>56</sup> A later phase II study by Heo *et al.* in patients with advanced hepatocellular carcinoma (HCC) reported similar findings.<sup>46</sup> Moreover, Enzyme-Linked ImmunoSpot (ELISPOT) analysis revealed T cell responses to  $\beta$ -galactosidase, a transgene encoded by Pexa-Vec.

Pexa-Vec has been shown to infect normal human endothelial cells [*i.e.* human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs)] activated with vascular endothelial growth factor (VEGF) or fibroblast growth factor 2 (FGF-2). Importantly, it was demonstrated to selectively infect tumour-associated vasculature as evidenced by reduction in tumour perfusion following treatment with Pexa-Vec in both murine models and in HCC patients with no concomitant reduction in adjacent normal tissue.<sup>57,58</sup> VEGF-mediated suppression of the innate antiviral response has been shown to be responsible for sensitizing tumour

endothelium to oncolytic VV infection.<sup>59</sup> In addition, the virus-induced inflammatory response within the tumour microenvironment has been shown to lead to the recruitment of neutrophils, which severely restrict blood flow to the tumour resulting in tumour vascular collapse.<sup>58</sup>

### **1.3.3 Tumour Selectivity**

A number of strains of VV have been shown to have inherent tumour tropism, for example, the Western Reserve, Lister, Tian Tan, and International Health Department (IHD) strains.<sup>60-64</sup> This may be due to the fact that many of the features of cancer cells provide optimal cellular conditions for VV replication.<sup>37</sup> VV replication has also been specifically associated with the activation of the epidermal growth factor receptor (EGFR)-Ras signalling pathway,<sup>53,65</sup> which is activated in many human cancers.<sup>5</sup> Chemotherapeutic agents that block the EGFR-Ras signalling pathway have been shown to block VV replication.<sup>66</sup> Nevertheless, research has focused on further augmenting the inherent tumour specificity. All clinical candidates have the TK gene deleted leading to a dependence on cellular TK,<sup>65,67,68</sup> which is constitutively expressed in many cancer cells,<sup>65,69-71</sup> but transiently expressed primarily during S phase of dividing normal cells.<sup>70</sup> However, under certain conditions, the deletion of TK may not be sufficient to reduce replication in normal cells.<sup>63</sup> VV also expresses vaccinia growth factor (VGF), which is an epidermal growth factor (EGF) homolog. VGF binds EGFR, which induces proliferation of infected and nearby non-infected cells thus increasing TK levels. Thorne *et al.* demonstrated that serum-starved Beas-2B cells (shown to have reduced levels of TK) pretreated with VGF and infected with a TK-deleted VV or wild-type VV exhibited equivalent viral gene expression for both viruses.<sup>63</sup> The clinical



candidate vvDD, which lacks TK and VGF, under these same conditions, had the greatest reduction in gene expression. Other mechanisms of tumour selectivity have been explored such as the deletion of VV anti-apoptotic genes<sup>72,73</sup> and the deletion of B18R, a type I interferon (IFN)-binding protein, which sequesters extracellular IFN.<sup>52</sup>

#### **1.3.4 Enhancing Oncolytic Vaccinia Virotherapy**

The ideal OV candidate is one that can be easily manufactured, efficiently delivered, undergo rapid intratumoural spread, induce potent antitumour immunity and selectively and robustly destroy cancer growths while posing no safety risk to the patient or the population with the added capacity to encode transgenes that may provide further therapeutic enhancement.<sup>74</sup> VV possesses many of these attributes. It can, for example, be easily manufactured as it can be grown to high titres, and virus preparations are stable.<sup>75</sup> It is also highly immunogenic with the capacity to stimulate antitumour immunity.<sup>76</sup> It has a good overall safety profile as it was used to safely vaccinate hundreds of millions of people against smallpox in the last century, and there is a large body of knowledge regarding its replication in humans including knowledge of populations at risk for adverse reactions with the availability of immunoglobulin and antiviral drugs to treat adverse reactions if necessary.<sup>38,65,75</sup> Furthermore, VV completes its life cycle in the cytoplasm guarding against the possibility of viral DNA integrating into the cellular genome and, as a DNA virus, it offers genomic stability. Additionally, it has the capacity to carry as much as 25-50kb<sup>76</sup> of foreign DNA enabling the insertion of transgenes.

While VV is endowed with many features that make it an attractive OV, efforts are ongoing to further enhance it. For example, complement inhibition<sup>77,78</sup> and the use

of cell carriers<sup>79-81</sup> have been examined and successfully tested as means to improve delivery in preclinical studies. Disrupting the extracellular matrix, which can hinder spread, by engineering VV to express matrix metalloproteinase 9 (MMP-9) was found to increase virus titres in infected murine tumours and improve tumour regression.<sup>82</sup> Engineering VV to express a mutated version of A34R, which was shown to produce more extracellular enveloped virions (EEVs) and infectious progeny, was found to enhance spread and prolong survival in tumour-bearing mice.<sup>83</sup> The first approach employed to enhance antitumour immunity was to encode GM-CSF in VV to increase the induction of tumour-specific cytotoxic T lymphocytes.<sup>51</sup> More recent work has entailed altering toll-like receptor (TLR) signalling to augment antitumour T cell responses,<sup>84</sup> engineering VV to target prostaglandin E2 (PGE<sub>2</sub>) and thereby deplete myeloid-derived suppressor cells (MDSCs)<sup>85</sup> and, in addition, combining VV treatment with the  $\alpha$ -PD-L1 checkpoint inhibitor.<sup>86</sup> Deletion of the TK gene has been a prime means of conferring tumour selectivity for current clinical candidates in addition to deleting VGF for vvDD,<sup>62,63</sup> but other strategies have been explored including the deletion of anti-apoptosis genes, specifically SPI-1 and SPI-2<sup>72,73</sup> and the small ribonucleotide reductase subunit.<sup>87</sup> Encoding VV to target the tumour vasculature and reduce angiogenesis,<sup>88</sup> and combining VV administration with chemotherapy<sup>89,90</sup> are other approaches that have been undertaken to enhance tumour destruction. None of these approaches can be taken in isolation, as enhancing any one aspect is likely to enhance another. It is also likely that combining different approaches will lead to the greatest results.

## **1.4 Vaccinia Virus Virology**

### **1.4.1 Origin and Strains**

Even though VV was used to vaccinate over 200 million people against smallpox in the last century in the World Health Organization (WHO) global eradication campaign, which ended in 1980, its origin has long been debated. In 1796, Edward Jenner first vaccinated an eight-year-old boy with a cowpox lesion from a milkmaid.<sup>91</sup> At some point, vaccination with cowpox virus was replaced by VV. Jenner is reported to have obtained his later inocula from horses.<sup>92</sup> As early as the late 1930s, VV was known to differ from cowpox virus.<sup>93</sup> Recently, Schrick *et al.* sequenced a VV vaccine manufactured by the Philadelphia H.K. Mulford Company for use in 1902. Their genomic and phylogenetic analysis revealed that it had the highest degree of similarity (99.7%) to horsepox virus.<sup>93</sup>

The first VV strains received their names from the country or region of origin, or the health agency. There are numerous VV strains. Here we describe the ones alluded to in this work. All vaccine strains in the United States were derived from The New York City Board of Health (NYCBH) strain.<sup>91</sup> The Wyeth strain was derived from the NYCBH strain by 22 to 28 passages in cows in the Wyeth Laboratories.<sup>91</sup> The Western Reserve (WR) strain was derived from the Board of Health (BH) strain, which was originally derived from the NYCBH strain, by 18 passages in mouse brains.<sup>94</sup> The IHD strain was derived from the NYCBH strain by 51 passages in mouse brains and four passages on egg chorioallantoic membranes.<sup>95</sup> The IHD-J strain is a variant of the IHD strain isolated in Japan.<sup>95,96</sup> It is believed that the Lister strain was isolated from a soldier suffering from smallpox in Cologne (Germany) in 1870 but was transferred to the Lister Institute

(France) and used for vaccination in Europe.<sup>91,97</sup> Tian Tan was widely used in China and was isolated in Beijing's Temple of Heaven where the Central National Epidemic Bureau was located.<sup>91</sup> The Modified Vaccinia Ankara (MVA) strain was derived from the Choriolallantois Vaccinia Ankara (CVA) strain, a vaccine strain originally used in Turkey, by passaging more than 500 times in chicken embryo fibroblasts leading to the loss of more than 30 kb of the genome and the resulting inability to complete its replication cycle in human cells and nearly all mammalian cells.<sup>98</sup> It was safely used to vaccinate more than 100 000 people in Germany with no reported complications and is currently regarded as a favourable platform for constructing recombinant poxvirus vectors and the next generation of smallpox vaccines.<sup>98</sup>

#### **1.4.2 Characteristics and Stages of Replication**

VV belongs to the *Poxviridae* family. The *Poxviridae* family consists of enveloped, double-stranded DNA viruses that replicate in the cytoplasm.<sup>99</sup> The *Poxviridae* family is divided into two subfamilies: *Chordopoxvirinae*, which consists of poxviruses with a vertebrate host range; and *Entomopoxvirinae*, which consists of poxviruses with an insect host range. VV belongs to the former and is in the *Orthopoxvirus* genus. VV is a large, brick-shaped virus with dimensions of ~360 x 270 x 250 nm.<sup>100</sup> It has a linear genome of ~200 kilobases (kb) in length encoding over 200 genes (The WR strain has 223 putative open reading frames).<sup>101</sup> VV has two basic infectious forms: the mature virion (MV) and the extracellular enveloped virion (EV).<sup>99</sup> MVs are the most abundant form; they have a single membrane and are released upon cell lysis.<sup>102</sup> EVs have an additional membrane derived from a *trans*-Golgi or early endosomal compartment with a different protein complement than MVs.<sup>103</sup> EVs are transported to the surface via

microtubules and are released at the cell periphery following fusion with the plasma membrane.<sup>102,104</sup>

MVs enter by fusing with the cell membrane or by an endocytic pathway (*i.e.* fluid-phase uptake or macropinocytosis).<sup>105-111</sup> MVs have a minimum of four proteins that mediate attachment (*i.e.* A27, A26, D8 and H3) and eleven to twelve (*i.e.* A16, A21, A28, F9, G3, G9, H2, I2, J5, L1, L5, O3) that mediate membrane fusion and core entry, and comprise the entry fusion complex (EFC).<sup>99,112</sup> No receptor is known, but cell surface glycosaminoglycans (*i.e.* chondroitin, heparin sulfate) and laminin have been shown to mediate attachment of MVs to the cell surface.<sup>112</sup> It has been difficult to study the entry of EVs as the membrane is fragile, but studies suggest that EVs enter by fusing with the plasma membrane upon shedding of the outer membrane<sup>113</sup> and by macropinocytosis.<sup>114</sup> Following entry of the virus cores into the cytoplasm, they are transported on microtubules to transcription sites.<sup>115</sup> Early gene transcription occurs within the virus core before uncoating. Early mRNAs encode growth factors, molecules that mediate resistance to host antiviral defences, factors important for DNA replication, and intermediate transcription factors.<sup>99</sup> All mRNAs are capped, methylated and polyadenylated.<sup>99</sup> Roughly half of the genes are transcribed early.<sup>116,117</sup> The cessation of early gene transcription is associated with disassembly of the virus core or uncoating.<sup>118-120</sup> DNA replication, which leads to the formation of concatemeric DNA intermediates, takes place following uncoating and occurs in discrete regions of the cytoplasm referred to as virus factories.<sup>99</sup> Two models have been proposed for DNA replication: a self-priming model and a rolling hairpin strand displacement model akin to that of parvovirus DNA replication. Intermediate genes encoding late transcription

factors are transcribed from the replicated DNA. Translation of the late transcription factors leads to transcription of late mRNA, which encodes late enzymes, structural proteins and early transcription factors. The concatemeric DNA intermediates formed during replication are decatenated and packaged as immature virions to later form MVs and EVs. (*Fields Virology* served as source for much of this material.<sup>99</sup>)

## **1.5 RNA Interference**

### **1.5.1 The Discovery of RNA Interference**

In 1998, just as the modern era of OV research was unfolding, Fire and Mello reported on their seminal discovery that double-stranded RNA (dsRNA) could trigger sequence-specific gene silencing, which they termed “RNA interference” (RNAi).<sup>121</sup> Antisense RNA had been previously shown to interfere with gene expression.<sup>122,123</sup> Fire and Mello were seeking to understand this phenomenon when they discovered that dsRNA more potently down-regulated gene expression in *Caenorhabditis elegans* (*C. elegans*) than antisense or sense RNA strands on their own. RNAi mediated gene silencing was subsequently found to operate in mammalian cells as well.<sup>124</sup> This groundbreaking discovery has paved the way for studying the function of genes on a genome-scale level providing insights in multiple fields, including the study of host-virus interactions.

### **1.5.2 RNAi Pathways**

In the almost 20 years since the discovery of RNAi, much work has been done to understand the mechanism and machinery involved in post-transcriptional, sequence-specific gene silencing by RNAi. There are three types of small silencing RNAs that have been described in Metazoa: microRNA (miRNA), small interfering RNA (siRNA) and

PIWI-interacting (piRNA). The piRNA pathway is considered distinct from the miRNA and siRNA pathways and seems to most clearly impact the germline.<sup>125,126</sup> This overview will only focus on the microRNA and siRNA pathways, as they are relevant to this work.

#### *1.5.2.1 The miRNA Pathway*

Endogenous miRNAs are encoded in the genomes of animals, plants and viruses.<sup>127,128</sup> Most miRNAs in human cells are transcribed by RNA polymerase II to form long primary transcripts referred to as primary miRNAs (pri-miRNAs).<sup>129,130</sup> These pri-miRNAs are processed in the nucleus by the Drosha-DiGeorge syndrome critical region 8 (Drosha-DGCR8) microprocessor complex to form ~70 nucleotide hairpin loops termed pre-miRNAs.<sup>131-133</sup> These pre-miRNAs are bound by Ran-(guanosine triphosphate)GTP and exported into the cytoplasm by exportin-5.<sup>134,135</sup> Once in the cytoplasm, Dicer in complex with transactivating RNA-binding protein (TRBP)<sup>136</sup> and protein activator of protein kinase PKR (PACT),<sup>137</sup> process the pre-miRNA into short RNA duplexes ~22 nucleotides in length.<sup>138</sup> This mature miRNA is incorporated into the RNA-induced silencing complex (RISC) minimally consisting of Argonaute 2 (AGO2) in addition to Dicer, PACT, and TRBP.<sup>139,140</sup> The strand with the thermodynamically less stable 5' end, referred to as the guide strand, is preferentially retained in RISC<sup>141</sup> where it binds through imperfect base pairing to its target mRNA leading to translational repression and/or mRNA decay.<sup>142-149</sup> miRNAs exert their effects predominantly by binding the 3' untranslated regions (UTRs) of target mRNAs, but they also bind within the coding region of mRNAs and in the 5' UTRs.<sup>150-152</sup> A perfect match in the seed region, which consists of the first 2-8 nucleotides at the 5' end

of the guide strand, is considered the most reliable predictor of miRNA targets as it is considered the key determinant of miRNA specificity,<sup>153-156</sup> but other regions can influence target binding.<sup>157,158</sup> miRNAs are involved in post-transcriptional regulation of mRNAs and have been shown to have roles in development, aging, health, and disease.<sup>127</sup>

#### 1.5.2.2 *The siRNA Pathway*

In nature, siRNA is derived from dsRNA precursors that are generated from exogenous sources such as viruses or from endogenous sources such as transposons,<sup>159</sup> bidirectional transcription,<sup>160</sup> and duplexes formed by the pairing of pseudogene-derived antisense transcripts and sense mRNAs.<sup>126,161,162</sup> In the animal kingdom, endogenous sources of siRNA have been described in nematodes, *Drosophila* and mouse oocytes.<sup>163</sup> Dicer processes the dsRNA into siRNA duplexes that are 21-23 nucleotides in length with 3' terminal dinucleotide overhangs.<sup>164</sup> As with mature miRNA, the siRNA duplexes are loaded into the RISC complex where the guide strand is retained<sup>165,166</sup> and base-pairs with its target mRNA. Unlike miRNAs, siRNAs have perfect complementarity to their intended targets; this leads to cleavage of the target mRNA.

#### 1.5.3 **High-throughput RNAi Screening**

RNAi tools have been developed to take advantage of the cellular RNAi machinery to conduct loss-of-function studies including libraries of synthetically derived siRNAs and short hairpin RNAs (shRNAs) able to target genes across the human genome. shRNA resembles pre-miRNA and is processed in a similar manner to endogenous pre-miRNA.<sup>167</sup> However, unlike endogenous miRNAs, shRNAs can be



designed to have perfect complementarity with their target mRNAs, thus triggering the cleavage of the mRNA. The advantage of shRNA is that it can be delivered into cells by means of a retrovirus, which allows for the shRNA sequence to integrate into the genome and provides a stable, heritable source of siRNA. It can also be used for difficult to transfect cells. The advent of RNAi technology has made feasible the study of the function of genes on a genome-wide level.

The first genome-scale RNAi screens were conducted with dsRNAs in *C. elegans*,<sup>168,169</sup> and *Drosophila* cells.<sup>170</sup> Initially, loss-of-function studies with dsRNAs could not be performed in mammalian cells as the introduction of dsRNA of longer than 30 nucleotides was found to trigger the interferon response.<sup>171</sup> The finding that siRNAs designed to mimic endogenous 21-nucleotide siRNAs could mediate RNAi without triggering the interferon response enabled studies in mammalian cells.<sup>124</sup> The first large-scale RNAi screens in human cells were reported in the early 2000s.<sup>172-174</sup> This technology has since been applied to basic and translational research and has provided valuable insights enabling the deciphering of cellular pathways and gene networks implicated in myriad biological processes as well as the discovery of drug targets and the development of biotherapeutics.<sup>175-177</sup>

In the past decade, numerous groups have undertaken cell-based high-throughput RNAi screening (genome-wide and targeted subsets) to probe host-virus interactions for a multitude of viruses including human immunodeficiency virus type 1 (HIV-1),<sup>178-181</sup> West Nile virus (WNV),<sup>182,183</sup> influenza virus,<sup>182,184-186</sup> hepatitis C virus,<sup>187-189</sup> dengue virus (DENV),<sup>182,190</sup> HSV-1,<sup>191</sup> VSV,<sup>192-194</sup> Maraba virus,<sup>195</sup> and VV.<sup>196-199</sup> The strength of large-scale RNAi screening is that it has enabled the identification of a

plethora of unanticipated factors that dictate the intricate interplay between host and virus interactions in an unbiased and comprehensive fashion. For example, Brass *et al.*<sup>182</sup> via a genome-wide screen discovered the previously unrecognized role of a family of antiviral restriction factors, namely interferon-inducible transmembrane proteins (*e.g.* IFITM1, 2 and 3). They found that one or all three of these proteins inhibited the early replication of influenza virus, WNV and DENV. Subsequent studies revealed that IFITMs play a role in the inhibition of other viruses including Ebola,<sup>200</sup> Marburg,<sup>200</sup> SARS coronavirus (SARS-CoV),<sup>200</sup> reovirus,<sup>201</sup> and HIV-1.<sup>202</sup>

In recent years, Mercer *et al.*,<sup>196</sup> Sivan *et al.*,<sup>197</sup> and Beard *et al.*<sup>198</sup> have conducted RNAi screens in arrayed format with VV to elucidate host factors that modulate VV replication in human tissue culture cells, specifically HeLa cells. Mercer *et al.* and Beard *et al.* screened with a subset of the human genome. Sivan *et al.* have conducted the only VV human genome-wide RNAi screen published to date.

Mercer *et al.*<sup>196</sup> published the first RNAi screen with VV using a druggable genome library (6 979 genes), an siRNA library targeting genes that express proteins that have the potential to serve as drug targets.<sup>203</sup> They concentrated on identifying host genes required for VV replication. They examined the effect of siRNA knockdown following 8 h incubation with VV-eGFP (a TK- WR strain of VV expressing enhanced GFP). Following a secondary validation screen, their final hit list comprised 188 genes required for replication, which included ribosomal proteins as might be expected for any virus, but, also nuclear factors [*e.g.* nucleoporin 98 (NUP98), nucleoporin 62 (NUP62) and nucleoporin 153 (NUP153)], which might not be anticipated for VV given that it replicates in the cytoplasm. Further studies revealed roles for proteasomes and

ubiquitination in genome uncoating and virus assembly; moreover, they discovered that VV genome replication in HeLa cells is dependent on Cullin3, a component of the E3 ubiquitin-protein ligase complex.

Sivan *et al.*<sup>197</sup> conducted two human genome-wide screens in parallel with two independent libraries targeting approximately 22 000 genes in total with VV IHD-J/GFP (IHD-J strain of VV expressing GFP). This strain produces copious amounts of EEV due to a point mutation in the VV A34R gene. They investigated genes that impacted on the spread of IHD-J/GFP after 18 h incubation. Following primary and secondary screening, they compiled a hit list of 576 genes that limited spread upon siRNA silencing (*i.e.* proviral hits). Pathway analysis of proviral hits found that the most significantly enriched terms were related to translation. They also found enrichment of nuclear pore components (*i.e.* nucleoporins). Detailed investigation of one of their top candidates, NUP62, a component of the nuclear pore complex, revealed a role in poxvirus morphogenesis, providing evidence for nuclear factors being involved in VV replication.

In the most recently published RNAi screen in arrayed format, Beard *et al.*<sup>198</sup> examined genes that impacted on the spread of VV using a druggable siRNA library (6 719 genes), WR A5-eGFP (Western Reserve strain of VV with a GFP tagged A5 core protein) and a 48 h incubation period. Based on the primary screen data analysis, they identified 153 proviral host factors and 149 antiviral host factors. When they retested a small subset of these genes, they found that 69% (24/35) of the proviral hits were successfully validated whereas only 29% (7/24) of the antiviral hits passed secondary validation. They posited that this may have been due to the dynamic range of their assay that was likely more suited to detect proviral hits, noting that at 48 hours post-

infection (hpi) effectively all cells in the well were infected. As might be expected, functional and pathway analyses of their proviral hits found translation to be the most highly enriched theme. Their follow-up work on tumour necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), one of their proviral hits, demonstrated that it promoted efficient VV entry.<sup>204</sup> As with the other two screens, no follow-up studies were performed with the antiviral hits.

While each of the previous studies offered insights into VV replication, there is little overlap of hits across the screens. Beard *et al.*<sup>198</sup> performed an analysis and found that there was no overlap of proviral hits between all three screens. There were 12 proviral hits in common between Beard *et al.* and Sivan *et al.*, and 13 between Mercer *et al.* and Sivan *et al.*<sup>196-198</sup> As has been described, Mercer *et al.* and Beard *et al.* conducted screens with only a subset of the genome, which may be one contributing factor to the low degree of overlap as many of the genes tested, for example, in the Sivan *et al.* genome-wide study were not even tested in the other studies.

The lack of correspondence between large-scale RNAi screens with the same virus has been well-documented.<sup>205-207</sup> This can be attributed to different experimental approaches including differences in viral strains, cell types, siRNA libraries, transfection protocols, virus incubation time, data analysis and hit selection criteria. It has been noted that while there might not be an overlap in hits, functional and pathway analysis often reveal an overlap in commonly enriched themes.<sup>198,206</sup> The low degree of overlap underscores the importance of follow-up studies to confirm true hits.

While the aforementioned screens were conducted in an arrayed format, Filone *et al.* conducted a genome-wide pooled RNAi screen to identify host factors required for

VV replication.<sup>199</sup> They transduced A549 cells with a pooled lentivirus shRNA library targeting over 17 000 genes and subsequently infected cells with a VV expressing Venus yellow fluorescent protein fused to the A4L core protein at an MOI of 5 with a 12 h incubation period. They identified that proteins implicated in the heat shock response, and, in particular heat shock factor 1 (HSF1), as critical for VV infection.

While the screens described thus far have entailed knockdown of host genes, Kilcher *et al.*<sup>208</sup> performed the first published RNAi screens silencing VV genes using a custom-made library targeting 80 VV genes. They were interested in identifying VV genes involved between early and intermediate gene expression. Therefore, they conducted two screens in parallel: one with a VV expressing eGFP under the control of an early promoter (VV E EGFP) and one with a VV expressing eGFP under the control of an intermediate promoter (VV I EGFP). The ratio of relative VV I EGFP/VV E EGFP infection for each gene was used as a readout. With this approach, they identified D5R, which encodes AAA+ ATPase, as the VV uncoating factor.

The focus of all the host screens conducted with VV thus been far has been to catalogue gene products required for VV replication. While these screens have provided insights into VV biology, none have been conducted to identify and validate targets that could be manipulated to enhance oncolytic vaccinia virotherapy, specifically antiviral host gene targets, which could enable the identification of small molecule inhibitors that could be administered in combination with VV or the engineering of VV to encode an shRNA or a dominant negative to inhibit these genes and thereby enhance replication.

There is precedence for conducting RNAi screens to identify host targets to enhance oncolytic virotherapy. We conducted genome-wide RNAi screens with Maraba virus in three tumour cell lines and were able to identify inositol-requiring enzyme 1 alpha (IRE1 $\alpha$ ) whose inhibition with a small molecule inhibitor resulted in a dramatic enhancement in oncolytic efficacy.<sup>195</sup> Mossman *et al.*<sup>191</sup> also conducted a genome-wide pooled shRNA screen where they identified and validated the host gene target serine/arginine-rich splicing factor 2 (SRSF2) whose inhibition with a DNA topoisomerase inhibitor reduced the phosphorylation of SRSF2 and, ultimately, in combination with HSV-1 prolonged survival of tumour-bearing mice.

## **1.6 Project Overview**

### **1.6.1 Rationale**

Great strides have been taken in the field of oncolytic vaccinia virotherapy with a phase III clinical trial currently underway, but it is clear there are still more avenues that could be explored to identify ways to further enhance its efficacy and consequently benefit more patients.

While genome-wide RNAi screens have been conducted to identify VV host genes required for replication, no comprehensive studies have been undertaken to identify host/tumour genes that could be manipulated to enhance oncolytic VV spread and thereby enhance efficacy. As viruses are obligate intracellular parasites that depend on the host to propagate and spread, one might expect there to be a complement of tumour genes that modulate the spread of VV, which could serve as biotherapeutic targets.

Another strategy that also has not been extensively investigated is identifying alternate mechanisms of tumour specificity. Current clinical candidates primarily rely on the deletion of TK and/or VGF factor to ensure tumour specificity. There are possibly alternative mechanisms of tumour specificity that could be exploited to generate more potent oncolytic VVs.

In this work we set out to identify novel host/tumour target genes that could be manipulated to enhance oncolytic VV spread and also to identify alternate mechanisms of tumour specificity using functional genomic screening—in particular human and VV genome-wide RNAi screening.

#### **1.6.2 Hypotheses**

1. There are host/tumour genes that can be manipulated to enhance the spread and thereby increase the efficacy of oncolytic vaccinia virotherapy, and these genes can be identified using functional genomic screening.
2. There are alternative mechanisms of tumour specificity that could be exploited to enhance the therapeutic index and ultimately the efficacy of oncolytic vaccinia virotherapy.

#### **1.6.3 Objectives**

Project 1: Identification of host modulators of VV spread using functional genomic screening

1. To perform primary genome-wide RNAi screens to comprehensively identify host/tumour genes that modulate oncolytic VV spread.
2. To conduct *in vitro* validation of hits identified in the screens.
3. To perform *in vivo* validation of a select candidate.

Project 2: Discovering alternative mechanisms of VV tumour specificity using functional genomic screening

1. To perform primary genome-wide RNAi screens targeting the VV genome to identify genes that can be deleted without affecting potency in tumour cells.
2. To engineer novel single deletion mutants based on the findings of the RNAi screens.
3. To test the deletion mutants *in vitro* and *in vivo*.



## **CHAPTER 2: Materials and Methods**

### **2.1 General**

#### **2.1.1 Cells**

NCI-H226, 786-O, SF268, and A549 cells were obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). U2OS, 293T, 4T1, EMT6 and B16F10 cells were obtained from the American Type Tissue Collection (ATCC). CT26 LacZ, MC38 and HeLa cells were gifts from Dr. J.C Bell. CV-1 cells were a gift from Dr. J.A. McCart. GM38 cells were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository (Camden, NJ). All tumour cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS), except U2OS cells which were maintained in DMEM supplemented with 8% new born calf serum and 2% FBS when used for plaque assays and plaque purification. GM38 fibroblasts were maintained in DMEM and supplemented with 2% heat-inactivated FBS. Cells were incubated at 37°C with 5% CO<sub>2</sub> and propagated using standard tissue culture practices.

#### **2.1.2 Viruses**

vvDD-eGFP, vvDD-R2RLUC, vv-ΔN1L, rWT-F13L+, vvWT-RFP and VSC20 were gifts from Dr. J.A. McCart.<sup>62,89,209-211</sup> JX-594 B5R (P189S) was a gift from Dr. J.C. Bell. The construction, rescue and purification of other VVs generated for this project are described in detail in the material and methods sections specific for each chapter (see sections below).

### **2.1.3 Preparation of virus stocks**

Viruses were propagated in HeLa or U2OS cells, harvested following 48 -72 h incubation and centrifuged at 2000 x g for 10 min at 4°C. Pellets were resuspended in 1mM Tris buffer (pH 9.0), subjected to 3 freeze-thaw cycles and centrifuged at 2000 x g for 10 min at 4°C following which the supernatant was collected. Further purification was conducted by centrifugation (Avanti J-25; Beckman Coulter) through a 36% sucrose cushion at 20 700 x g for 1.5 h at 4°C in a swinging bucket rotor (JS-13.1; Beckman Coulter). Pellets were resuspended in 1mM Tris (pH 9.0).

### **2.1.4 Plaque assays**

U2OS ( $7.0 \times 10^5$ ) cells were infected with serial dilutions of virus in 6-well plates for 2 h after which the inoculum was removed, and the cells were overlaid with culture medium containing 1.5% (w/v) carboxymethyl cellulose (CMC) and incubated for 72 h. To visualize plaques, cells were washed with 1X PBS and then incubated with 10% (v/v) formaldehyde and stained with crystal violet (1.3 mg/mL) for 30 min.

### **2.1.5 Statistical analyses**

GraphPad Prism 7.0d software was used for all statistical analyses. When comparing the means of two groups, two-tailed student's t tests were performed. Multiple independent groups were compared with one-way ANOVA (Analysis of variance) followed by Tukey's multiple comparison post-test. Survival curves were compared with Mantel-Cox log rank analysis. For all experiments: \* $p < 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

## **2.2 Materials and methods specific to Chapter 3**

### **2.2.1 Human genome siRNA screens**

Genome-wide screens were performed with three different tumour cell lines (*i.e.* NCI-H226, SF268, 786-O) using the siGENOME SMARTPool library (Dharmacon, a Horizon Discovery Group Co.) consisting of individually pooled siRNA (4 siRNAs/gene/well) targeting 18 120 genes. The pools were spotted onto black clear-bottom 384-well plates using the JANUS Automated Workstation (PerkinElmer, Cat. no.: AJI4M01). Each screen was conducted in triplicate. Cells were reverse transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) with a final siRNA concentration of 10 nM. Cell densities of 1250 cells/well for the NCI-H226 screen, 2250 cells/well for the SF268 screen and 1500/well for the 786-O screen were used. At 48 h post-transfection, the cells were inoculated with vvDD-eGFP at an MOI of 0.05 for the NCI-H226 and SF268 screens and at an MOI of 0.06 for the 786-O screen. NCI-H226, SF268 and 786-O cells were incubated with virus for 40h, 30h and 24h, respectively. Following incubation, cells were fixed with 4% formaldehyde and stained with Hoechst 33342 (7.5ug/mL) (Thermo Fisher Scientific). Images were acquired with the Opera imaging system (PerkinElmer, Cat. no.: HH100000115; 15 fields/well; 10X magnification). All screening plates had non-targeting (NT) negative controls [*i.e.* NCI-H226: custom NT: 5'-GGAUCCUUGACAAUACCAA[dT][dT]-3' (Invitrogen); SF268: Luciferase GL2 (Qiagen); 786-O: AllStars Negative Control siRNA (Qiagen)] and positive controls targeting PLK1 (siGenome SMARTpool PLK1; Dharmacon) to assess transfection efficiency. The MicroFlo Select (Fisher Scientific; Cat. no. 11120621) was used for liquid dispensing of transfection reagent, cells, virus, and fixing and staining solution. Automated image

analysis was conducted to determine the nuclear area and GFP area per well using a script developed with the Acapella High Content Imaging and Analysis Software (PerkinElmer). The robust Z-score was calculated for each siRNA pool (*i.e.* gene target) using the formula:

$$\text{Robust Z-score} = x_{ij} - \text{plate median}(x) / \text{plate MAD}$$

where  $x$  = GFP area per individual sample well

where plate MAD (median absolute deviation) =  $1.4826 * \text{median}(|x_{ij} - \text{plate median}(x)|)$

The siRNA pools were ranked based on the median robust Z-score across the replicates.

### **2.2.2 Secondary screen with custom shRNA lentivirus library**

A custom library of hairpin-pLKO.1 vectors targeting 116 genes (384 vectors; *i.e.* 1-5 shRNAs/gene) was obtained as glycerol stocks (Sigma-Aldrich). Transfection quality DNA was prepared from the glycerol stocks using the NucleoSpin Multi-96 Plus Plasmid kit (Macherey-Nagel). For lentivirus production, 293T cells were seeded in 96-well plates (22,000 cells/well) and forward transfected with the hairpin-pLKO.1 vectors (100 ng/well) targeting the genes of interest (1 vector/well), the packaging plasmid (pCMV-dR8.74; 100 ng/well) and the envelope plasmid (pMD2-G; 10 ng/ $\mu$ l) using MIR 2300 TransIT-LT1 (Mirus Bio). pDY-GFP (lentivirus expression plasmid encoding GFP) was used as a transduction control. At 18 h post-transfection, FBS was added to the cells (for a final concentration of 30%). 24 h post the addition of FBS, lentiviruses were harvested (*i.e.* the supernatant from wells was transferred to polypropylene deep wells) and fresh growth media (30% FBS) was added back to the plates. A second harvest of virus was performed again at 24 h. Both harvests were combined, filtered (0.45 $\mu$ m; Millipore Sigma; Cat. no. MSHVS4510) and used to

transduce 786-O cells seeded (7000 cells/well) in 96-well plates. 72 h post-transduction, 786-O cells were infected with vvDD-eGFP. At 20 hpi, 786-O cells were fixed with 4% formaldehyde and stained with Hoechst 33342 (5 µg/mL). Prior to imaging the plates with the Cellomics Arrayscan VTI (Thermo Scientific), plates were washed with 1X PBS (phosphate-buffered saline) using the ELx405 Select CW microplate washer (Biotek). The GFP area per well was calculated. Each sample well measurement was normalized to the median measurement of the negative control (*i.e.* shRNA targeting lac-Z/luciferase) wells on a per-plate basis. The screens were conducted in triplicate and the median of the normalized values for each replicate was used to rank gene targets.

### **2.2.3 siRNA knockdown assays**

NCI-H226 ( $1.5 \times 10^5$  cells/well), SF268 ( $2.6 \times 10^5$  cells/well), and 786-O ( $2 \times 10^5$  cells/well) cells were reverse transfected in 6-well plates with non-targeting siRNA (NCI-H226: siGENOME Non-Targeting siRNA Pool #2, Dharmacon; SF268, 786-O: AllStars Negative control, Qiagen) or siRNA targeting MRAS (siGENOME Human MRAS, Dharmacon) (final concentration 10nM). Cells were inoculated with vvDD-eGFP (NCI-H226: 900 PFU/well; SF268: 1000 PFU/well; 786-O: 500 PFU/well). Whole wells were imaged live with the Cellomics Arrayscan VTI (Thermo Scientific). Spread was measured by the GFP area per well. Cell lysates were collected in RLT lysis buffer (Qiagen) at 0 hpi. RNA was extracted using the RNeasy Mini Kit (Qiagen). Standard qPCR was performed using the one-step QuantiTect SYBR Green RT-PCR Kit (Qiagen) and the Mastercycler EP Realplex (Eppendorf). Primers: GAPDH, Hs\_GAPDH\_2\_SG

(Qiagen, Cat. no. QT01192646); MRAS, Hs.PT.58.21543015 (Prime Time qPCR Primers, Integrated DNA technologies)

#### **2.2.4 shRNA knockdown assays**

Confluent wells ( $5 \times 10^5$  cells/well) of stable 786-O cell lines expressing non-targeting shRNA or shRNA targeting MRAS were infected with vvDD-eGFP at an MOI of 0.002. Whole wells were imaged live at 72 hpi with the Cellomics Arrayscan VTI (Thermo Scientific). Spread was measured by the GFP area per well. Cell lysates were collected in RLT lysis buffer (Qiagen) at 0 hpi. RNA was extracted and qPCR performed as described in 2.2.3.

#### **2.2.5 CRISPR-Cas9 knockout assays**

Confluent wells of 4T1 ( $3 \times 10^5$  cells/well) or A549 ( $6 \times 10^5$  cells/well) with wild-type MRAS or with MRAS disrupted using CRISPR-Cas9 technology were infected at MOIs of 0.1 or 0.001, respectively, with vvDD-eGFP or JX-594. For the JX-594 assay, cells were fixed with 4% formaldehyde and stained with 33342 Hoechst (5 mg/mL) at 144 hpi. Whole wells were imaged live with the Cellomics Arrayscan VTI (Thermo Scientific). Spread was measured by the GFP area per well. Cell death was measured by the Hoechst area per well.

#### **2.2.6 Generation of knockout cell lines with CRISPR/Cas9n**

The Feng Zhang lab protocol was used to generate the cell lines.<sup>212</sup> In brief, guide RNA sequences were designed using the Feng Zhang LabCRISPR design portal (<http://crispr.mit.edu>). As Cas9n was used, pairs of forward and reverse guides were designed to target MRAS. Oligonucleotide sequences for the human MRAS guide pair (h-gRNA#1) were: 5'-GTACGGAGATTGACAATCAAT-3' (forward guide) and 5'-GTCAATGGTGGGGTCATAGTC-3' (reverse guide). For mouse MRAS, the guide pair (m-

gRNA#1) sequences were: 5'-GTGACAATCAGTGGGCCATCT-3' (forward guide) and 5'-GTCAATGGTGGGGTCGTAGTC-3' (reverse guide). The complementary oligonucleotides for each guide pair including additional bases for cloning purposes (see Appendix A for sequences) were annealed and the resulting inserts were cloned into pSpCas9n (BB)-2A-Puro (pX462) (a gift from Feng Zhang; Addgene plasmid #48141).<sup>212</sup> A549 and 4T1 cells were transfected with h-gRNA#1/pX462 or m-gRNA#1/pX462, respectively, along with pcDNA3-eGFP (ThermoFisher) for cell sorting purposes. After 48 h, cells were dissociated and GFP positive cells were sorted using fluorescent-activating cell sorting (FACS) individually into 96-well round bottom plates (MoFlo Cell Sorter; Beckman Coulter). Genomic DNA was extracted from confluent wells of individual clones using QuickExtract (Epicentre) and screened for the presence of indels by PCR. To screen the A549 clones, the following primers were used: forward primer 5'-CACCATCCAGTTTTTCCAGAAG-3' and reverse primer 5'-TATCCCACCCAATGCAGG-3'. To screen the 4T1 clones, the following primers were used: forward primer 5'-CAAGAGTGCGCTCACTATTC -3' and reverse primer 5'-CACCCCATGCAAGACTTGTC -3'. The 4T1 primers were also used to sequence the indel region of the 4T1 clone used in this study.

### **2.2.7 Multi-step growth curve assay**

Confluent wells ( $6 \times 10^5$  cells/well) of A549 and  $\Delta$ MRAS A549 cells were incubated with vvDD-eGFP (MOI = 0.001) for 2 h following which the inoculum was removed and replaced with culture medium. At 12 h intervals, infected cells from whole wells were scraped into the culture medium and subsequently subjected to 3 rounds of

freeze thaws. Plaque assays (see 2.1.4) were performed to quantify virus at each time point.

#### **2.2.8 One step growth curve assay**

Confluent wells ( $6 \times 10^5$  cells/well) of A549 and  $\Delta$ MRAS A549 cells were incubated with vvDD-eGFP (MOI = 10) for 2 h following which the inoculum was removed and replaced with culture medium. At 4 h intervals, infected cells from whole wells were scraped into the culture medium and subsequently subjected to 3 rounds of freeze thaws. Plaque assays (see 2.1.4) were performed to quantify virus at each time point.

#### **2.2.9 Quantification of EEV assay**

Confluent wells ( $6 \times 10^5$  cells/well) of A549 and  $\Delta$ MRAS A549 cells were incubated with vvDD-eGFP (MOI = 0.001) for 2 h following which the inoculum was removed and replaced with culture medium. At 72 hpi, infected cells and infected culture medium were collected separately. The cell fraction was scraped into fresh culture medium. Each sample was subsequently subjected to 3 rounds of freeze thaws. Plaque assays (see 2.1.4) were performed to quantify virus. The total PFU per fraction was calculated by multiplying the yield (*i.e.* PFU/mL) by the volume collected for each sample.

#### **2.2.10 Testing of a VV defective in actin tail formation**

Confluent wells ( $6 \times 10^5$  cells/well) of A549 and  $\Delta$ MRAS A549 were infected with JX-594 (with a GFP reporter gene) or JX-594 B5R P189S (with a YFP reporter gene) at an MOI of 0.001. Whole wells were imaged live with the Cellomics Arrayscan VTI (Thermo Scientific). Spread was measured by the GFP or YFP area per well.



### 2.2.11 Construction of vvDD-MRAS (S27N)

To construct vvDD-MRAS (S27N) (*i.e.* vvDD encoding human or mouse MRAS dominant negative) and vvDD-FLUC, first cassettes were constructed with firefly luciferase (FLUC) alone or with FLUC followed by an intervening 2A porcine teschovirus-1 virus sequence (see Appendix A for 2A sequence) and mouse or human MRAS dominant negative (S27N) [*i.e.* the FLUC-2A-MRAS (S27N) cassette]. To construct the FLUC-2A-MRAS (S27N) cassettes, RNA was extracted from GM38 cells or mouse embryonic fibroblasts to generate human and mouse cDNA of MRAS, respectively. MRAS was amplified from the mouse and human cDNA. The 2A-MRAS cassettes were each ligated into the expression plasmid pDY-FLUC using SbfI and BamHI restriction enzymes (Before ligation of the 2A-MRAS cassette into pDY-FLUC, the stop codon of FLUC was mutated to an SbfI site). Mutagenesis was performed to generate MRAS (S27N). The FLUC cassette was amplified from pDY-FLUC and the FLUC-2A-MRAS (S27N) cassette from pDY-FLUC-2A-MRAS (S27N) and subcloned into the pVx-R2RLUC shuttle plasmid<sup>89</sup> replacing the R2RLUC cassette with FLUC-2A-MRAS (with mouse or human MRAS) or FLUC using In-Fusion (Clontech). Following homologous recombination of the new shuttle vectors with the parental virus VSC20 that has the LacZ gene inserted into the VGF loci, the new viruses were subject to eight rounds of purification in selective medium (DMEM supplemented with heat inactivated 10% FBS) containing mycophenolic acid (2.5 mg/mL), hypoxanthine (1.49 mg/mL) and xanthine (10 mg/mL) to eliminate any parental wild-type virus following which they were expanded in HeLa cells and purified through a sucrose cushion (see 2.1.3). MRAS was

sequenced following purification (sequencing primer: 5'-AGGAGCCACGAACTTCTCTC-3'). See Appendix A for all the primers used in the construction of vvDD-MRAS (S27N).

### **2.2.12 *In vitro* vvDD-MRAS (S27N) assay**

NCI-H226 ( $3.6 \times 10^5$  cells/well), 786-O ( $5 \times 10^5$  cells/well), SF268 ( $6 \times 10^5$  cells/well), A549 ( $6 \times 10^5$  cells/well), 4T1 ( $4 \times 10^5$  cells/well), MC38 ( $6 \times 10^5$  cells/well), EMT6 ( $4.5 \times 10^5$  cells/well), B16F10 ( $5 \times 10^5$  cells/well) and CT26 ( $6 \times 10^5$  cells/well) cells were seeded in 6-well plates and the following day were infected with 60 PFU/well of vvDD-FLUC or vvDD-MRAS (S27N) [with mouse or human MRAS (S27N) depending on the cell line]. At 48 hpi, luciferin was added to each well (200  $\mu$ g/mL) and the bioluminescence signal was captured using the IVIS 200 (Xenogen). The fold change in radiance (p/sec/cm<sup>2</sup>/sr) of vvDD-MRAS (S27N) relative to vvDD-FLUC was quantified using the IVIS 200 Image 2.5 software.

### **2.2.13 Mouse studies**

All mouse studies were approved by the Animal Care Committee of the University of Ottawa. Female BALB/c mice, 6-8 weeks in age were used for all experiments (Charles River Laboratories).

4T1 tumours were established by subcutaneous injection of  $1 \times 10^5$  into the left mammary fat pad. Tumours were treated by intratumoural injection with a single injection of  $2.5 \times 10^7$  PFUs/mouse when they reached 50-100 mm<sup>3</sup>. Tumour growth was measured with callipers, and the tumour volume was calculated with the formula: length x width<sup>2</sup>/2. Mice were euthanized when they reached  $\geq 1000$  mm<sup>3</sup>.

CT26 tumours were established by subcutaneous injection of  $1 \times 10^6$  into the right flank. When the tumours reached 50-100 mm<sup>3</sup>, they were treated by intratumoural

injection with two injections of  $5 \times 10^7$  PFUs/mouse 48 h apart. Tumour growth was measured with callipers, and the tumour volume was calculated with the formula: length x width x height/2.

#### **2.2.14 Wound-healing assay**

A wound was made in confluent wells of A549 and  $\Delta$ MRAS A549 cells seeded in Essen Bioscience ImageLock 96-well plates with the WoundMaker (Essen Bioscience) following the protocol provided by the manufacturer. Plates were imaged every 2 h with the Incucyte Zoom (Essen Bioscience) (10X objective; one image per well). The wound width in each well was calculated using the Incucyte software (version: 2015A).

### **2.3 Materials and methods specific to Chapter 4**

#### **2.3.1 Vaccinia genome siRNA screens**

A custom library (Dharmacon, a Horizon Discovery Group Co.) consisting of siRNA targeting 173 vaccinia genes (1-8 siRNAs/gene; 637 oligonucleotides) was arrayed in 96-well plates (1 siRNA/well). The siRNAs (final concentration 20nM) were reverse transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) into U2OS (12 000 cells/well) or NCI-H226 (10 000 cells/well) cells. At 16 h post-transfection, NCI-H226 cells were infected with vvWT-RFP at an MOI of 0.05; U2OS cells were infected with vvWT-RFP or vvDD-eGFP at an MOI of 0.01. At 30 hpi (or 48 hpi for screens with vvDD-eGFP), cells were fixed with formaldehyde (4%) and stained with Hoechst 33342 (5 ug/mL), and subsequently washed with 1X PBS using the ELx405 Select CW microplate washer (Biotek). Images of each well were acquired with the ArrayScan VTI HCS Reader (36 fields/well; 10X magnification). Automated image analysis was conducted to determine the nuclear area and RFP or GFP area per well using a script

developed using Acapella High Content Imaging and Analysis Software (PerkinElmer). Each sample well was normalized on a per-plate basis to the median RFP or GFP area of the negative control wells transfected with non-targeting siRNA [vvWT screens: siGENOME Non-targeting siRNA Pool #2 (Dharmacon); vvDD screen: Luciferase GL2 (Qiagen)]. The screens were conducted in triplicate and the median score across the replicates was used to score each siRNA. siRNA targeting GFP (Ctrl\_GFP\_3; Cat. no. SI03650346; Qiagen) was used as a positive transfection control in the vvDD-eGFP screen, and siRNA targeting J6R [custom: 5'-CGUUAAGAUGGUAGACUAUU-3' (Dharmacon)] was used in the vvWT-RFP screens.

### **2.3.2 Construction, rescue and purification of single deletion mutants**

The shuttle vector pVx-R2RLUC, which has a portion of the 5'end (*i.e.* left arm) and the 3'end (*i.e.* right arm) of the TK gene flanking the R2RLUC cassette in addition to encoding the GPRT (xanthine-guanine phosphoribosyltransferase) gene (for drug selection), was used for cloning. The R2RLUC cassette contains RFP (*i.e.* mCherry) and Renilla luciferase reporter genes with an intervening foot-and-mouth disease viral 2A sequence (see Lun *et al.*<sup>89</sup> for a further description). The TK portions were replaced with the corresponding portions of the candidate genes by first amplifying 300-500 bp portions of the 5' and 3' ends of each gene with 15 bp homology regions to pVx-R2RLUC and restriction enzyme sequences on each end (see Appendix A for primers used) to enable In-Fusion cloning (Clontech). pVx-R2RLUC was prepared for the insertion of the left arms by double digest with Esp3I and SpeI followed by gel extraction and purification of the linearized vector using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel). The left arms were inserted using In-Fusion HD cloning (Clontech)

following the Clontech protocol. pVx-R2RLUC with left arm inserts were subsequently linearized for the insertion of the right arms with *PauI* and *BpII*. Rescue experiments entailed infecting confluent wells of CV-1 cells (6-well plate) with rWT-F13L+, followed by transfection of the newly constructed shuttle vectors. This was followed by 4-8 rounds of purification using selective medium (DMEM supplemented with 10% FBS) containing mycophenolic acid (2.5 mg/mL), hypoxanthine (1.49 mg/mL) and xanthine (10 mg/mL) to eliminate any parental wild-type virus. PCR was used to confirm the absence of parental wild-type virus and the presence of the R2RLUC cassette in the recombinants (for primers see Appendix A).

### **2.3.3 Images of infectious foci of vv- $\Delta$ A22R, vv- $\Delta$ D4R, vv- $\Delta$ G5R, vv- $\Delta$ I2L and vv- $\Delta$ L4R**

Confluent wells of U2OS cells were infected with vv- $\Delta$ A22R, vv- $\Delta$ D4R, vv- $\Delta$ G5R, vv- $\Delta$ I2L or vv- $\Delta$ L4R following 5 rounds of purification. After a 2 h incubation period, infected cells were overlaid with medium (DMEM; 10% FBS) containing 0.5% agarose. Images of infectious foci were taken with the Olympus microscope (DP72; 4X objective) at 4 days post-infection.

### **2.3.4 *In vitro* testing of the single deletion mutants**

Confluent wells (96-well plate; n=3) of murine (*i.e.* CT26, MC38, EMT6) and human (*i.e.* U2OS, A549, NCI-H226) tumour cells were infected with the single deletion mutants and control virus (*i.e.* vvDD-R2RLUC and/or vvWT) and imaged (4 fields/well) with the Incucyte Zoom (Essen Bioscience) (10X objective). Normal human fibroblasts (*i.e.* GM38 cells) were also infected and imaged in the same manner, but they were infected 6-days post seeding to allow the cells to become quiescent. The mean RFP area and/or percent confluency was calculated (*i.e.* 4 fields/well) per well using the Incucyte

software (version: 2016B). The RFP area was quantified using the red channel images, and the percent confluency was quantified using the phase contrast images.

### **2.3.5 Plaque-size measurements**

Confluent wells of U2OS were incubated with virus for 2 h. The inoculum was subsequently removed, and the wells were overlaid with 1.5% (w/v) CMC and DMEM (10% FCS) media. 3 days post-infection, the cells were fixed with formaldehyde (10%) and stained with crystal violet (0.1%). Whole wells were scanned with the EVOS FL Auto Cell Imaging System using bright-field illumination (10X objective). The plaque sizes were quantified using Fiji (Version 1.0)<sup>213</sup> by outlining each plaque and calculating the area.

### **2.3.6 Mouse studies**

All mouse studies were approved by the Animal Care Committee of the University of Ottawa. MC38 tumours were established by the subcutaneous injection of  $1 \times 10^5$  cells into the right flanks of C57BL/6, 6-8 weeks in age (Charles River Laboratories). Mice were treated with Tris buffer (n=4), vvDD (n=3), or vv-ΔA50R (n=3) by intratumoural injection on days 13, 16 and 20 post tumour cell implantation with  $1 \times 10^7$  PFU/mouse. Tumour volume was calculated based on caliper measurements using  $\text{length} \times \text{width}^2/2$ .

A549 tumours were established by the subcutaneous injection of  $2 \times 10^6$  cells into the right flanks of CD-1 Nude mice, 6-8 weeks in age (Charles River Laboratories). When the tumours reached 30-70 mm<sup>3</sup>, they were treated by intratumoural injection with 3 doses of virus ( $1 \times 10^7$  PFU/mouse; vvDD-R2RLUC or vv-ΔA50R) or Tris buffer

72 h apart. Tumour growth was measured with callipers, and the tumour volume was calculated with the formula: length x width x height/2.

### **2.3.7 Construction and rescue of combination VV mutants**

#### *2.3.7.1 Construction of double deletion mutants with $\Delta A50R$ or $\Delta N1L$*

The R2RLUC cassette of pVX-R2RLUC-GeneX was replaced with a cassette containing FLUC (see Appendix A for the primers for amplifying FLUC from pVx-FLUC; see also 2.2.11 for the construction of pVx-FLUC) in the shuttle vectors that were previously made for each candidate gene (see 2.3.2). To rescue the double deletion mutants, CV-1 cells were first infected with vv- $\Delta A50R$  (RFP) or vv- $\Delta N1L$  (RFP) and then transfected with each of the new shuttle vectors (*i.e.* pVx-FLUC-GeneX).

#### *2.3.7.2 Construction of vv- $\Delta A50R$ -MRAS (S27N)*

The R2RLUC cassette in the pVx-R2RLUC-A50R (see 2.3.2) shuttle vector was replaced with the previously made cassette containing FLUC or FLUC-2A-MRAS (S27N) [see 2.2.11; see also Appendix A for the primers used to amplify the FLUC-2A-MRAS (S27N) cassette from pVx-FLUC-2A-MRAS (S27N) and FLUC from pVx-FLUC]. To rescue vv- $\Delta A50R$ -MRAS (S27N) and vv- $\Delta A50R$ -FLUC, CV-1 cells were first infected with vv- $\Delta A50R$  (RFP) and then transfected with the new shuttle vectors.

#### *2.3.7.2 Construction of vv- $\Delta N1L$ -MRAS (S27N)*

A PCR based cloning method was used. The FLUC or FLUC-2A-MRAS (S27N) cassettes along with the Pse/I promoter were amplified from pVx-FLUC or pVX-FLUC-2A-MRAS (S27N), respectively, using primers with 80 bp N1L homology sequences on each end (see Appendix A for primers). To rescue vv- $\Delta N1L$ -MRAS (S27N) and vv- $\Delta N1L$ -

FLUC, CV-1 cells were first infected with vv- $\Delta$ N1L (RFP) and then transfected with the gel purified PCR amplicon.



## **CHAPTER 3: Genome-wide RNAi screens uncover MRAS as a novel target to enhance oncolytic vaccinia virotherapy**

### **3.1 Abstract**

Oncolytic vaccinia virotherapy has been used to treat a spectrum of cancers, yielding encouraging results in phase I and II clinical trials. While patients have clearly benefited, the possibility exists that innovative modifications made to the therapy could lead to superior outcomes. We hypothesized that enhancing the spread of vaccinia virus (VV) within tumours by manipulating host tumour genes could increase the therapeutic efficacy. Thus, we employed high-throughput genome-wide RNAi screening in three tumour cell lines to identify genes that modulate VV spread. We identified 237 genes that when silenced increased VV spread in at least two out of the three tumour cell lines tested and 23 that increased spread across all three cell lines. Mouse RAS oncogene homolog (MRAS) emerged as a promising candidate following *in vitro* validation studies. To test the impact of inhibiting this gene *in vivo*, we engineered an oncolytic VV encoding a dominant negative of MRAS. Treatment of tumour-bearing mice with this virus resulted in delayed tumour progression, prolonged survival and durable responses compared to oncolytic VV alone. This study offers a novel approach to improving clinical outcomes—that of targeting host tumour genes in combination with oncolytic vaccinia virotherapy. Moreover, we demonstrate the value of functional genomic screening as a means of identifying novel biotherapeutic targets.

### **3.2 Introduction**

Despite improvements in conventional care, cancer remains the second leading cause of death worldwide,<sup>1</sup> underscoring the critical need for innovative therapies.

Oncolytic virotherapy is one such therapy that has the potential to dramatically alter the disease trajectory and patient outcomes. Oncolytic virotherapy employs viruses that have the ability to instigate the targeted destruction of local and disseminated tumour cells with minimal side effects and no known late effects. A distinct advantage of many oncolytic viruses (OVs) is their ability to target tumour cells on multiple fronts making it more difficult for cancer cells to evolve resistance.<sup>12</sup> Some of their mechanisms of action include direct tumour cell lysis, the induction of antitumour immunity and the destruction of tumour vasculature.<sup>18,27,50</sup>

Oncolytic virotherapy has been making great strides in recent years. In 2015, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved Imlygic (talimogene laherparepvec or T-Vec), a herpes simplex virus type 1 (HSV-1) engineered to be tumour-specific and to express granulocyte-macrophage colon-stimulating factor (GM-CSF), for the treatment of melanoma.<sup>214</sup> This was the first FDA- and EMA-approved OV. Pexa-Vec (pexastimogene devacirepvec; JX-594), a thymidine kinase (TK)-negative oncolytic VV also expressing GM-CSF with a Wyeth backbone has also progressed a great distance down the clinical pipeline with a phase III clinical trial currently underway. In a landmark phase II trial of Pexa-Vec in patients with advanced hepatocellular carcinoma (HCC), the overall median survival was significantly better for patients in the high dose treatment group: 14.1 months compared to 6.7 months for the low dose treatment group (hazard ratio 0.39;  $p = 0.020$ ).<sup>46</sup> Early clinical trials have also been undertaken with other strains of VV including the Western Reserve (WR) strain. Recent evaluations of phase I trials with vvDD (vaccinia virus double-deleted), a TK- and vaccinia growth factor (VGF)-negative

WR strain, showed that it was tumour-specific with no dose-limiting toxicities.<sup>47,48</sup> Notwithstanding the documented clinical success of OV<sub>s</sub>, there is a need to further augment this therapy to achieve greater benefits for patients.

In the present study, we undertook human genome-wide RNAi screening to comprehensively identify host genes that modulate VV spread with the aim of manipulating these genes to enhance its therapeutic efficacy. A number of studies in mice suggest that heightened spread and replication of VV at the tumour site correlate with improved efficacy.<sup>215-217</sup> For example, combination treatment with radiation<sup>217</sup> or with histone deacetylase inhibitors<sup>215</sup> led to increased intratumoural spread and ultimately better outcomes in mice. Thorne *et al.*<sup>85</sup> found that this correlation applied specifically to resistant murine tumour models. Additionally, preclinical and clinical studies with OV<sub>s</sub> in general have demonstrated that treatment with higher doses of virus, which likely means greater replication at the tumour site, results in improved responses.<sup>218-220</sup> Conversely, it has been observed in *in vitro* and *in vivo* models of cancer that efficacy is reduced or abrogated when defective-replicating viruses are employed.<sup>221-223</sup> Furthermore, it has been shown with VV specifically that replication is required for the shutdown of tumour vasculature and induction of antitumour immunity<sup>58</sup>—two key mechanisms of action for VV in addition to the direct lysis of cancer cells.

VV produces two basic types of infectious virions that spread by different mechanisms: mature virions (MV<sub>s</sub>) and extracellular enveloped virions (EV<sub>s</sub>).<sup>103</sup> MV<sub>s</sub> have a single membrane and are the most abundant form of infectious virions.<sup>102</sup> They are released only upon cell lysis and spread slowly from cell to cell.<sup>99,224</sup> EV<sub>s</sub> evolve

from MVs by acquiring an additional membrane and traffic to the cell periphery along microtubule networks.<sup>102,104</sup> EVs are sometimes distinguished into two different types: cell-associated enveloped virions (CEVs) and extracellular enveloped virions (EEVs).<sup>103</sup> CEVs, the predominant form of EVs produced by the WR strain,<sup>225</sup> remain attached at the cell surface and are primarily responsible for cell-to-cell spread via actin tails.<sup>209,225-228</sup> The accumulation of the VV protein A36 and its phosphorylation by Src kinases in the plasma membrane beneath CEVs induce actin tail polymerization that propel CEVs away from the cell surface into neighbouring cells.<sup>229</sup> These actin tails also enable CEVs to spread rapidly across cells as they are able to propel them over infected cells to reach uninfected cells by a mechanism termed “repulsion of superinfecting virions.”<sup>224,229</sup> EEVs, which constitute a very small percentage (*e.g.* 0.2 -0.8 % depending on the cell line)<sup>103,230</sup> of virions produced by the WR strain, are CEVs that have been released from the cell into the culture medium and are capable of long-range dissemination in the host.<sup>102,231,232</sup>

Knowledge is increasing regarding the involvement of host genes in VV spread and replication in general. For example, actin, myosin II, Rac family small GTPase 1 (RAC1), cell division cycle 42 (CDC42), p21 activated kinase 1 (PAK1), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), ADP ribosylation factor 6 (ARF6), epidermal growth factor receptor (EGFR), SRC proto-oncogene (c-SRC), growth factor receptor bound protein 2 (GRB2), NCK adaptor protein 1(NCK), and neural Wiskott-Aldrich syndrome protein (N-WASP) have been identified as important host factors involved in macropinocytosis, a mechanism of VV entry, and/or actin tail formation.<sup>109,110,233-237</sup> Abelson tyrosine-protein kinase 1 (c-ABL)<sup>238</sup> and SH2 domain-containing inositol 5-

phosphatase 2 (SHIP-2)<sup>239</sup> have been found to play key roles in EEV release. Other studies have explored the role of ubiquitin-proteasome system in VV replication.<sup>196,240,241</sup> In recent years, a number of large-scale functional genomic screens have been conducted to more broadly probe host factors involved in VV replication.<sup>196-199</sup> Intriguingly, even though VV replicates in the cytoplasm, Sivan *et al.*<sup>197</sup> found that nuclear pore proteins impacted on poxvirus morphogenesis.<sup>197</sup> This finding was supported by Postigo *et al.*<sup>242</sup> who found evidence of nuclear factors, in particular components of the host DNA replication and repair machinery, playing a role in VV genome replication in the cytoplasm.

While our understanding regarding the involvement of host factors in VV replication is increasing, there is still much to be discovered. Little is known regarding tumour-specific host factors that modulate oncolytic vaccinia virotherapy. Thus, we conducted high-throughput RNAi screens in three tumour cell lines to comprehensively identify host factors that modulate the spread of VV in tumour cells. Ultimately, we focused primarily on the genes that enhanced spread upon silencing (*i.e.* antiviral genes) as we reasoned that these might be more clinically tractable targets as we could enhance spread by administering VV in combination with a small molecule inhibitor of the identified gene target, or we could engineer VV to express a shRNA or a dominant negative to inhibit the gene target. MRAS was the top hit among 23 host factors that upon knockdown enhanced the spread of VV across all three cell lines. MRAS is a member of the Ras family of small GTPases that includes the classic Ras oncogenes HRAS, KRAS and NRAS which act as molecular switches in the cell alternating between active GTP-bound and inactive GDP-bound states. We engineered VV to express a

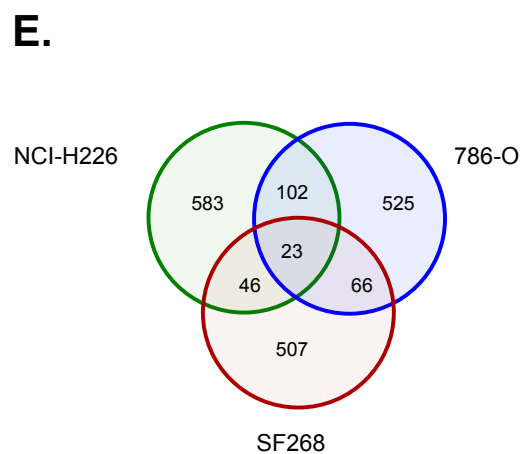
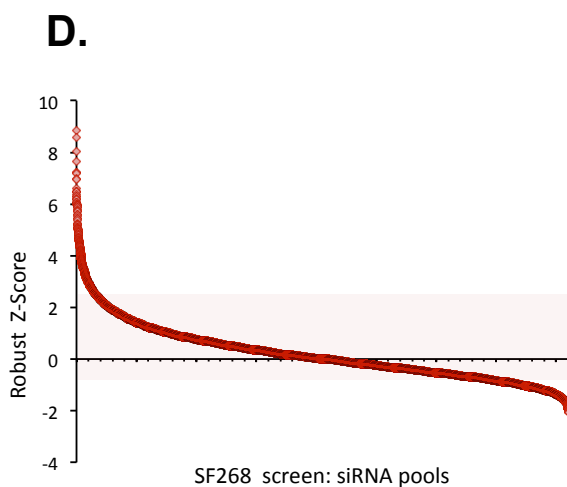
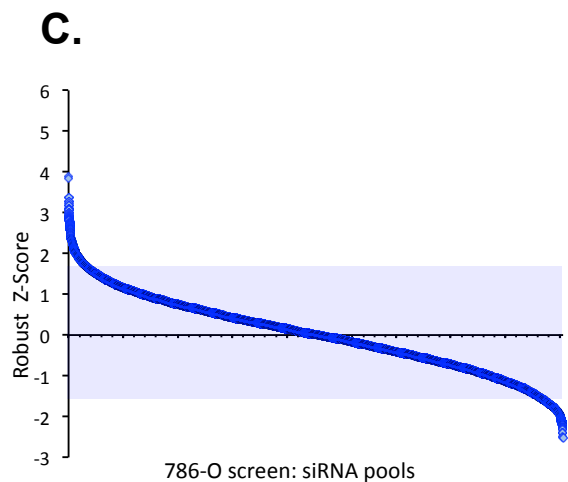
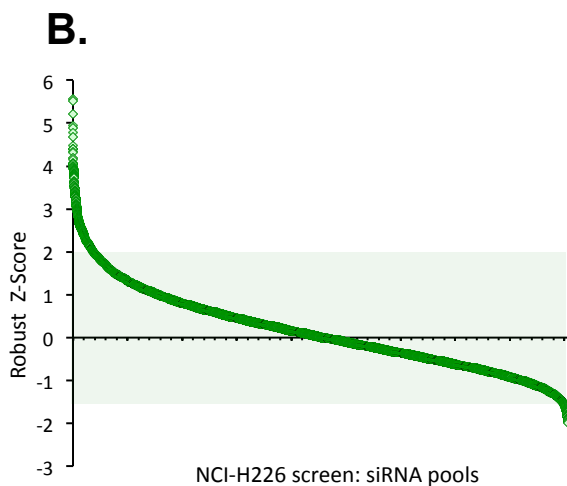
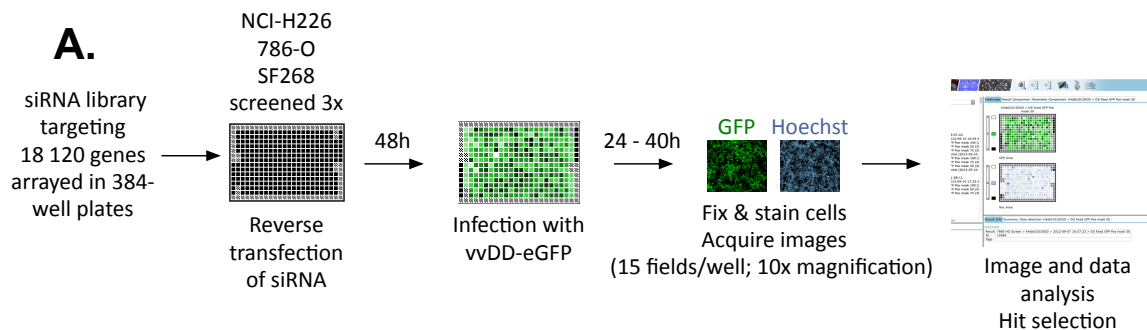
dominant negative mutant of MRAS and found that treatment with this virus significantly delayed tumour progression, prolonged survival and led to durable responses in tumour-bearing mice compared to those treated with oncolytic VV alone. This is the first study to uncover MRAS as a target for augmenting oncolytic vaccinia virotherapy and the first to utilize functional genomic screening as a tool to identify novel host targets in this context.

### **3.3 Results**

#### **3.3.1 Primary genome-wide siRNA screens identify 237 genes that upon silencing enhance the spread of VV.**

To identify host genes that modulate the spread of VV, we conducted human genome-wide siRNA screens targeting 18 120 genes (Fig. 3.1A). To offset cell intrinsic bias, we screened in three different tumour cell lines in triplicate, namely NCI-H226 (non-small cell lung carcinoma), 786-O (renal cell adenocarcinoma) and SF268 (glioblastoma). We specifically selected these cell lines as we had found them to be moderately to strongly resistant to VV, and, in addition, were amenable to high-throughput handling. We screened with vvDD-eGFP, the vvDD clinical candidate that also expresses enhanced GFP. We chose this strain of VV due to the fact that it is undergoing clinical testing<sup>47,48</sup> and that it has a WR backbone. A previous study comparing ten strains of VV demonstrated that it had the greatest therapeutic index of all the strains tested including Wyeth, the backbone of Pexa-Vec.<sup>63</sup>

We developed and validated a high-throughput screening assay in a 384-well plate format for each cell line. This included optimizing each experimental element. The overall increase and decrease in GFP area per well was used as a surrogate measure for



**Figure 3.1: Primary genome-wide RNAi screens with vvDD identify 237 genes that enhance spread upon gene silencing**

(A) Schematic overview of primary high-throughput screens. A Dharmacon siRNA library consisting of individually pooled siRNA (*i.e.* 4 siRNAs/gene/well) targeting 18 120 host genes was arrayed in 384-well plates. The screens were conducted in 3 tumour cell lines (*i.e.* NCI-H226, 786-O and SF268) in triplicate. Cells were reverse transfected with siRNA and, 48 hours post-transfection, infected with vvDD-eGFP (MOI=0.05: NCI-H226, SF268; or MOI=0.06: 786-O). Following incubation with virus (24 - 40h; time dependent on the cell line), cells were fixed with formaldehyde and stained with Hoechst, and subsequently imaged. (B-D) Results of each high-throughput screen as conducted in (A). The median robust Z-score (RZS) of each siRNA pool is plotted from the highest (increased spread) to the lowest (decreased spread). Genes outside of the shaded areas were considered as hits. (E) Venn diagram showing the number of overlapping hits (*i.e.* siRNA pools with RZS greater than the shaded area indicated on each plot). MOI: multiplicity of infection; vvDD-eGFP: oncolytic vaccinia virus expressing GFP with thymidine kinase and vaccinia growth factor deleted.



spread as we established that this directly correlated with increases and decreases in virus foci area in each well. First, we monitored spread over time in order to determine the multiplicity of infection (MOI) and the incubation time that would allow for the detection of both increases and decreases in spread as previously described.<sup>243</sup> For all three cell lines the MOI was 0.05 (*i.e.* NCI-H226, SF268) or 0.06 (*i.e.* 786-O), and the incubation times for NCI-H226, SF268 and 786-O were 40, 30 and 24 hours post-infection (hpi), respectively. For data analysis, we employed the robust Z score (or median absolute deviation) method<sup>244,245</sup> on a plate-wise basis. For hit calling, depending on the desired stringency, scores of >2 or 3 and <-2 or -3 have been suggested as upper and lower cut-offs, respectively.<sup>244</sup> Two recent vaccinia human genome-wide screens<sup>196,197</sup> set -1.5 as the lower cut-off with no upper cut-offs described, as only genes that decreased spread upon silencing (*i.e.* proviral hits) were the focus of these screens. As previously alluded, our focus was on genes that enhanced spread upon knockdown (*i.e.* antiviral hits); based on the controls and available metrics, we set our upper cut-offs as 2, 1.65 and 2.5 for the NCI-H226, 786-O and SF268 screens, respectively. With these cut-offs, we identified 237 antiviral hits in at least two out of the three cell lines and 23 in all three cell lines (Fig. 3.1B-E).

Although identifying host factors required for replication was not our focus, in agreement with the findings of previous VV RNAi screens,<sup>196-198</sup> bioinformatic analysis of proviral hits with The Database for Annotation, Visualization, and Integrated Discovery (DAVID)<sup>246</sup> revealed high enrichment of proteasome components, genes involved in ubiquitination, and genes involved in translation. It was evident that many of these genes were also critical for cell function, as their knockdown was cytotoxic. We

also identified nuclear pore proteins (*e.g.* NUP62, NUP98), which were identified in the primary VV RNAi screens conducted by Mercer *et al.*,<sup>196</sup> Sivan *et al.*,<sup>197</sup> and Beard *et al.*<sup>198</sup> with NUP62 being validated as a true hit by Sivan *et al.*<sup>197</sup>

### **3.3.2 Secondary screen validation with a custom shRNA library**

As a secondary validation strategy, we examined the 237 candidates that were hits in at least 2 out of the 3 screens and curated a subset of these for a secondary validation screen based on the magnitude of the hit and on biological interest. We employed a custom shRNAi library consisting of individually arrayed shRNAs targeting 116 selected genes with one to five shRNAs per gene (*i.e.* 384 shRNAs total). We conducted the screen in the 786-O cell line as we found this cell line to be the most conducive to lentiviral transduction out of the primary screen cell lines. For data analysis, we used negative scaling: samples were scaled to the median of the negative controls on a plate-wise basis. To prevent edge effect from impacting the controls or samples, we positioned them all in the inner wells of the microtiter plates and filled the perimeter wells with media. The screen was conducted in triplicate and the median score of the three replicates for each sample was used for hit calling. The cut-off for hit calling was a 1.5-fold increase over the negative controls. Out of the 116 genes that were tested in the secondary screen, 83 were scored as hits with a minimum of one shRNA (72%). Out of the 23 genes that enhanced spread upon silencing in all three cell lines in the primary screen, 21 were included in the secondary validation screen. 15 out of the 21 genes (71%) had a least one shRNA that enhanced spread, and seven of these genes had as many as two or three shRNAs (Table 1).

While no genome-wide screens have been specifically conducted to identify

**Table 1: Secondary screen validation of the hits that enhanced spread across all three cell lines in the primary screens**

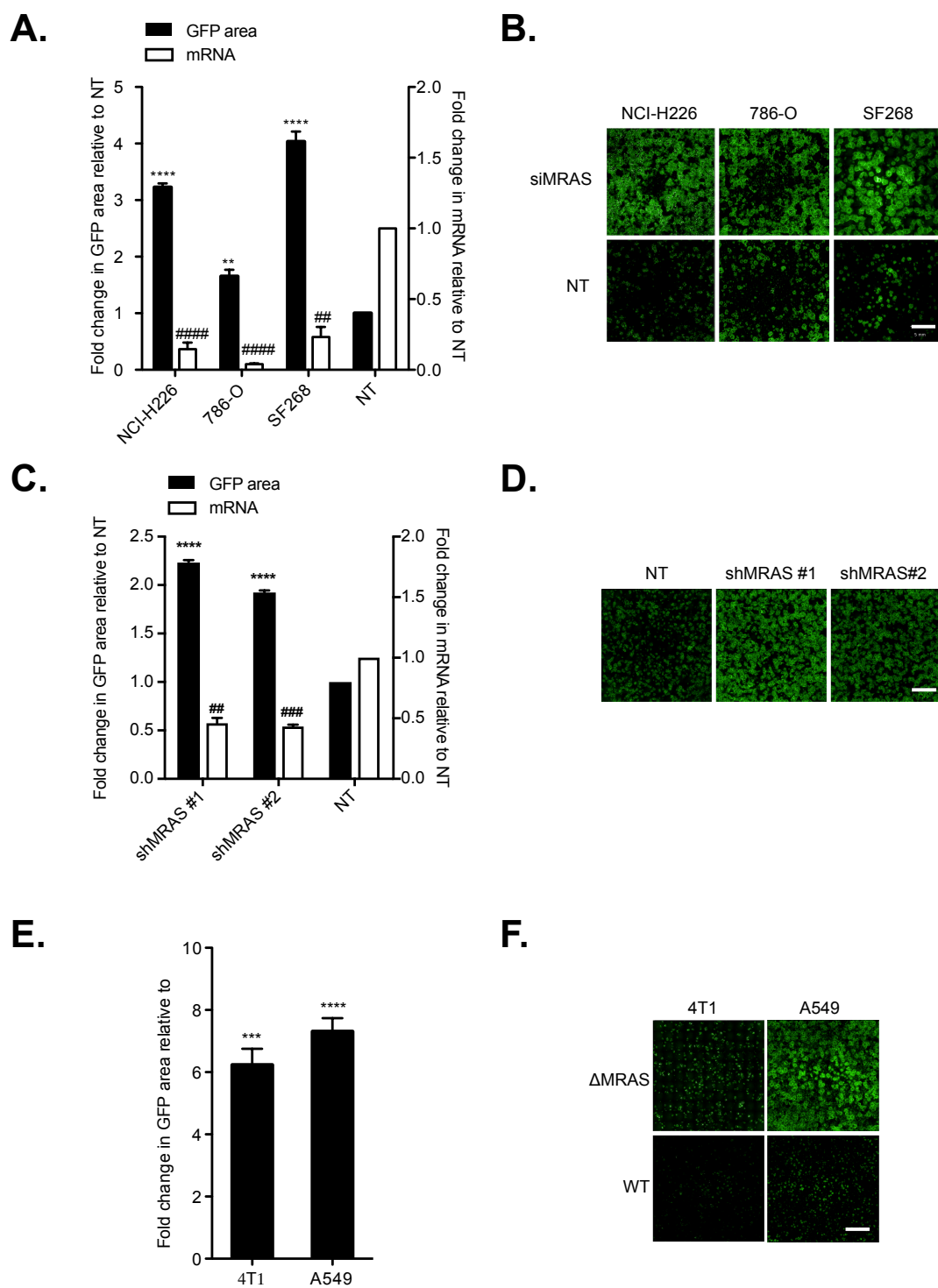
| Symbol  | Gene ID | Clone ID       | Fold change* | Gene Description                                     |
|---------|---------|----------------|--------------|------------------------------------------------------|
| ACP6    | 51205   | TRCN0000052701 | 2.0          | Acid phosphatase 6,                                  |
| APOO    | 79135   | TRCN0000072703 | 3.5          | lysophosphatidic<br>Hypothetical protein MGC4825     |
| DLX5    | 1749    | TRCN0000007447 | 3.3          | Distal-less homeobox 5                               |
|         |         | TRCN0000007446 | 2.2          |                                                      |
| IRX5    | 10265   | TRCN0000018302 | 1.8          | Iroquois homeobox protein 5                          |
| MCF2    | 4168    | TRCN0000038169 | 2.5          | MCF.2 cell line derived                              |
| MRAS    | 22808   | TRCN0000036989 | 1.6          | transforming sequence<br>Muscle RAS oncogene homolog |
| NRAP    | 4892    | TRCN0000178952 | 1.9          | Nebulin-related anchoring<br>protein                 |
| OBFC1   | 79991   | TRCN0000128123 | 3.2          | Oligonucleotide/oligosaccha-                         |
|         |         | TRCN0000128703 | 1.5          | ride-binding fold containing 1                       |
| PCDHGB3 | 56102   | TRCN0000056395 | 3.2          | Protocadherin gamma                                  |
|         |         | TRCN0000056394 | 2.3          | subfamily B, 3                                       |
|         |         | TRCN0000056396 | 1.7          |                                                      |
| RAB3IL1 | 5866    | TRCN0000140361 | 2.9          | RAB3A interacting protein                            |
|         |         | TRCN0000139844 | 1.5          | (rabin3)-like 1                                      |
| RAMP3   | 10268   | TRCN0000060982 | 2.4          | Receptor (calcitonin) activity                       |
|         |         | TRCN0000060980 | 1.5          | modifying protein 3                                  |
| RFC3    | 5983    | TRCN0000330991 | 3.0          | Replication factor subunit 3                         |
|         |         | TRCN0000330990 | 2.7          |                                                      |
| SOD2    | 6648    | TRCN0000005942 | 2.6          | Superoxide dismutase 2,                              |
|         |         |                |              | mitochondrial                                        |
| TM2D1   | 83941   | TRCN0000167182 | 2.2          | TM2 domain containing 1                              |
|         |         | TRCN0000167213 | 2.1          |                                                      |
|         |         | TRCN0000167893 | 1.9          |                                                      |
| USP31   | 57478   | TRCN0000004550 | 2.7          | Ubiquitin specific peptidase 31                      |

\*Score = fold change in GFP area relative to non-targeting controls

antiviral VV genes, Panda *et al.*<sup>247</sup> recently published the results of genome-wide RNAi screens in two different cell lines with a serine protease inhibitor 1 (SPI-1) rabbitpox virus mutant. One of their very top antiviral hits that they also validated with VV was replication factor C subunit 3 (RFC3). In agreement with their work, RFC3 was among the 23 genes that was a hit across all three cell lines and was a strong hit in the secondary screen with two shRNAs enhancing spread by three-fold (Table 1).

### **3.3.3 *In vitro* validation of MRAS as a true hit using siRNA, shRNA and CRISPR-Cas9**

We selected MRAS for further investigation as it was the top hit among the 23 hits that enhanced spread across all three cell lines in the primary screen, and it was validated in the secondary screen. To confirm that the phenotype we observed upon loss of MRAS was due to an on-target effect, we performed multiple orthogonal assays in multiple tumour cell lines. We first quantified spread and gene silencing upon knockdown with siRNA in all three primary screen cell lines. NCI-H226, 786-O and SF268 were reverse transfected with siRNA targeting MRAS or non-targeting siRNA control in a six-well plate format. At 48 h post-transfection, cells were infected with vvDD-eGFP; cell lysates were also collected at this time-point to assess knockdown. Knockdown of MRAS across all three cell lines resulted in a two- to four-fold increase in spread relative to the non-targeting control. This corresponded with a 77-96% knockdown of mRNA (Fig. 3.2A,B). Next, we generated stable cell lines of 786-O expressing non-targeting shRNA or different shRNAs targeting MRAS. Confluent wells were infected with vvDD-eGFP. There was a two-fold increase of spread with both shRNAs, and the corresponding knockdown was greater than 50% for both shRNAs



**Figure 3.2: Targeting of MRAS using siRNA, shRNA and CRISPR-Cas9 enhances spread in multiple tumour cell lines**

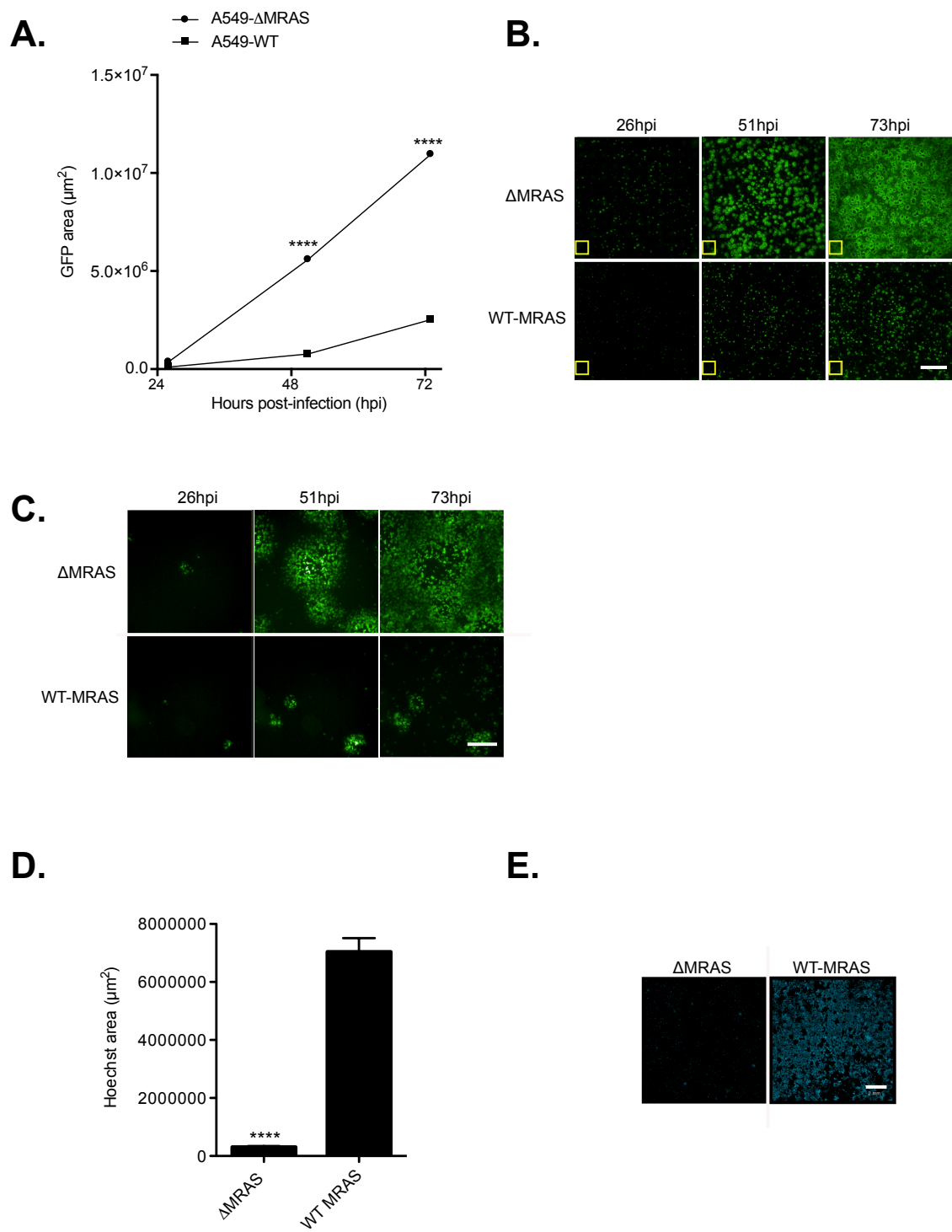
(A) NCI-H226, 786-O and SF268 cells were reverse transfected with siRNA targeting MRAS or with a non-targeting siRNA control and subsequently infected with vvDD-eGFP (NCI-H226: 900 PFU/well; 786-O: 500 PFU/well SF268: 1000 PFU/well) 48 h post transfection; results at peak time-points (*i.e.* 120 hpi, 96 hpi, and 72 hpi, respectively) are shown on the plot (n=3). Relative mRNA expression of MRAS following siRNA knockdown (48 hours post-transfection) is depicted by white bars (right y axis) (n=3). (B) NCI-H228, 786-O and SF268 cells were treated as in (A). Images of representative wells corresponding with the times shown on the plot. (C) Confluent wells of 786-O cells stably expressing shRNA targeting MRAS or non-targeting shRNA were infected with vvDD-eGFP (MOI of 0.002). Results at 72 hpi are shown on the plot (n=4). Relative mRNA expression of MRAS at 0 hpi is depicted by white bars (right y axis) (n=2). (D) Cells were treated as in (C). Images of representative wells at 72 hpi are shown. (E) Confluent wells of 4T1 and A549 cells with wild-type MRAS (WT) or with MRAS disrupted ( $\Delta$ MRAS) using CRISPR-Cas9 technology were infected with vvDD-eGFP (MOIs of 0.1 and 0.001, respectively). Results for 4T1 and A549 at 96 hpi and 72 hpi respectively are shown on the plot (n=3). (F) Cells were treated as in (E). Images of representative wells are shown. NT: non-targeting; siMRAS: siRNA targeting MRAS; shMRAS: shRNA targeting MRAS; hpi: hours post-infection; MOI: multiplicity of infection; Scale bar: 5 mm; \*\*  $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ , ## $p \leq 0.01$ , ### $p \leq 0.001$ , #### $p \leq 0.001$  by t-test; Data presented as means  $\pm$  SEM.

targeting MRAS (Fig. 3.2C,D).

As a final validation, we engineered both a human and mouse cell line, namely A549 (human lung carcinoma) and 4T1 (murine mammary carcinoma) with MRAS disrupted (*i.e.*  $\Delta$ MRAS) using clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology. We chose to engineer these specific cell lines as they could be engrafted in mice and thus used for *in vivo* testing if desired. We used the mutant endonuclease Cas9n in order to limit off-target effects as well as increase the likelihood of repair via error-prone non-homologous end joining.<sup>248</sup> Confluent wells of both wild-type and 4T1  $\Delta$ MRAS and A549  $\Delta$ MRAS cells were infected with an MOI of 0.1 or 0.001, respectively. There was a six- to seven-fold increase in spread (Fig. 3.2E,F) for both 4T1  $\Delta$ MRAS and A549  $\Delta$ MRAS cells over their wild-type counterparts. The fact that we observed an enhancement of spread of vvDD upon the loss of MRAS using siRNA, shRNA and CRISPR-Cas9 in multiple cell lines provides solid evidence that MRAS is a true hit.

#### **3.3.4 Loss of MRAS increases the spread and oncolytic activity of JX-594**

We were also interested in determining whether a similar enhancement of spread could be observed with the leading clinical candidate, JX-594. We infected confluent wells of both A549 and A549  $\Delta$ MRAS cells with JX-594 expressing GFP and conducted a time-course. There were seven- and four-fold increases in spread in A549  $\Delta$ MRAS infected cells over wild-type A549 cells at 51 and 73 hpi, respectively (Fig. 3.3A,B). Upon a close inspection of individual fields, a clear enhancement in infectious focus area is observed in A549  $\Delta$ MRAS infected cells over time (Fig. 3.3C). We also fixed the plates at 144 hpi and stained the cells with Hoechst to determine whether the increase





### **Figure 3.3: Loss of MRAS increases the spread and oncolytic activity of JX-594**

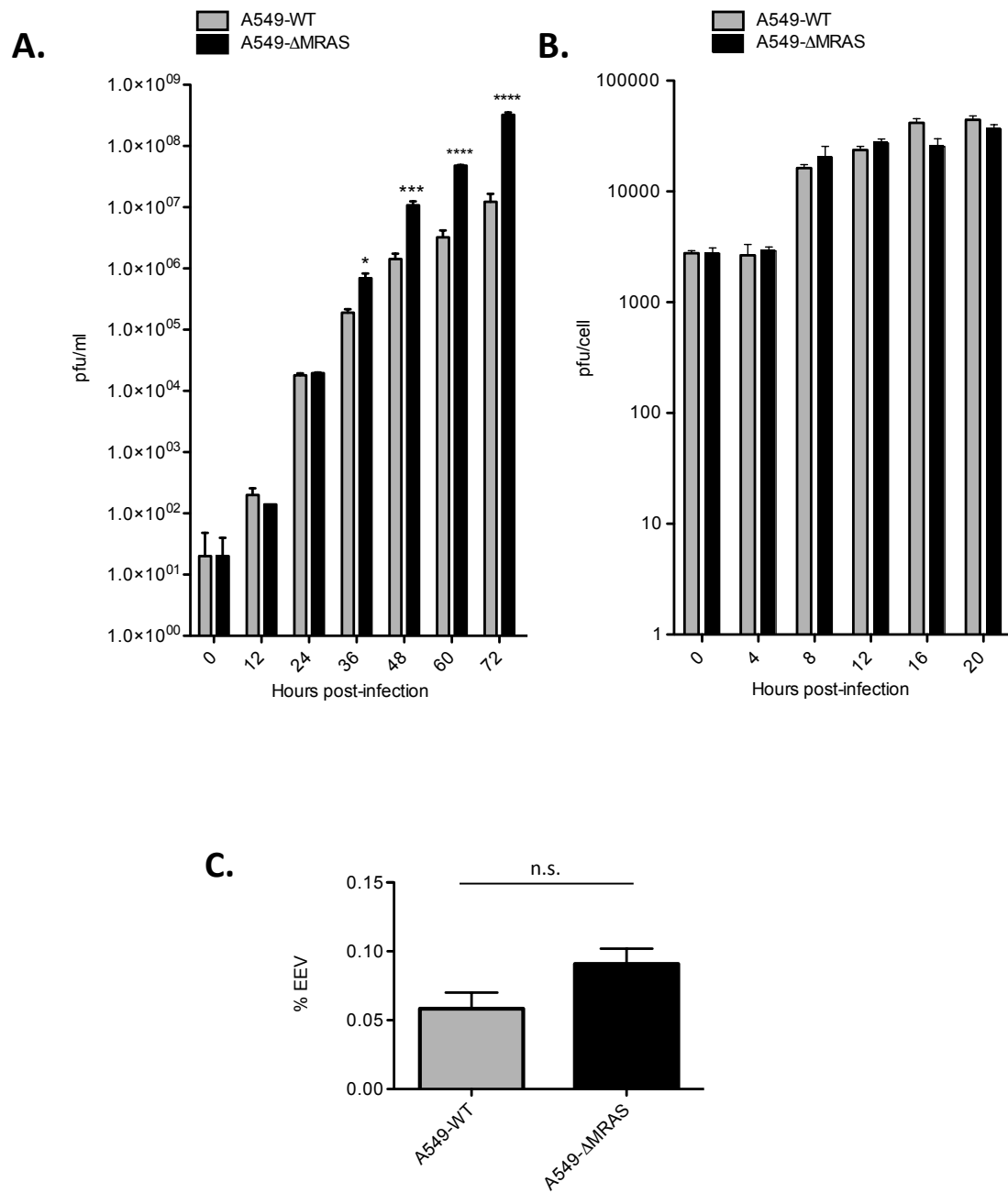
Confluent wells of A549 and A549  $\Delta$ MRAS cells were infected with JX-594 at an MOI of 0.001. Results of a 72 h time-course are depicted on the plot (n=3). (B) Cells were treated as in (A). Images of one representative well is shown over time. Scale bar: 5 mm. (C) Cells were treated as in (A). Images of one field is shown over time. Field is shown as a yellow box in (B). Scale bar: 500  $\mu$ m. (D) Cells were treated as in (A). Results are shown on the plot of cells fixed and stained with Hoechst at 144 hpi (n=3). (E) Cells were treated as in (D). Images of representative wells are shown. Scale bar: 2 mm; yellow boxes: fields displayed in (C);  $\Delta$ MRAS cells: A549 cells with MRAS disrupted using CRISPR-Cas9 technology; hpi: hours post-infection; MOI: multiplicity of infection; \*\*\*\*  $p \leq 0.0001$ , by t-test; Data presented as means  $\pm$  SEM

in spread resulted in increased cell death. The A549  $\Delta$ MRAS cells had 96% less cells as measured by the Hoechst area compared to wild-type cells (Fig. 3.3D,E). These results demonstrate that the loss of MRAS not only enhances the spread of vvDD, but also JX-594 and that an enhancement in spread results in increased cell death.

### **3.3.5 Enhancement of VV spread upon loss of MRAS is not due to increased burst size, the percentage of EEV produced, or actin tail formation**

Once we established MRAS as a true hit and that it enhanced the spread of both vvDD and JX-594, we more closely examined the impact of the loss of MRAS on VV spread. In our previous assays, we used GFP area as a measure for quantifying spread. As another means to quantify spread, we conducted a multi-step growth curve. We infected confluent wells of both A549 and A549  $\Delta$ MRAS cells with vvDD-eGFP at an MOI of 0.001. We collected infected cells and culture medium from whole wells at 12 h intervals and quantified the amount of virus at each time point by plaque assay. From 48 to 72 hpi, 13- to 26-fold more virus was recovered from A549  $\Delta$ MRAS infected cells compared to A549 (Fig. 3.4A). These results further confirm that the loss of MRAS leads to the enhancement of cell-to-cell spread.

To determine whether the increase in spread was due to a greater number of virions produced per cell (*i.e.* burst size), we conducted a one-step growth curve by infecting A549 and A549  $\Delta$ MRAS cells at an MOI of 10 and collecting infected cells and culture medium every four hours. We quantified the amount of virus generated at each time point by plaque assay and found that there was no significant difference in burst size in virus grown in A549  $\Delta$ MRAS cells versus those grown in A549 at any time-point (Fig. 3.4B). We, therefore, concluded that the enhancement of spread was not due to



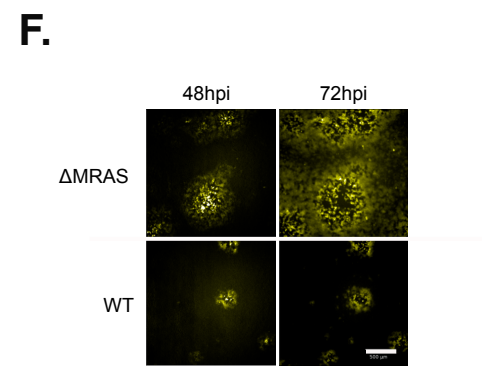
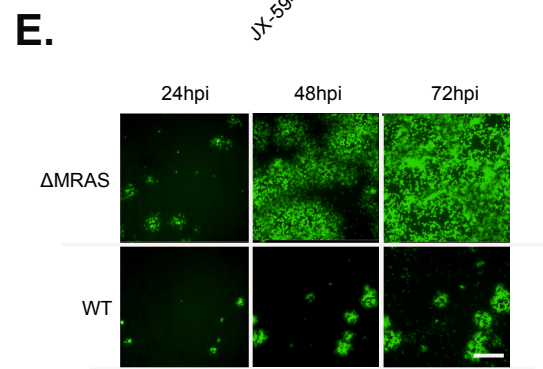
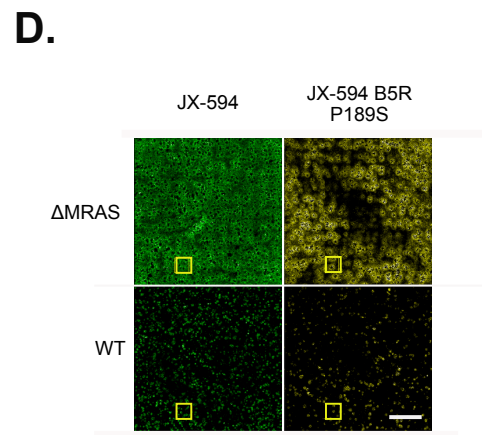
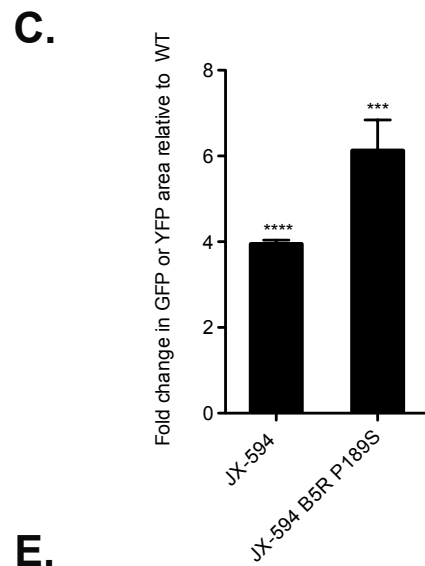
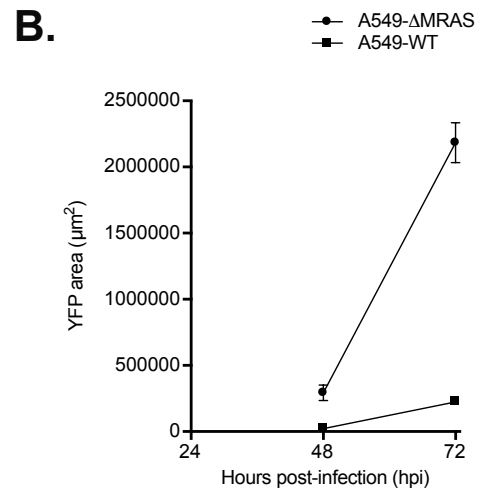
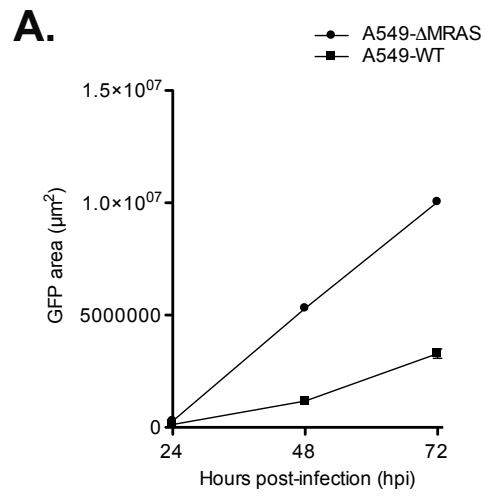
**Figure 3.4: Loss of MRAS enhances cell-to-cell spread, but not the burst size or the percentage of EEV produced**

(A) Confluent wells of A549 and A549  $\Delta$ MRAS cells were infected with vvDD-eGFP (MOI = 0.001). At 12 h intervals, infected cells from whole wells were scraped into the culture medium, and the amount of virus was quantified by plaque assay (n=2-6). (B) Confluent wells of A549 and A549  $\Delta$ MRAS cells were infected with vvDD-eGFP (MOI = 10). At 4 h intervals, infected cells from whole wells were scraped into the culture medium, and the amount of virus was quantified by plaque assay (n=2-3). (C) Confluent wells of A549 and  $\Delta$ MRAS A549 cells were infected with vvDD-eGFP (MOI of 0.001). The amount of virus from infected culture medium alone or from infected cells alone was quantified by plaque assay at 72 hpi (n=2-3). Shown on the plot is the percentage of EEV produced [PFU in culture medium/PFU of infected cells alone (%)]. A549  $\Delta$ MRAS cells: A549 cells with MRAS disrupted using CRISPR-Cas9 technology; MOI: multiplicity of infection; PFU: plaque forming units; n.s.: non-significant, \*p < 0.05, \*\*\*p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, by t-test; Data presented as means  $\pm$  SEM.

more virions being produced on a per cell basis.

We next assessed whether there was an increase in the percentage of EEVs produced. A549 and A549  $\Delta$ MRAS cells were infected with vvDD-eGFP (MOI of 0.001) and, at 72 hpi, culture medium and cells were collected separately from whole wells. The number of total plaque forming units (PFUs) in each fraction was quantified by plaque assay, and the percentage of EEV produced was calculated. The percentage of EEV recovered from A549  $\Delta$ MRAS cells was not significantly different from that of A549 (0.1% versus 0.06%, respectively)(Fig. 3.4C) indicating that a greater percentage of EEV was not responsible for the MRAS-mediated enhancement of VV spread.

As actin tails have been shown to play a role in the rapid spread of VV,<sup>229</sup> we also explored whether the spread enhancement mediated by the knockout of MRAS was dependent on actin tail formation. As previously described, VV can spread via the formation of actin tails that form late during infection.<sup>249-251</sup> We tested a VV mutant (*i.e.* JX-594 B5R P198S) previously shown to be defective in actin tail formation, specifically one with a P189S mutation in the short consensus repeat 4 (SCR4) domain of the B5 protein.<sup>229</sup> B5 is found on the surface of EVs and is a type I membrane glycoprotein having an extracellular portion comprised of four consensus repeat (SCRs) domains. We infected confluent wells of A549 and A549  $\Delta$ MRAS cells with JX-594 expressing GFP and with JX-594 B5R P198S expressing yellow fluorescent protein (YFP) and monitored spread over time. The YFP signal was more difficult to detect, especially at the 24 h time point; therefore, no data is available for this time point. We observed a significant enhancement of spread with JX-594 in A549  $\Delta$ MRAS cells compared to A549 as previously observed (Fig. 3.3), but also with JX-594 B5R P198S (Fig. 3.5A-F)



**Figure 3.5: Enhancement of VV spread mediated by the loss of MRAS is not dependent on actin tail formation**

(A) Confluent wells of A549 and A549  $\Delta$ MRAS cells were infected with JX-594 (with a GFP reporter gene) at an MOI of 0.001. Results of a live time course are plotted on the graph (n=3). (B) Confluent wells of A549 and A549  $\Delta$ MRAS cells were infected with JX-594 B5R P189S (with a YFP reporter gene) at an MOI of 0.001. Results of a live time course are plotted on the graph (n=3). (C) Cells were treated as in (A,B) and fixed at 72 hpi. The relative fold change in fluorescent protein area of infected A549  $\Delta$ MRAS over A549 cells is plotted on the graph. (D) Cells were treated as in (C). Representative images at 72 hpi are shown. Yellow box: field displayed in (E,F); Scale bar: 5mm. (E) Cells were treated as in (A). Images of one field over time are shown. Scale bar: 500 $\mu$ m. (F) Cells were treated as in (B). Images of one field over time are shown. Scale bar: 500 $\mu$ m. MOI: multiplicity of infection; hpi: hours post-infection; A549  $\Delta$ MRAS cells: A549 cells with MRAS disrupted using CRISPR-Cas9 technology; WT: wild-type; GFP: green fluorescent protein; YFP: yellow fluorescent protein; \*\*\*p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, by t-test; Data presented as means  $\pm$  SEM.

demonstrating that the enhancement of spread was not contingent on actin tail formation and that further studies are required to decipher the mechanism of MRAS-mediated enhancement of VV spread.

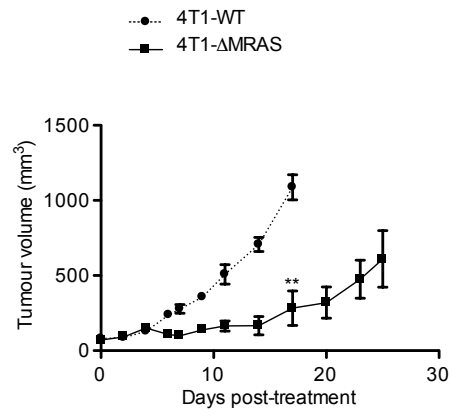
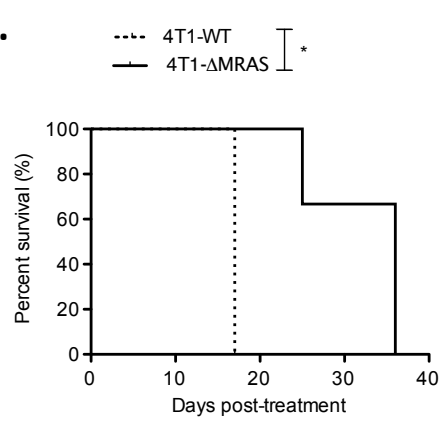
### **3.3.6 Treatment with vvDD coincident with the knockout of MRAS delays tumour progression and prolongs survival *in vivo***

As the inhibition of MRAS clearly enhanced VV spread *in vitro*, we sought to study the impact of inhibiting MRAS *in vivo* on the efficacy of VV-based therapy. As we had already generated 4T1  $\Delta$ MRAS cells, we elected to conduct a pilot study in an orthotopic 4T1 breast tumour model. We implanted BALB/c mice with 4T1 and 4T1  $\Delta$ MRAS cells and treated them with vvDD-R2RLUC [vvDD expressing mCherry red fluorescent protein]. Tumour progression was significantly delayed in the mice implanted with 4T1  $\Delta$ MRAS (Fig. 3.6A) as illustrated by the significant difference in tumour size measured at day 17 when all the mice reached endpoint in the 4T1 tumour-bearing group (280 vs. 1090 mm<sup>3</sup>, respectively;  $p = 0.005$ ). The survival of mice bearing 4T1  $\Delta$ MRAS tumours was also significantly longer compared to the 4T1 tumour-bearing mice (median survival 36 vs. 17 days, respectively,  $p = 0.03$ ) (Fig. 3.6B). This study served as proof of concept for moving forward with further *in vivo* investigation in a more clinically relevant context as the knockout of MRAS upon treatment with vvDD was shown to significantly delay tumour progression and prolong survival *in vivo*.

### **3.3.7 vvDD engineered to inhibit MRAS enhances spread *in vitro* and efficacy *in vivo***

A more clinically relevant method to test MRAS would be to administer VV in combination with a small molecule inhibitor of MRAS or to engineer VV to inhibit



**A.****B.**

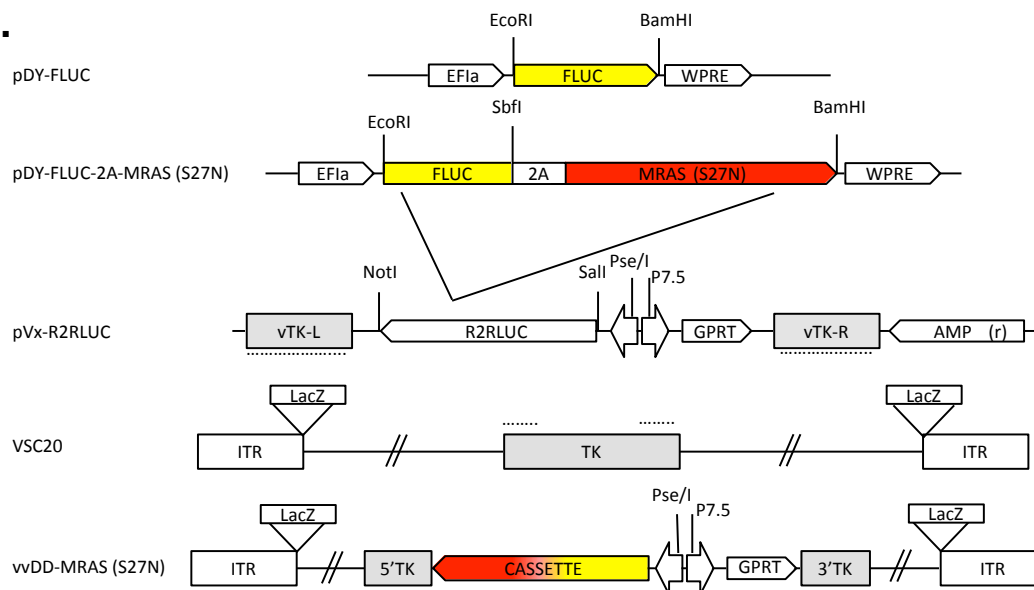
**Figure 3.6: Loss of MRAS delays tumour progression and prolongs survival in mice treated with vvDD**

(A) 4T1 or 4T1  $\Delta$ MRAS cells ( $1 \times 10^5$  cells) were implanted in the left mammary fat pad of BALB/c mice ( $n = 3/\text{group}$ ). Tumours were treated by a single intratumoural injection ( $2.5 \times 10^7$  PFUs/mouse) with vvDD-FLUC when they reached 50-100 mm<sup>3</sup> in volume. Tumour growth was assessed by caliper measurements. The mean tumour volume of each group is plotted until the first mouse in each group reaches endpoint (*i.e.*  $\geq 1000$  mm<sup>3</sup>). (B) Kaplan-Meier survival plot of mice treated as in (A). 4T1  $\Delta$ MRAS cells: 4T1 cells with MRAS disrupted using CRISPR-Cas9 technology; \*\*  $p \leq 0.01$ , by t-test; \* $p = 0.03$  by Log-rank Mantel-Cox test. Data presented as means  $\pm$  SEM.

MRAS. Testing a small molecule inhibitor of MRAS was considered, but none were readily available. We, therefore, elected to engineer a VV expressing a dominant negative mutant of MRAS. An MRAS dominant negative mutant with a substitution of asparagine (N) for serine (S) at position 27 (*i.e.* S27N), corresponding to the RAS (S17N) mutant, had previously been studied.<sup>252,253</sup> The RAS (S17N) dominant-negative mutant inhibits RAS by outcompeting RAS for binding to guanine-nucleotide-exchange factors (GEFs), which results in the formation of unproductive complexes thereby interfering with RAS activation.<sup>254</sup> We engineered vvDD expressing both a mouse and human dominant negative mutant of MRAS (S27N) in the TK locus. To engineer vvDD-MRAS (S27N), we constructed a cassette containing firefly luciferase and MRAS dominant negative (S27N) separated by an intervening porcine teschovirus-1 virus 2A sequence to allow for the bicistronic expression, and cloned it into the pDY expression vector (Fig. 3.7A). The cassette was then subcloned into the pVx-R2RLUC shuttle vector used for recombination at the TK locus of the VV genome. Following eight rounds of purification, we sequenced the MRAS gene of vvDD-MRAS (S27N) for both human [*i.e.* vvDD-hMRAS (S27N)] and mouse [*i.e.* vvDD-mMRAS (S27N)] genes and confirmed the substitution of asparagine at position 27 for serine (Fig. 3.7B,C).

We next compared the spread of vvDD to vvDD-MRAS (S27N) *in vitro* on a panel of mouse and human tumour cell lines as well as on a normal human fibroblast cell line (*i.e.* GM38). To assess spread, we infected confluent wells of six-well plates with vvDD or vvDD-MRAS (S27N) both expressing firefly luciferase and quantified the fold change in bioluminescence (*i.e.* radiance) of vvDD-MRAS (S27N) relative to vvDD at 48 hpi. In the three human tumour cell lines tested, we observed a relative two-fold to three-fold

**A.**



**B.**

|              |     |              |      |      |      |                  |     |     |      |      |      |        |      |       |       |
|--------------|-----|--------------|------|------|------|------------------|-----|-----|------|------|------|--------|------|-------|-------|
| hMRAS (S27N) | 1   | ATSAVPSDNLP  | TYKL | VVVG | DGGV | GK <sup>27</sup> | N   | ALT | IQFF | QKIF | VPDY | DPTIED | SYLK | HTEID | NQW   |
| hMRAS ref    | 1   | MATSAVPSDNLP | TYKL | VVVG | DGGV | GKS              | A   | LT  | IQFF | QKIF | VPDY | DPTIED | SYLK | HTEID | NQW   |
| hMRAS (S27N) | 61  | AILDVLD      | TAGQ | EEFS | SAMR | EQYMR            | TG  | DG  | FLIV | SV   | TDK  | ASFE   | HVDR | HFQL  | LILRV |
| hMRAS ref    | 61  | AILDVLD      | TAGQ | EEFS | SAMR | EQYMR            | TG  | DG  | FLIV | SV   | TDK  | ASFE   | HVDR | HFQL  | LILRV |
| hMRAS (S27N) | 121 | MILVANK      | V    | DL   | MHLR | KITR             | EQG | K   | EMAT | KHN  | IPYI | ETSA   | KD   | PP    | LN    |
| hMRAS ref    | 121 | MILVANK      | V    | DL   | MHLR | KITR             | EQG | K   | EMAT | KHN  | IPYI | ETSA   | KD   | PP    | LN    |
| hMRAS (S27N) | 181 | EKSQ         | K    | K    | K    | K                | K   | K   | K    | K    | K    | K      | K    | K     | K     |
| hMRAS ref    | 181 | EKSQ         | K    | K    | K    | K                | K   | K   | K    | K    | K    | K      | K    | K     | K     |

**C.**

|              |     |          |      |      |      |       |      |                  |      |      |      |      |      |        |       |
|--------------|-----|----------|------|------|------|-------|------|------------------|------|------|------|------|------|--------|-------|
| mMRAS (S27N) | 1   | ATSAVPS  | ENL  | P    | TYKL | VVVG  | DGGV | GK <sup>27</sup> | N    | ALT  | IQFF | QKIF | VPDY | DPTIED | SYLK  |
| mMRAS ref    | 1   | MATSAVPS | ENL  | P    | TYKL | VVVG  | DGGV | GKS              | A    | LT   | IQFF | QKIF | VPDY | DPTIED | SYLK  |
| mMRAS (S27N) | 61  | AILDVLD  | TAGQ | EEFS | SAMR | EQYMR | TG   | DG               | FLIV | SV   | TDK  | ASFE | HVDR | HFQL   | LILRV |
| mMRAS ref    | 61  | AILDVLD  | TAGQ | EEFS | SAMR | EQYMR | TG   | DG               | FLIV | SV   | TDK  | ASFE | HVDR | HFQL   | LILRV |
| mMRAS (S27N) | 121 | MILVANK  | V    | DL   | MHLR | KVTR  | DQ   | G                | K    | EMAT | KYN  | IPYI | ETSA | KD     | PP    |
| mMRAS ref    | 121 | MILVANK  | V    | DL   | MHLR | KVTR  | DQ   | G                | K    | EMAT | KYN  | IPYI | ETSA | KD     | PP    |
| mMRAS (S27N) | 181 | EKNQ     | K    | K    | K    | K     | K    | K                | K    | K    | K    | K    | K    | K      | K     |
| mMRAS ref    | 181 | EKNQ     | K    | K    | K    | K     | K    | K                | K    | K    | K    | K    | K    | K      | K     |

### Figure 3.7: Engineering of vvDD-MRAS (S27N) to inhibit MRAS expression *in vivo*

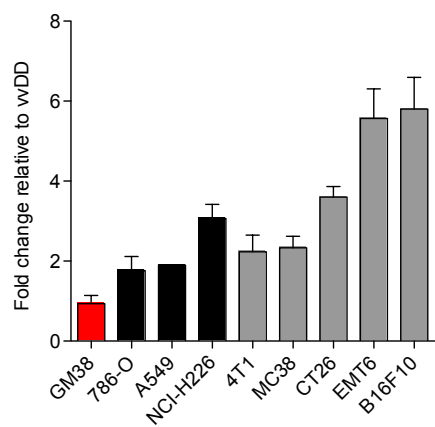
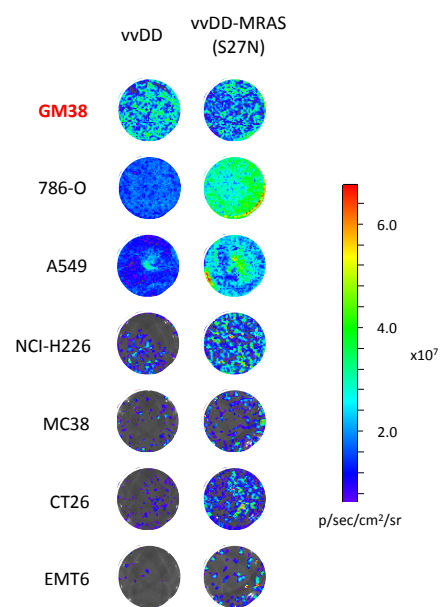
(A) Linear diagrams of the lentivirus expression plasmids of pDY-FLUC and pDY-FLUC with the FLUC-2A-MRAS cassette, pVx-R2RLUC plasmid (shuttle vector for cloning into vaccinia), parental VSV20 vaccinia virus [*i.e.* VV with the vaccinia growth factor (VGF) genes disrupted by the insertion of LacZ within the inverted terminal repeats], and a linear diagram of the recombinant vvDD-MRAS (S27N). vTK-L and vTK-R consist of the left and right segments of the thymidine kinase (TK) gene. Dotted lines indicate sites of predicted recombination between the pVx-R2RLUC vector and VSC20. (B) Sequence alignment of the hMRAS (*i.e.* human MRAS) gene from vvDD-hMRAS (S27N) with the NCBI reference sequence (*i.e.* hMRAS ref). (C) Sequence alignment of mMRAS (*i.e.* mouse MRAS) from vvDD-mMRAS (S27N) with the NCBI reference sequence (*i.e.* mMRAS ref). R2RLUC = cassette containing red fluorescent protein (mCherry) and renilla luciferase gene separated by the porcine teschovirus-1 virus 2A sequence motif; Pse/I = synthetic early/late promoter; P7.5 = 7.5 promoter; AMP (r) = ampicillin resistance gene; GPRT = xanthine-guanine phosphoribosyl transferase gene (*i.e.* drug selectable marker); ITR = inverted terminal repeat; WPRE = Woodchuck hepatitis posttranscriptional regulatory element; EF1a = EF-1 alpha promoter.

enhancement of spread (Fig. 3.8 A,B) over vvDD. In the five mouse tumor cell lines tested, we observed a two-fold to six-fold increase in spread over vvDD (Fig. 3.8 A,B). Notably, there was no change in spread in the normal human fibroblast cell line, GM38, indicating that vvDD-MRAS (S27N)-mediated spread enhancement was tumour-specific.

As enhancement was observed with vvDD-MRAS (S27N) *in vitro*, we pursued *in vivo* testing. We chose an ectopic CT26 colon carcinoma murine model as it is a moderately resistant model and a marked enhancement of spread was observed *in vitro* with this cell line (Fig. 3.8A,B). We subcutaneously implanted BALB/c mice with CT26 cells in the right flanks and treated intratumourally with two doses of vvDD, vvDD-MRAS (S27N), or vehicle control (*i.e.* Tris buffer) 48 h apart. Tumour volume data was collected and analyzed at day 10 post-treatment when the first mouse reached endpoint ( $\geq 1000 \text{ mm}^3$ ). Mice treated with vvDD-MRAS (S27N) had significantly lower tumour burden compared to mice treated with vvDD (110 vs. 310  $\text{mm}^3$ ;  $p \leq 0.01$ , respectively) and mice treated with vehicle control (100 vs. 420  $\text{mm}^3$ ;  $p \leq 0.001$ , respectively) (Fig. 3.9A). Mice treated with vvDD-MRAS (S27N) also had significantly prolonged survival compared to vvDD-treated mice ( $p = 0.03$ ) and vehicle control mice ( $p = 0.0001$ ) (Fig. 3.9B) with two durable responses in the vvDD-MRAS (S27N) treatment group. These results demonstrate that the inhibition of MRAS enhances the efficacy of VV *in vivo*.

### 3.4 Discussion

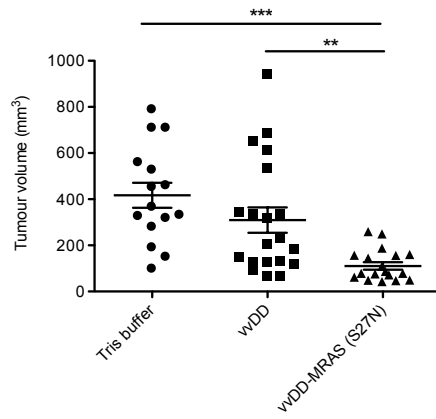
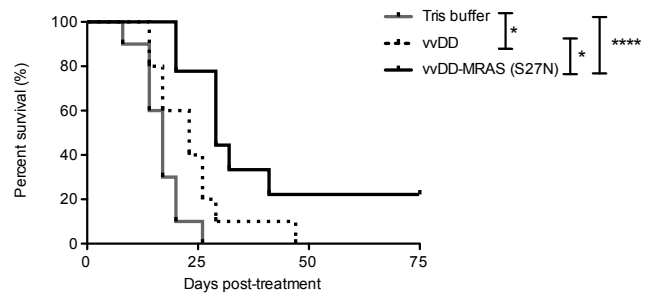
To comprehensively identify host tumour genes that could be manipulated to enhance oncolytic VV virotherapy, we employed human genome-wide RNAi screening

**A.****B.**

**Figure 3.8: vvDD-MRAS (S27N) enhances spread in multiple tumour cell lines, but not in normal human fibroblasts**

(A) Cells were infected with 60 PFUs/well (corresponding to an MOI of ~ of 0.001 as cell densities varied for each cell line) of vvDD or vvDD-MRAS (S27N) each expressing firefly luciferase in a 6-well plate. At 48 hours post infection, luciferin was added to each well and the bioluminescence was quantified. The fold-change in average radiance (p/sec/cm<sup>2</sup>/sr) of vvDD-MRAS (S27N) relative to vvDD is shown on plot. (B) Cells were treated as in (A). Bioluminescent images of representative wells are shown. PFUs: plaque forming units; red bar: normal human fibroblasts; black bars: human tumour cell lines; grey bars: murine tumour cell lines.



**A.****B.**

**Figure 3.9: Treatment with vvDD-MRAS (S27N) increases therapeutic efficacy *in vivo***

(A) CT26 or CT26  $\Delta$ MRAS cells ( $1 \times 10^6$  cells) were implanted in the right flanks of BALB/c mice ( $n = 15-20/\text{group}$ ). When tumours reached 50-100 mm<sup>3</sup> in volume, they were administered with two doses ( $5 \times 10^7$  PFUs/dose) of vvDD, vvDD-MRAS (S27N) or vehicle control (*i.e.* Tris buffer) 48 h apart by intratumoural injection. Tumour growth was assessed at day 10 when the first mouse reached endpoint (*i.e.*  $\geq 1000$  mm<sup>3</sup>). \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , one-way ANOVA, followed by Tukey's post-test (B) Kaplan-Meier survival plot of mice treated as in (A), but monitored for overall survival ( $n = 9-10$ ). \*  $p < 0.05$ , \*\*\*\*  $p \leq 0.0001$ , by Log-rank Mantel-Cox test. Data presented as means  $\pm$  SEM.

in three different tumor cell lines in triplicate. We identified 237 genes that enhanced spread upon siRNA silencing in at least two out of the three cell lines tested and 23 across all three cell lines. We successfully validated one of our top candidates, namely MRAS. Inhibition or knockout of MRAS led to the enhancement of VV spread *in vitro* in all nine tumour cell lines tested namely, NCI-H226, SF268, 786-O, A549, 4T1, CT26, B16F10, EMT6, and MC38. Treatment with a VV encoding a dominant negative mutant of MRAS to inhibit MRAS *in vivo* led to enhanced efficacy with delayed tumour progression, prolonged survival and durable responses in tumour-bearing mice compared to treatment with vvDD alone. This study serves as a proof-of-concept for manipulating host tumour genes to enhance oncolytic vaccinia virotherapy and for employing RNAi screens as a means for identifying host targets.

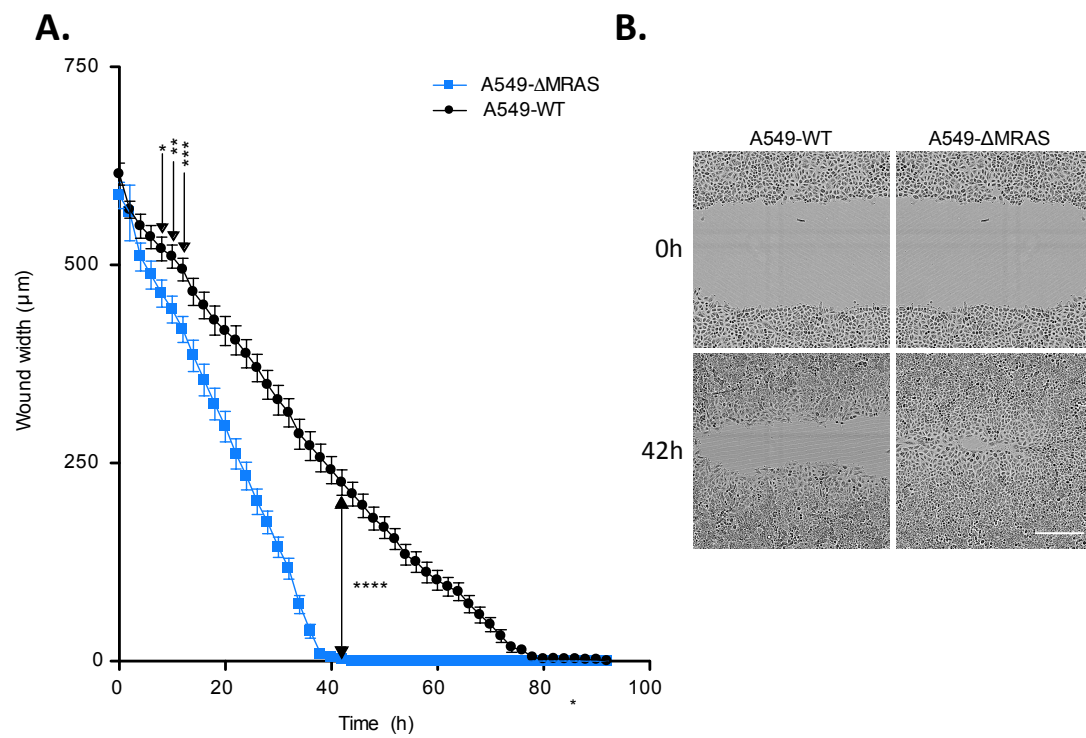
The advantage of RNAi screening is the ability to uncover genes that one might not predict to have an impact on VV spread such as MRAS, which we demonstrated to be a good target for improving VV-based oncolytic treatment. MRAS is a member of the RAS family of small GTPases that includes the prototypic Ras oncogenes, namely HRAS, NRAS and KRAS,<sup>255</sup> which have been well-characterized and are mutated in roughly 30% of cancers.<sup>256</sup> Studies are limited regarding MRAS in relation to cancer. Yang *et al.*<sup>257</sup> found that it was overexpressed in the majority of tumour tissue samples tested of the breast, uterus, stomach, ovary, thyroid, prostate, and small intestine. An analysis of publicly available data by Castro *et al.*<sup>258</sup> from three independent studies found MRAS to be significantly overexpressed in estrogen receptor (ER)-negative breast carcinomas versus ER-positive.<sup>258-261</sup> Expression of a constitutively active form of MRAS has been shown to result in the cellular transformation of NIH 3T3 mouse fibroblasts.<sup>262</sup>

MRAS shares approximately 40-50% overall sequence homology with the prototypic RAS oncogenes.<sup>255</sup> While the GDP/GTP-binding domains are nearly identical and the core effector domains are in fact identical, the extended effector domain of MRAS diverges appreciably by seven amino acids.<sup>252</sup> MRAS signalling pathways and effectors have not been well-characterized, but given the divergence in the extended effector domain, MRAS might be expected to have some different downstream effectors and signalling pathways than the classic RAS proteins. The two best-characterized RAS signalling pathways are the RAF-MEK-ERK and PI3K-AKT pathways, but others include the RALGDS-RAL-PLD and RIN1-ABL pathways.<sup>263</sup> In one yeast two-hybrid study, MRAS was found to interact weakly with known RAS effectors in these pathways such as Raf-1 proto-oncogene (RAF1), A-Raf proto-oncogene (ARAF), B-Raf proto-oncogene (BRAF), phosphoinositol-3 kinase  $\delta$  (PI3K  $\delta$ ), Ral guanine nucleotide dissociation stimulator (RALGDS), and ras interaction protein 1 (RIN1).<sup>262</sup> However, in another study, MRAS was found to bind BRAF in PC12 (rat pheochromocytoma) cells and stimulate ERK activity, but not in NIH 3T3 (murine fibroblast) cells,<sup>264</sup> suggesting that the cellular context also influences signalling. This is supported by a Castro *et al.* study that found that MRAS signalled via the classic Raf/MEK/ERK pathway to activate ELK1 in HeLa and HEK293T cells, but by a RAF/MEK/ERK independent pathway in MCF-7 cells.<sup>258</sup> MRAS has been found to participate in neuronal differentiation,<sup>253,264</sup> osteoblast differentiation,<sup>265</sup> dendrite development,<sup>266</sup> actin reorganization<sup>252</sup> and cell migration.<sup>267</sup>

As described, VV can spread rapidly via the formation of actin tails that form late during infection.<sup>224,249-251</sup> We did not find that MRAS-mediated enhancement of VV

spread was dependent on actin tail formation; however, another mechanism of spread that has been described is by virus-induced cell motility, which is independent of actin tail formation.<sup>224,268-271</sup> Virus-induced motility encompasses cell migration and cell contractility as well as the formation of long cellular projections late during infection (distinct from the formation of actin projections).<sup>268-271</sup> Cell migration follows cell rounding and cell flattening in the sequence of VV-induced cytopathic effects (CPE) and is dependent on early gene expression.<sup>268</sup> Morales *et al.*<sup>271</sup> found that the VV gene F11L promoted cell migration and that VV lacking F11L formed smaller plaques, implicating cell migration in cell-to-cell spread. We did examine the impact of the loss of MRAS in the absence of virus on cell migration as MRAS has been shown to modulate cell migration.<sup>267</sup> In our preliminary work, we found in a wound-healing assay that A549  $\Delta$ MRAS cells filled in the wound area more quickly than the A549 cells (Fig. 3.10A,B), suggesting that the loss of MRAS in A549 cells enhances migration. Investigating cell migration with the 4T1 tumour cell lines (*i.e.* WT and  $\Delta$ MRAS) as well as with the GM38 normal human fibroblast cells would be important in establishing whether migration could play a role in the enhancement of VV spread in this context. We would anticipate that migration would not be increased in MRAS-depleted GM38 cells as we did not observe an enhancement of VV spread upon inhibition of MRAS, but we would expect it to be increased in 4T1  $\Delta$ MRAS cells compared to 4T1. More work is needed to delineate the mechanism of MRAS in enhancing VV spread.

Our work is the first study harnessing the power of RNAi technology to enhance oncolytic vaccinia virotherapy. In recent years, Mercer *et al.*,<sup>196</sup> Sivan *et al.*,<sup>197</sup> Beard *et al.*<sup>198</sup> and Filone *et al.*<sup>199</sup> have conducted RNAi screens focused on elucidating host



**Figure 3.10: Knockout of MRAS enhances migration in wound healing assay**

(A) A wound was generated in confluent wells of A549 and A549  $\Delta$ MRAS cells seeded in a 96-well plate. The wound width is plotted on the graph at 2 h intervals (n=48). (B) Cells were treated as in (A). Representative images at 0 h and at 42 h upon closure of the wound in  $\Delta$ MRAS A549 cells. A549  $\Delta$ MRAS cells: A549 cells with MRAS disrupted using CRISPR-Cas9 technology; Scale bar: 300  $\mu$ m; \*  $p < 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , by t-test compared to A549; Data presented as means  $\pm$  SEM.

factors required for VV replication in human cells. All screens were conducted with HeLa cells except the Filone *et al.*<sup>199</sup> study where A549 cells were used. The importance of our screens in the context of oncolytic vaccinia virus therapy is that they were conducted with a clinically relevant oncolytic VV, namely vvDD, that they were specifically focused on identifying antiviral genes that modulate spread, and that they were performed with three different tumour cells lines minimizing cell-specific bias and broadening the scope of the study.

A variety of other approaches designed to enhance oncolytic vaccinia virotherapy and overcome limitations have or are currently being investigated. One of the first approaches was to manipulate the tumour microenvironment by encoding GM-CSF into VV.<sup>51</sup> More recent work in this area has included engineering VV to alter toll-like receptor (TLR) signalling<sup>84</sup> and thereby increase antitumour T cell responses, engineering VV to target prostaglandin E2 (PGE<sub>2</sub>)<sup>85</sup> and thereby deplete myeloid-derived suppressor cells (MDSCs), combining VV treatment with a checkpoint inhibitor<sup>86</sup> and studying the role of tumour-associated fibroblasts in augmenting VV growth.<sup>272</sup> Other approaches to enhancing VV therapy have included enhancing delivery to the tumour site via complement inhibition<sup>77,78</sup> or cell carriers,<sup>79-81</sup> dampening the antiviral response of tumour cells using histone deacetylase inhibitors (HDIs),<sup>215</sup> encoding therapeutic transgenes such as the sodium iodide symporter (NIS) membrane protein to enable uptake of radioactive iodine-131 (I<sup>131</sup>) at the tumour site and also to permit *in vivo* monitoring of viral biodistribution in human trials,<sup>273,274</sup> encoding VV to target the tumour vasculature and reduce angiogenesis,<sup>88</sup> and combining chemotherapy with VV.<sup>89,90</sup>



It is unlikely that one single strategy to enhancing oncolytic vaccinia virotherapy will be able to surmount all barriers and a multi-pronged approach will be required. Functional genomic screening is one resource that has not been fully exploited. In our present study, we found that inhibiting MRAS alone led to the enhancement of VV spread *in vitro* and improved therapeutic efficacy *in vivo*. However, our screens uncovered other intriguing gene targets, and we anticipate that further studies may lead to the discovery of additional targets to improve VV-based antitumour therapy. Moreover, we envisage designing optimal gene combinations to achieve greater effect. We also anticipate combining our work with some of the other promising endeavours (*e.g.* checkpoint inhibitors), which could increase the therapeutic effect considerably and, ultimately, lead to significantly better outcomes for patients.

## CHAPTER 4: Discovering alternative mechanisms of tumour specificity

### 4.1 Abstract

Clinical testing has shown oncolytic vaccinia virus to be safe and beneficial to cancer patients; however, this therapy could be improved. The tumour selectivity of the current clinical candidates is primarily based on deletions of thymidine kinase (TK) and/or vaccinia growth factor (VGF). While these deletions add a greater measure of safety to these viruses, they also in some cases reduce their potency in tumour cells. TK and VGF were selected for deletion based on their known function. Vaccinia virus (VV) has over 200 genes with many whose function(s) is still unknown.<sup>275</sup> Only a small number of VV genes have been studied in the context of enhancing oncolytic virotherapy. We hypothesized that there were other VV genes that could be manipulated to increase the therapeutic efficacy of oncolytic vaccinia virus while still maintaining tumour specificity. To identify them, we conducted VV genome-wide screens using a custom siRNA library targeting 173 VV genes. We found 35 VV gene candidates and engineered deletion mutants of 13 of these. We demonstrated that a deletion mutant of the DNA ligase gene (vv-ΔA50R) to be as attenuated as vvDD in normal human fibroblast cells *in vitro*. Importantly, it was more potent in multiple tumour cell lines than vvDD. Furthermore, we showed it provided a therapeutic advantage over vvDD in a pilot study in MC38 tumour-bearing mice, and it had the same safety profile as vvDD in an immunocompromised mouse model. This study provides a resource for building the next generation of oncolytic VVs.

## 4.2 Introduction

Vaccinia virus is a large, enveloped, double-stranded DNA virus belonging to the *Poxviridae* family. It completes its entire life cycle in the cytoplasm. VV enters by fusing with the plasma membrane or following endocytosis.<sup>105-111,113,114</sup> Following entry into the cytoplasm, but prior to uncoating, early mRNAs are synthesized. Early mRNAs encode molecules that mediate resistance to host antiviral defence mechanisms, growth factors, factors essential for viral DNA replication and transcription factors for intermediate genes.<sup>99</sup> Following uncoating, DNA is replicated to form concatemers.<sup>99</sup> DNA replication is required for intermediate gene transcription.<sup>99</sup> Intermediate transcripts encode factors important for late gene expression, while late genes encode early transcription factors, structural proteins and late enzymes.<sup>99</sup> Virus assembly is initiated in distinct structures referred to as virus factories (*i.e.* restricted, granular, electron-dense regions of the cytoplasm containing VV DNA).<sup>99</sup> As part of assembly, the concatameric VV DNA is decatenated into individual genomes and packaged into immature virions that evolve to become mature infectious virions (MVs), which are released upon cell lysis.<sup>99</sup> MVs can acquire an additional membrane to become wrapped virions which are transported to the cell surface on microtubules where they are released or propelled by actin tails into neighbouring cells; these virions are referred to as extracellular enveloped virions (EVs).<sup>99</sup>

VV was used as a vaccine to eradicate smallpox in the 20<sup>th</sup> century. There are a considerable number of strains of VV, but only a few have been tested as oncolytic agents, namely Wyeth [a vaccine strain derived from the New York City Board of Health (NYCBH) strain],<sup>91</sup> Western Reserve (derived from the NYCBH strain by passaging in

mouse brains),<sup>94</sup> and Lister (a European vaccine strain). The genomes of these strains are approximately 200 kilobases (kb) in length with over 200 genes. Prior to whole genome sequencing, genes were identified by the HindIII restriction endonuclease DNA segment letter,<sup>276</sup> followed by the number of the open reading frame (ORF) within the segment and the direction of transcription [*i.e.* left (L) or right (R)]; the direction of transcription is omitted for polypeptide names (*e.g.* A50, K1, O1). Now with whole genome sequencing, most poxvirus genome sequences ORFs are numbered consecutively from the beginning to the end of the genome; however, common names are still employed in the literature to maintain continuity.<sup>99</sup>

Some strains of VV have been shown to be naturally oncotropic.<sup>60-62,64</sup> For example, in several preclinical studies in tumour-bearing mice treated intraperitoneally or intravenously with the wild-type Western Reserve strain of VV, the highest yields of virus were recovered from tumours, followed by the ovaries with low detection in other organs.<sup>60-62,277</sup> Similarly, tumour-bearing mice treated intravenously with the Lister-Institute for Viral Preparations (LIVP) vaccine strain expressing *Renilla* luciferase and GFP in a nonessential region of the genome demonstrated tumour-specific amplification using real-time imaging.<sup>64</sup> Nevertheless, to enhance tumour selectivity and increase the therapeutic index, genetic modifications have been made to the VV genome in particular deletions of thymidine kinase (TK) and vaccinia growth factor (VGF).<sup>60-62,64,277</sup>

All oncolytic vaccinia viruses currently undergoing clinical trial testing, namely Pexa-Vec (Wyeth strain), vvDD [Western Reserve strain (WR)] and GL-ONC1 (Lister strain) are TK deficient (TK-). TK catalyzes the conversion of thymidine to

deoxythymidine monophosphate (dTMP) in the nucleotide salvage pathway where nucleotides are synthesized from preformed purine or pyrimidine bases or nucleosides—intermediates of nucleotide degradation. Cellular TK (*i.e.* Thymidine kinase 1/TK1) is transiently expressed primarily during S-phase<sup>278</sup> as it is necessary for replenishing nucleotide pools in normal cells during DNA synthesis. Viral TK is, therefore, important for replication of viral DNA in normal cells, but dispensable in many tumour cell where TK is constitutively expressed.<sup>65,67-71</sup>

To further enhance the specificity and thereby the safety of VV, McCart *et al.*<sup>62</sup> engineered a double deletion mutant with both TK and VGF-deleted (*i.e.* vvDD). VGF is an epidermal growth factor (EGF) homolog expressed early during infection. It is a secreted protein that binds the EGF receptor on both infected and nearby non-infected cells thus inducing cell proliferation,<sup>211,279</sup> which is important for VV replication in normal cells, but not in highly proliferating cancer cells. McCart *et al.*<sup>62</sup> found that infection of resting, confluent NIH3T3 (murine fibroblast) cells with vvDD resulted in significantly less virus yield than WT or TK-deleted or VGF-deleted alone, but equivalent yield in synchronously infected dividing, non-confluent NIH3T3 cells. In *in vivo* studies, vvDD was also shown to be less pathogenic in immunocompromised and immunocompetent mice than WT, TK-deleted or VGF-deleted alone.<sup>62,63</sup> Most importantly, recent phase I clinical studies have found vvDD to be safe in cancer patients with evidence of antitumour activity.<sup>47,48</sup>

Clinical candidate GL-ONC1 (also known as GLV-1h68), which has a Lister backbone, in addition to being TK- is A56R- and F14.5L-deficient. GL-ONC1 was generated to be used as an oncolytic therapeutic and a diagnostic agent.<sup>280</sup> It has three

expression cassettes: a *Renilla* luciferase-*Aequorea* green fluorescent protein fusion cassette (*i.e.* RUC-GFP) inserted into the F14.5L locus,  $\beta$ -galactosidase inserted into the TK locus and  $\beta$ -glucuronidase inserted into the A56R locus. It appears that A56R and F14.5L loci were not initially selected because their disruption might enhance tumour specificity, but because they were considered to encode non-essential genes for replication in culture.<sup>280,281</sup> A56R was originally shown to have hemagglutination activity, but has since been shown to have other roles including forming a complex with K2 to decrease the amount of virus superinfecting cells and preventing the formation of syncytia.<sup>282</sup> In recent years, F14.5L has been revealed as a component of the intracellular mature virion; its C-terminal region is exposed on the surface of infected cells and plays a role in cell adhesion and virulence as VV lacking F14.5L has reduced virulence *in vivo*.<sup>283</sup>

There are a small number of oncolytic VVs made exploiting other mechanisms of tumour specificity. For example, Guo *et al.*<sup>72</sup> engineered a WR VV deletion mutant (*i.e.* vSP) with the anti-apoptotic/host range serine protease inhibitor 1 (SPI-1 *i.e.* B22R) and serine protease inhibitor 2 (SPI-2 *i.e.* B13R also known as CrmA) genes deleted based on the hypothesis that a VV with deletions of these genes would replicate selectively in cancer cells as one of the distinctive features of cancer cells is their resistance to apoptosis,<sup>6</sup> and studies had been successfully conducted with other OVVs with deletions of anti-apoptotic genes.<sup>284</sup> Guo *et al.*<sup>72</sup> were able to demonstrate enhanced tumour specificity with vSP compared to WT. Yang and Guo *et al.*<sup>73</sup> further improved tumour selectivity by engineering a triple deletion mutant with TK also

deleted; however, it came at the expense of reduced efficacy compared to vSP and a TK-negative single deletion mutant.<sup>73</sup>

In a recently published work, Potts *et al.*<sup>87</sup> tested F4L-deleted VVs [*i.e.* single deletion of F4L (vv-ΔF4L) or in combination with the deletion of J2R (ΔJ2R), the gene encoding TK]. F4L encodes the small subunit of the viral ribonucleotide reductase. Ribonucleotide reductase (RNR) is composed of a large and small subunit and catalyzes the *de novo* reduction of ribonucleoside diphosphates (rNDPs) to deoxynucleoside diphosphates (dNDPs), the rate-limiting step in deoxynucleoside triphosphate (dNTP) biosynthesis.<sup>285,286</sup> dNTPs are required for cellular and viral DNA replication. Protein levels of the large cellular subunit (RRM1) remain constant throughout the cell cycle, whereas the small subunit (RRM2) is primarily expressed during S phase.<sup>286-290</sup> Overexpression of both subunits of RNR has been observed in many cancers.<sup>291</sup> In particular, elevated levels of RRM2 have been observed in bladder cancer,<sup>292</sup> the focus of the Potts *et al.* study.<sup>87</sup> Potts *et al.* observed no signs of toxicity in immunocompromised tumour-bearing (*i.e.* RT-112-luc) mice treated with vv-ΔF4L and vv-ΔF4LΔJ2R whereas significant toxicity was observed upon treatment with vv-ΔJ2R as well as recovery of virus from multiple normal organs. In immunocompetent tumour-bearing (*i.e.* AY-27) rats, treatment with all three viruses (*i.e.* vv-ΔF4L, vv-ΔJ2R and vv-ΔF4LΔJ2R) generated statistically similar results for survival; however, only vv-ΔJ2R was recovered from normal organs.

The mechanisms of tumour specificity of TK-, VGF- and F4L-deleted VVs could be described as cell proliferation-based. The limitation with cell proliferation-based mechanisms is they may not provide a therapeutic advantage in solid human tumours

with low numbers of cells in S-phase. For this reason, Guo *et al.* investigated an alternative mechanism—that of deleting VVs anti-apoptotic genes.<sup>72</sup> Apoptosis has been described as a host antiviral defence. Exploiting the differences in antiviral defences between tumour and normal cells<sup>37</sup> has been successfully applied to other OV, in particular rhabdoviruses,<sup>219,223,293</sup> but this mechanism has not been extensively investigated with oncolytic VV. VV expresses an arsenal of immunomodulatory proteins to thwart components of the host antiviral defence that could potentially be manipulated including the complement system (*e.g.* C21), the interferon (IFN) system (*e.g.* B8, B18), nuclear factor kappa B (NFκB) signalling (*e.g.* A46, A52, K1, M2, N1), protein kinase R (PKR) activation (*e.g.* K3, E3), apoptosis (*e.g.* F1, B13), cytokines (*e.g.* A53, B13) and chemokines (*e.g.* A41, C23).<sup>294,295</sup> Kirn *et al.*<sup>52</sup> studied a double deletion VV mutant with TK and B18R deleted (*i.e.* vv-ΔB18RΔTK) as well as vv-ΔB18RΔTK expressing IFN-β from the TK locus (*i.e.* vv-ΔB18RΔTK-INF-β). B18 acts to neutralize secreted type I IFNs. Treatment of mice bearing JC murine mammary gland tumours with vv-ΔB18RΔTK-INF-β resulted in significantly enhanced tumour selectivity and survival compared with mice treated with vv-ΔB18RΔTK. Pexa-Vec, in addition to being TK-negative also expresses a truncated version of B18R.<sup>296</sup>

To discover alternate mechanisms of tumour specificity, we employed functional genomic screening (*i.e.* siRNA screening) to identify genes in an unbiased and comprehensive manner, whose deletion could impart tumour specificity. According to one estimate, VV has approximately 25% genes whose function has not been well-characterized;<sup>275</sup> we hypothesized that one or a combination of these genes and/or genes with known function could be manipulated to convey pronounced tumour

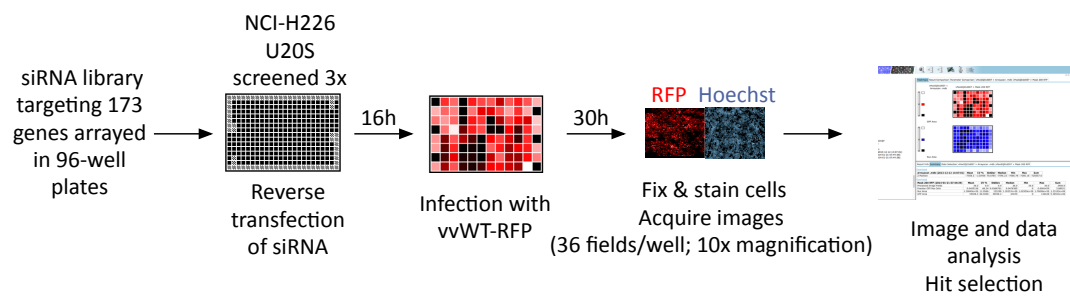


specificity without compromising efficacy. We performed VV genome-wide RNAi screens using a custom siRNA library targeting 173 VV genes with a wild-type WR VV in two different cancer cell lines. From these screens, we identified 35 genes that upon knockdown did not attenuate spread in tumour cells. We elected to engineer deletion mutants of 13 of these candidates. Following *in vitro* testing, we found that the VV with a deletion of the DNA ligase gene (vv-ΔA50R) was as attenuating as vvDD in normal cells, but more potent than vvDD in multiple tumour cell lines. A pilot *in vivo* study demonstrated that it had greater efficacy than vvDD in tumour-bearing MC38 mice, but was as safe as vvDD in immunocompromised mice.

## **4.3 Results**

### **4.3.1 VV genome-wide siRNA screens**

To identify VV genes that could be deleted without diminishing potency in tumour cell lines, we conducted VV genome-wide screens with a custom library of individually arrayed siRNA targeting 173 VV genes (1-8 siRNAs/gene; 637 oligonucleotides) (Fig. 4.1). To avoid cell specific bias, we elected to screen with two different tumour cell lines. We selected the NCI-H226 (non-small cell lung carcinoma) tumour cell line as we found it to be strongly resistant to VV infection and U2OS (osteosarcoma) as it is sensitive. We infected cells with vvWT-RFP (*i.e.* wild-type VV with dsRed inserted into a non-coding region). For screens in both cell lines, we selected a multiplicity of infection (MOI) and an incubation period that would enable the detection of increases and decreases in spread. Knockdown efficiency was monitored using siRNA targeting a subunit of the VV DNA-dependent RNA polymerase with late and early expression, namely RNA polymerase subunit 147 (RPO 147 *i.e.* J6R). The red fluorescent protein



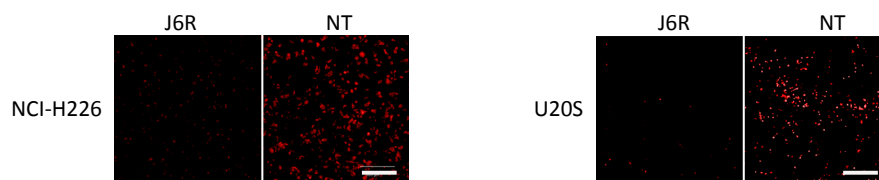
#### **Figure 4.1: Schematic of workflow for RNAi screens with vvWT-RFP**

(A) Schematic of workflow of RNAi screens conducted in triplicate. A custom vaccinia genome siRNA library targeting 173 vaccinia genes (1-8 siRNAs/gene in a non-pooled format) was arrayed in 96-well plates. U2OS and NCI-H226 cells were reverse transfected with siRNA. After 16 h incubation with siRNA, the cells were infected (U2OS: MOI of 0.01; NCI-H226: MOI of 0.05) with vvWT-RFP (wild-type vaccinia expressing RFP in a non-coding region). Following 30 h incubation with vvWT-RFP, U2OS and NCI-H226 cells were fixed with formaldehyde and stained with Hoechst. Each sample well was normalized on a per-plate basis to the median RFP area of the negative control wells transfected with non-targeting siRNA. The screens in each cell line were conducted in triplicate.

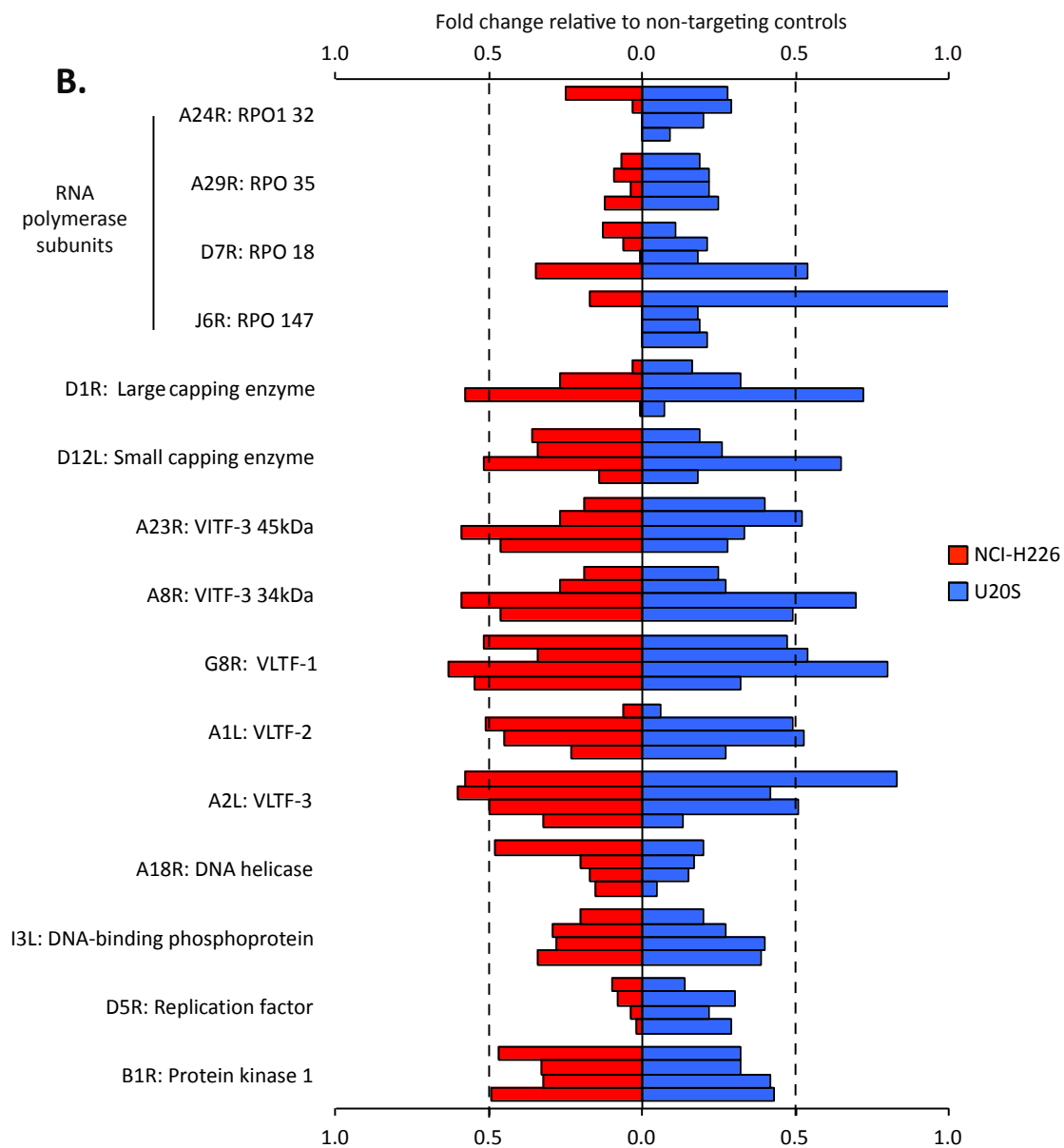
(RFP) area per well was used as a surrogate for quantifying spread as we had previously established a direct correlation between increases and decreases in infectious foci area with overall RFP area. Each sample well was normalized to the median RFP area of the negative controls wells on a per-plate basis. The screens were conducted in triplicate, and the median score across the replicates was used to score each siRNA.

Knockdown of genes critical for transcription and genome replication resulted in the marked inhibition or abrogation of VV spread. For example, knockdown of four out of the seven RNA polymerase subunits present in the library severely limited spread with the majority of the siRNAs tested, specifically RPO 132 (A24R), RPO 35 (A29L), RPO 18 (D7R) and J6R as well as the siRNA targeting J6R in the control wells (Fig. 4.2A,B). A minimum of one siRNA targeting RPO 22 (J4R), RPO 7 (G5.5R), and RPO 30 (E4L) also reduced or abrogated spread (data not shown). Knockdown of the large (D1R) and small (D12L) subunits of the capping enzyme inhibited replication (Fig. 4.2B). Silencing of transcription factors such as the two subunits of VITF-3 (*i.e.* A23R, A8R), an intermediate transcription factor (siRNA targeting VITF-1, the other VV intermediate transcription factor was not present in the library), and the late transcription factors, namely VLTF-1 (G8R), VLTF-2 (A1L), VLTF-3 (A2L) inhibited spread (Fig. 4.2B). Silencing of genes involved in replication also had an inhibitory effect on replication such as the DNA helicase (A18R), the single-stranded DNA-binding phosphoprotein (I3L), Protein Kinase 1 (B1R) as well as D5R, an essential component of the replication machinery having primase and NTPase activity<sup>297-299</sup> (Fig. 4.2B); D5R also serves as the virus genome-uncoating factor.<sup>208</sup> B1R has been shown to interact

**A.**



**B.**

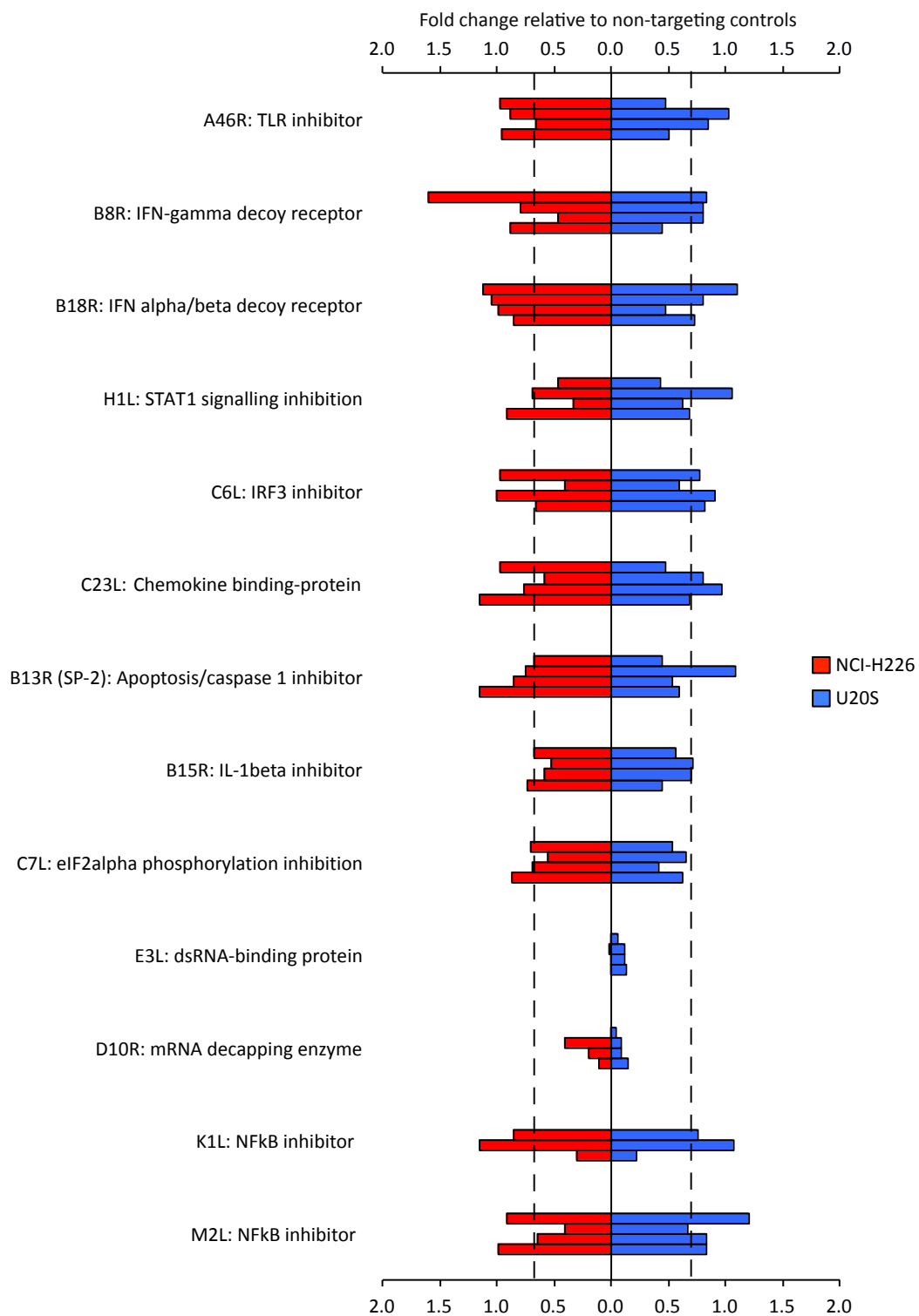


**Figure 4.2: The knockdown of VV genes critical for transcription and genome replication inhibits spread in RNAi screens**

(A) Representative images of fields from the positive (siRNA targeting J6R) and negative (non-targeting siRNA) control wells spotted on each plate. (B) Plotted on the graph are the vvWT-RFP screen results of siRNA-mediated silencing of the indicated genes (4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median RFP area of the negative control wells transfected with non-targeting siRNA. Dotted lines demarcate siRNAs deemed inhibitory (*i.e.*  $\leq 0.5$ ). NT: non-targeting siRNA; RFP: red fluorescent protein; VITF: vaccinia intermediate transcription factor; VLTF: vaccinia late transcription factor; Scale bar: 500  $\mu\text{m}$ .

with barrier-to-autointegration factor (BAF), a potent antagonist of VV replication whose DNA-binding activity is obstructed by B1-mediated phosphorylation.<sup>300-302</sup>

While we expected that the knockdown of VV genes critical for transcription or replication to severely limit spread in all instances, we anticipated that the silencing of VV genes involved in immune modulation to be inhibitory in some instances, but in many cases to have no impact as their role is exerted *in vivo* or due to the fact that the antiviral defence pathways in tumour cells are often dysfunctional<sup>37</sup> For example, the knockdown of B8R (IFN-gamma decoy receptor) and B18R (IFN-alpha/beta decoy receptor) had neutral impact on spread in one or both cell lines (Fig. 4.3). Genes were scored as neutral when the majority of siRNAs (*e.g.* 5/8, 3/4, 2/3, 2/2, 1/1) targeting a given gene had a score of greater or equal to 0.7. The knockdown of E3L, however, severely limited replication in both cell lines (Fig. 4.3). E3 is a dsRNA-binding protein that is able to bind and sequester dsRNA,<sup>303,304</sup> which is generated by VV convergent transcription.<sup>295,305</sup> The sequestration of dsRNA prevents the activation of PKR and 2'-5'-oligoadenylate synthetase (OAS),<sup>303,304,306</sup> enzymes activated by dsRNA that inhibit the translation and synthesis of proteins in infected cells and induce apoptosis,<sup>306,307</sup> effectively blocking virus replication. In addition, E3 has been shown to have other roles described in Perdiguero *et al.*<sup>295</sup> such as binding and interfering with the antiviral action of ISG15,<sup>308</sup> and preventing the DNA sensing molecule, DNA-dependent activator of IFN regulatory factors (DAI; also, referred to as DLM-1/ZBP1) from interacting with VV DNA.<sup>309</sup> E3L is not essential in all cells. For example, it is required for VV to replicate in HeLa cells, but not in BHK cells.<sup>310</sup>





**Figure 4.3: The knockdown of VV immunomodulatory genes has neutral or inhibitory impact in RNAi screens**

(A) Plotted on the graph are the RNAi vvWT-RFP screen results of siRNA-mediated silencing of the indicated genes (3-4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median RFP area of the negative control wells transfected with non-targeting siRNA. Dotted lines demarcate siRNAs deemed neutral (*i.e.*  $\geq 0.7$ ). IRF3: interferon regulatory factor 3; TLR: toll-like receptor; IFN: interferon; NF $\kappa$ B: nuclear factor kappa B; STAT1: signal transducer and activator of transcription 1.

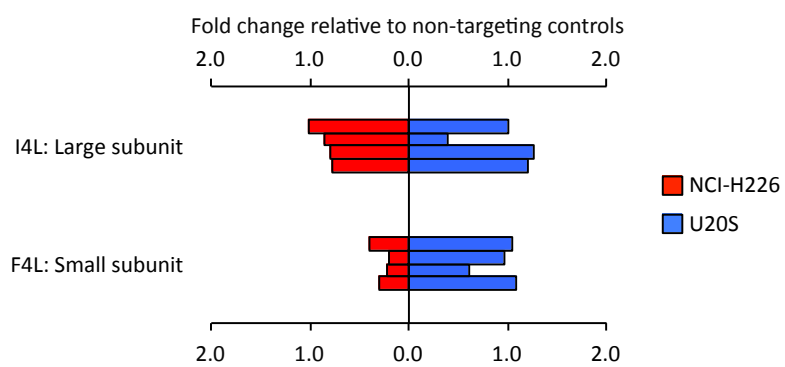
Based on the Potts *et al.*<sup>87</sup> study, we anticipated that knockdown of the small (F4L) and large ribonucleotide reductase subunits (I4L) to have no impact in U2OS cells, but that the knockdown of F4L might be inhibitory to VV replication in NCI-H226 cells as they have a long doubling time (*i.e.* 61h)<sup>311</sup> and likely have low levels of the cellular homolog to compensate for the loss of F4L. As alluded to previously, F4L-deleted VV was shown to be highly attenuated in normal cells, but not in human bladder cancer cells.<sup>87</sup> While silencing F4L had no effect in U2OS cells, its knockdown greatly reduced the spread of VV in NCI-H226 cells for all the siRNAs tested (Fig. 4.4 A,B).

While none of the screening results can be considered definitive until further validation, the fact that the screens identified numerous expected hits, especially genes critical for transcription and genome replication, serves to confirm the robustness of the screening assay.

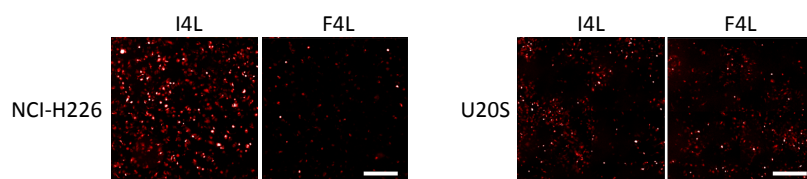
#### **4.3.1 Candidate genes selected for further validation following RNAi screens**

The aim of our screening endeavour was to identify genes that when silenced had no impact on or enhanced VV replication in tumour cells. We identified 35 genes in this class. We elected to make deletion mutants of 13 of these (Fig. 4.5A) based on their biology, their potential for combinations with the deletions of other genes and based on our findings with these genes from other screens. At the top of our list were immunomodulatory genes as the deletion of these genes would exploit a non-cell-proliferation-based mechanism of tumour specificity distinct from the current clinical candidates and might not attenuate VV replication in slowly dividing tumour cells. We also chose a number of genes whose knockdown produced highly unexpected results: these genes had been previously deemed to be required for replication, but their

**A.**

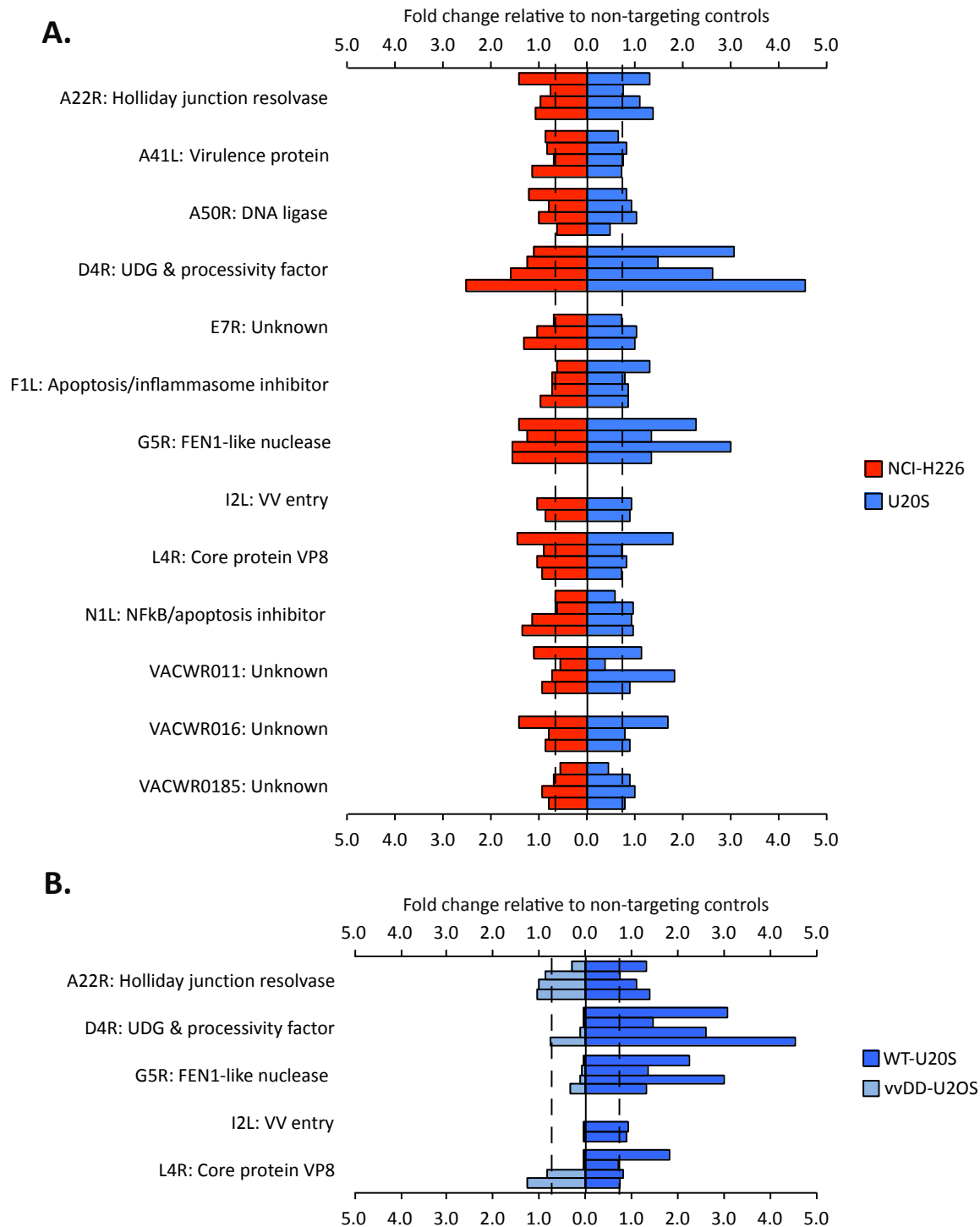


**B.**



**Figure 4.4: The impact of the knockdown of the VV small ribonucleotide reductase subunit is cell line specific**

(A) Plotted on the graph are the RNAi vvWT-RFP screen results of siRNA-mediated silencing of the ribonucleotide reductase subunits (4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median RFP area of the negative control wells transfected with non-targeting siRNA. (B) Representative images of fields from I4L (large subunit) and F4L (small subunit) wells from the RNAi screens. Scale bar: 500  $\mu\text{m}$ .



**Figure 4.5: Candidates selected for further validation following RNAi screens**

(A) Plotted on the graph are the RNAi vvWT-RFP screen results of siRNA-mediated silencing of the indicated genes (2-4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median RFP area of the negative control wells transfected with non-targeting siRNA. (B) Plotted on the graph are the RNAi vvWT-RFP and vvDD-GFP screen results of siRNA-mediated silencing of the indicated genes (2-4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median RFP or GFP area of the negative control wells transfected with non-targeting siRNA. Dotted lines demarcate siRNAs deemed neutral (*i.e.*  $\geq 0.7$ ); UDG: uracil-DNA glycosylase; FEN1: flap endonuclease 1; NF $\kappa$ B: nuclear factor kappa B.

knockdown did not impact VV replication in our screens. Genes whose functions are unknown were also of interest as there was the potential for novel findings.

The immunomodulatory genes that we elected to make deletion mutants of were N1L, A41L and F1L. N1L was our top contender, as a VV with this gene deleted had previously been shown to be highly attenuated in mice, particularly when delivered in the brain,<sup>312,313</sup> in addition to being highly immunogenic. N1 is a B-cell CLL/lymphoma-2 (BCL2)-like protein that inhibits both apoptosis and NF- $\kappa$ B activation.<sup>314</sup> Following intranasal administration, vv- $\Delta$ N1L was found to be two logs less virulent than wild-type VV,<sup>315</sup> and the LD<sub>50</sub> for vv- $\Delta$ N1L was 1.6 x 10<sup>4</sup>-fold higher following intracranial administration.<sup>312,313</sup> Mice immunized with vv- $\Delta$ N1L were shown to be highly protected against lethal challenge with wild-type VV including in the brain.<sup>313,315</sup> Furthermore, mice treated with vv- $\Delta$ N1L were demonstrated to have increased central and memory CD8<sup>+</sup> T-cell populations as well as increased CD8<sup>+</sup> T-cell cytotoxicity.<sup>315,316</sup>

Our interest in A41L was that a VV lacking A41L was previously shown to form normal sized plaques and replicate to the same titre as wild-type VV in immortalized cells (*i.e.* BS-C-1; African green monkey cells),<sup>317</sup> and in addition was demonstrated to have enhanced immunogenicity.<sup>318</sup> For example, following an intranasal infection of BALB/c mice with a VV lacking A41L, there was an increase in the relative number of VV-specific CD8<sup>+</sup> T cells in the spleen producing IFN- $\gamma$  or displaying cytolytic activity.<sup>318</sup> Furthermore, there was an enhancement of the antiviral CD8<sup>+</sup> memory cells in mice treated with A41L-deficient Modified Vaccinia Ankara (MVA) virus expressing a foreign antigen (*i.e.* *Plasmodium berghei* circumsporozoite antigen).<sup>318</sup> The amino acid sequence of A41 is similar to a number of secreted chemokine-binding poxvirus

proteins including vCKBP of the VV Lister strain that binds CC chemokines;<sup>317,318</sup> however, the ligand of A41 has not been identified.<sup>317</sup> A negative aspect of VV lacking A41L is that it was found to be slightly more virulent following intranasal administration in BALB/c mice<sup>318</sup> and also caused more severe lesions compared to WT.<sup>317</sup> These findings suggested that engineering a deletion mutant of A41 alone would not be useful for oncolytic virotherapy, but we believed that it might be a good candidate in combination with an attenuated deletion mutant such as  $\Delta$ N1L.

A key reason we were interested in pursuing the F1L gene is that vv- $\Delta$ F1L was shown to be attenuated *in vivo*.<sup>319</sup> F1 is an anti-apoptotic protein that localizes to the mitochondria where it prevents Bak and Bax activation and tBid-induced cytochrome c release.<sup>320,321</sup> It also prevents inflammasome activation that leads to the activation of caspase-1 and the ensuing proteolytic activation of interleukin (IL)-1 $\beta$  and IL-18 by binding to nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein 1 (NLRP1).<sup>319</sup>

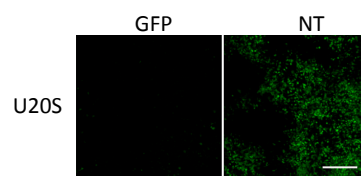
Surprisingly, five genes that we elected to further investigate were previously shown to be required for replication, namely A22R,<sup>322</sup> D4R,<sup>323-325</sup> G5R,<sup>326,327</sup> I2L<sup>328</sup> and L4R.<sup>329</sup> However, all of the studies with the exception of D4R were conducted in a TK-negative background, which could explain our conflicting results. In a screen with the same library but with vvDD-eGFP (*i.e.*  $\Delta$ TK $\Delta$ VGF) instead of vvWT-RFP in U2OS cells, knockdown of these genes was inhibitory for the majority of siRNAs tested as expected (Fig. 4.5B) with the exception of L4R where two siRNAs were highly inhibitory and two were neutral, and A22R where only one siRNA was inhibitory. The screen with vvDD-eGFP in U2OS cells was conducted and analyzed in the exact same manner as the



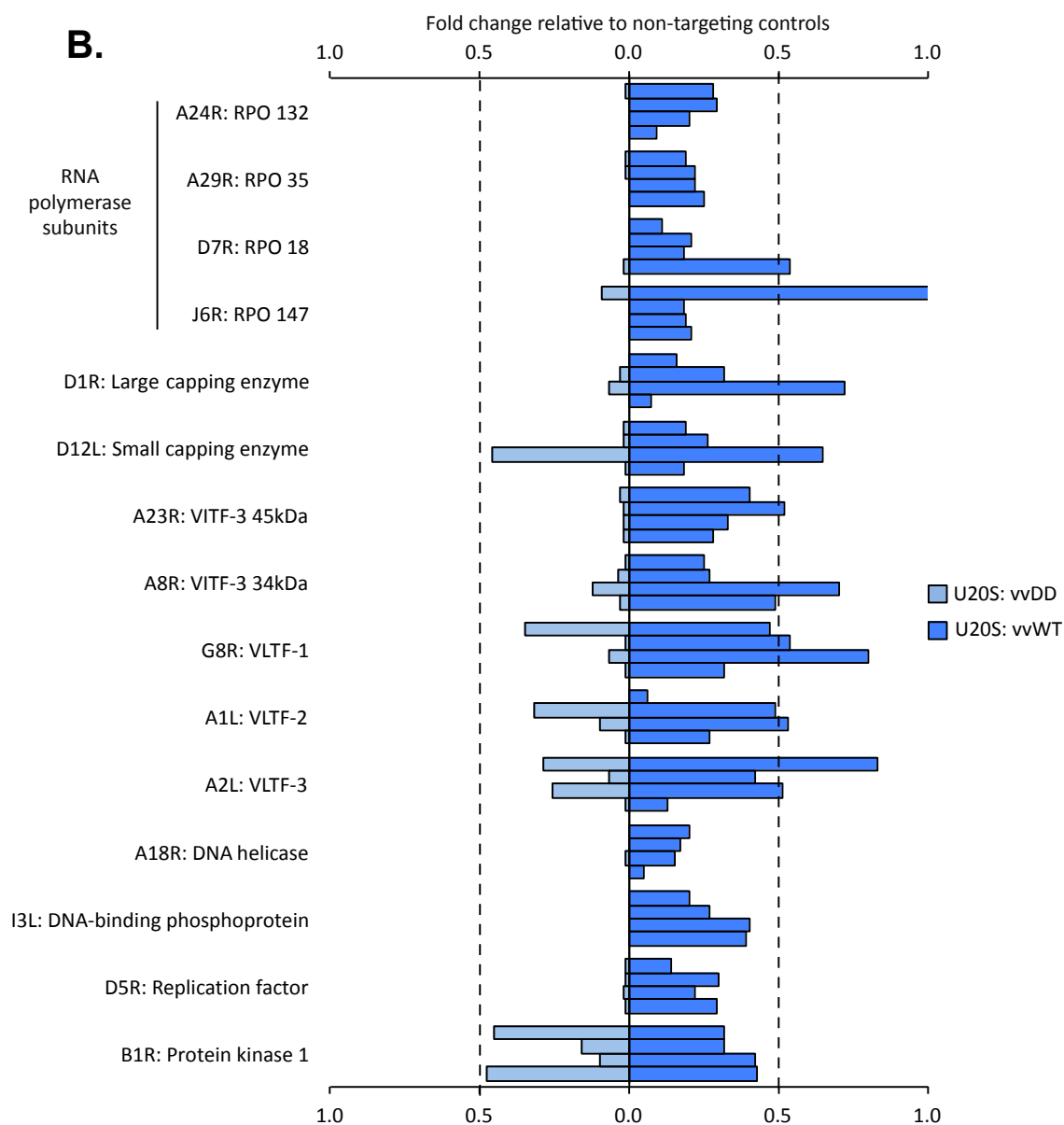
screens with vvWT-RFP. We had confidence in the results of the previous screens as knockdown with the positive control siRNA (siRNA targeting GFP) led to the robust knockdown of GFP expressed by vvDD-eGFP (Fig. 4.6A) and targeting of the essential or critical genes resulted in reduction in spread as anticipated (Fig. 4.6B). Another reason for further investigating these genes is that for four of these genes silencing of one or more siRNAs enhanced spread above the controls (Fig. 4.5B), namely A22R, D4R, G5R and L4R. A22R, D4R and G5R were also of interest as they have roles in DNA replication and/or repair, more specifically A22R is a Holliday junction resolvase, D4R is a uracil-DNA glycosylase (UDG) and processivity factor for the DNA polymerase, and G5R is a predicted flap endonuclease 1 (FEN1)-like nuclease required for homologous recombination, double-strand break repair and full-size genome formation.<sup>326,327</sup> In RNAi screens examining host genes implicated in modulating VV replication, we identified genes involved in DNA replication and repair as hits as did Beard *et al.*<sup>198</sup>

We were also interested in genes that were not well characterized such as E7R, VACWR011, VACWR016, and VACWR185. Little is known regarding E7R other than it has been classified as an intermediate protein<sup>330</sup> and is likely myristylated.<sup>331</sup> The modification of a protein with myristic acid can help in protein trafficking or targeting, or in stabilizing protein-protein and protein-membrane interactions.<sup>331-333</sup> VACWR011 encodes a truncated version of the variola virus E3 ubiquitin ligase p28 protein, but lacks the function of a ubiquitin ligase.<sup>334,335</sup> VACWR016 is annotated as an ankyrin-like protein and VACWR185 (B3L) as schlafen-like, but no literature is available regarding these VV genes.

**A.**



**B.**



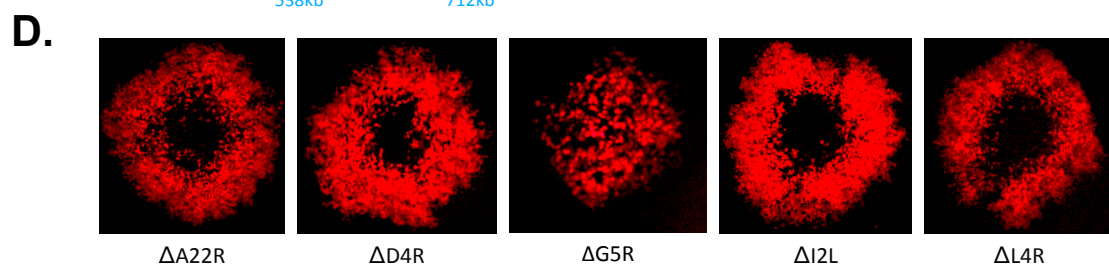
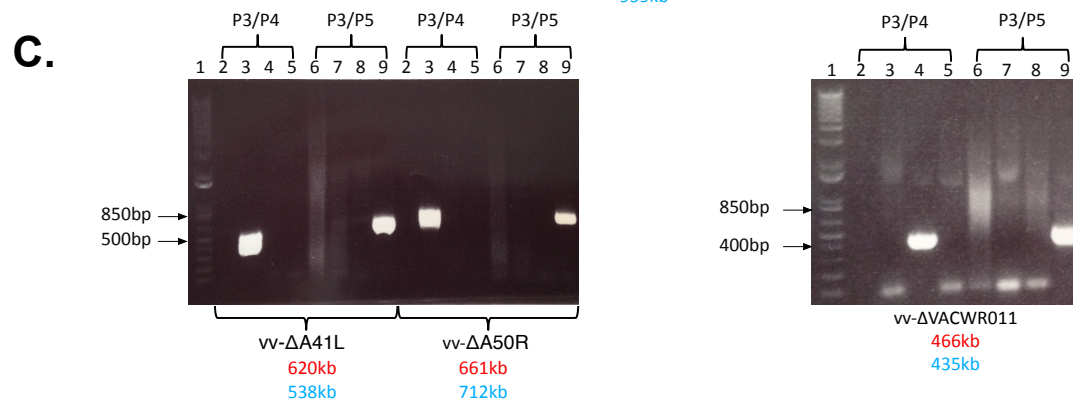
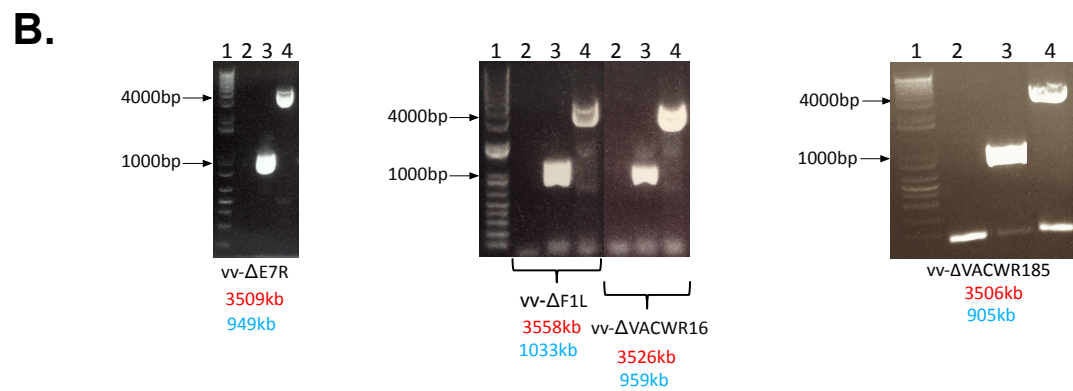
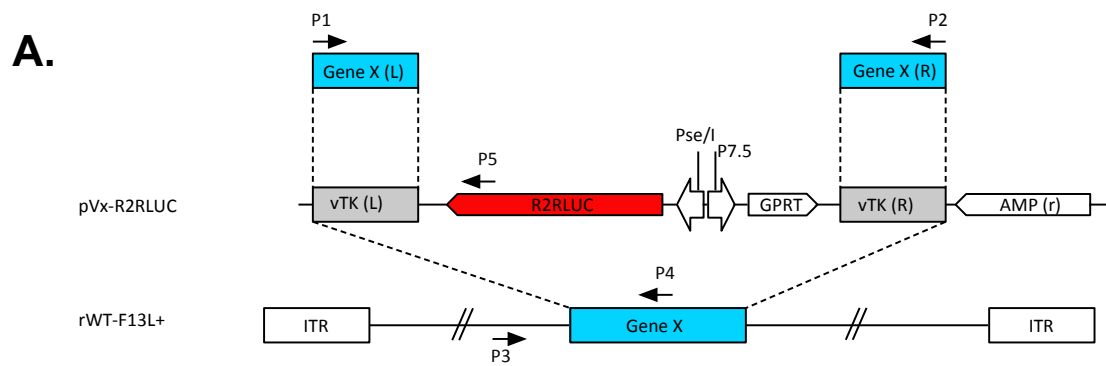
**Figure 4.6: The knockdown of genes critical for transcription and genome replication inhibits spread in RNAi screens with vvDD-eGFP**

(A) Representative images of fields from the positive (siRNA targeting GFP) and negative (non-targeting siRNA) control wells from the RNAi screens. (B) Plotted on the graph are the RNAi vvDD-eGFP screen results of siRNA-mediated silencing of the indicated genes (4 siRNAs/gene). Each sample well was normalized on a per-plate basis to the median GFP area of the negative control wells transfected with non-targeting siRNA. Dotted lines demarcate siRNAs deemed inhibitory (*i.e.*  $\leq 0.5$ ). NT: non-targeting siRNA; eGFP: enhanced green fluorescent protein; VITF: vaccinia intermediate transcription factor; VLTF: vaccinia late transcription factor; Scale bar: 500  $\mu\text{m}$ .

We also chose to make a mutant VV with A50R deleted, a gene that encodes a DNA ligase.<sup>336,337</sup> An A50R-deleted VV was shown *in vitro* to have reduced replication by as many as two logs in resting normal primary cells compared to proliferating cells,<sup>338</sup> in addition to having reduced pathogenicity in mice.<sup>339</sup> Based on these findings, we speculated that the deletion of this gene might work well in combination with non-attenuating deletions such as the deletion of A41L.

#### **4.3.2 Engineering, rescue and purification of deletion mutants**

To construct deletion mutants of our candidate genes, we used the shuttle plasmid pVx-R2RLUC (Fig. 4.7A), which has segments of the 5' end (*i.e.* left arm) and the 3' end (*i.e.* right arm) of the TK gene flanking the R2RLUC cassette and also encodes the xanthine-guanine phosphoribosyltransferase (GPRT) gene used for drug selection. The R2RLUC cassette contains RFP (*i.e.* mCherry) and *Renilla* luciferase reporter genes with an intervening foot-and-mouth disease viral 2A sequence,<sup>89</sup> which allows for bicistronic expression.<sup>340</sup> We replaced the TK segments with the corresponding segments of our candidate genes (Fig. 4.7A). We then performed rescue experiments where we infected CV-1 (African green monkey kidney cells) cells with wild-type VV followed by transfection with each shuttle plasmid to enable recombination. This was followed by multiple rounds of purification under drug selection to eliminate any parental wild-type virus. PCR was used to confirm the absence of parental wild-type virus and the presence of the R2RLUC cassette in the recombinants (Fig. 4.7B,C). For some of the deletion mutants (Fig. 4.7C) we could not amplify the ~4000 bp region of the single deletion mutant containing the R2RLUC cassette using primers P1 and P2 (Fig. 4.7A). As an alternate means to verify its insertion, we used one primer external to the site of



#### Figure 4.7: Engineering, rescue and purification of single VV deletion mutants

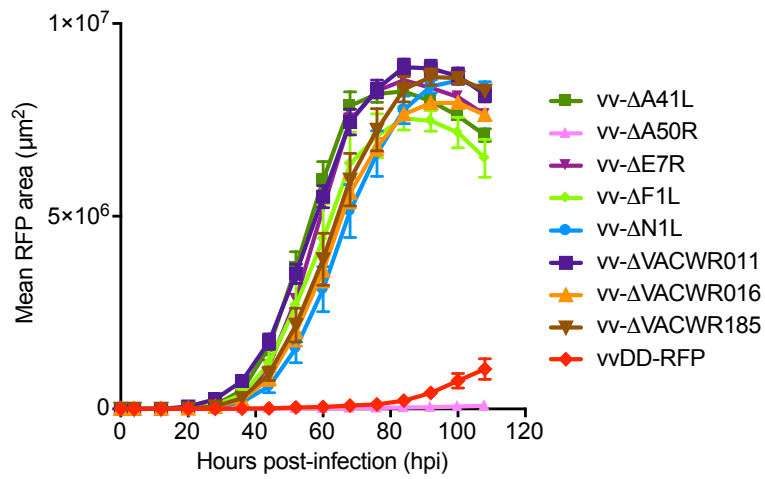
(A) Linear diagrams of pVx-R2RLUC plasmid (shuttle vector for cloning into vaccinia) and rWT-F13L+ (wild-type vaccinia virus). vTK (L) and vTK (R) consist of left and right segments of the thymidine kinase (TK) gene, respectively. For each deletion mutant, left (L) and right (R) segments of each gene (*i.e.* Gene X) were amplified and cloned in the place of vTK (L) and vTK (R) in pVx-R2RLUC. pVX-R2RLUC was then transfected with rWT-F13L+ to generate single deletion mutants of each gene. (B) Gel images of PCR amplicons with the primers P1 & P2. Lane 1: Kb+ DNA ladder; Lane 2: no template; Lane 3: rWT-F13L+ DNA; Lane 4: DNA of the indicated deletion mutant. (C) Gel images of PCR amplicons with the primers P3 & P4 (*i.e.* Lanes 2-5) or P3 & P5 (*i.e.* Lanes 6-9). Lane 1: Kb+ DNA ladder; Lanes 2 & 6: no template; Lanes 3 & 7 (left panel): rWT-F13L+ DNA; Lanes 4 & 8 (left panel): DNA extracted from U20S cells; Lanes 4 & 7 (right panel): rWT-F13L+ DNA; Lanes 3 & 8 (right panel): DNA extracted from U20S cells; Lanes 5 & 9: DNA of the indicated deletion mutant. (D) Images of infectious foci of VV with the indicated gene deleted (4X magnification). R2RLUC: Red fluorescent protein (mCherry) and renilla luciferase gene with the intervening the foot-and-mouth disease virus 2A sequence motif; Pse/l: synthetic early/late promoter; P7.5: VV 7.5 promoter; AMP (r): ampicillin resistance gene; GPRT: xanthine-guanine phosphoribosyl transferase gene (*i.e.* drug selectable marker); ITR: inverted terminal repeat; P1, P2, P3, P4, and P5: primers 1, 2, 3, 4 and 5; length in red: expected length of amplicon if cassette present; length in blue: expected length of amplicon if wild-type present.

recombination (*i.e.* P3) and one primer binding to the end of the R2RLUC cassette (*i.e.* P5) (Fig. 4.7C). To verify the absence of wild-type parental virus, we used the primer external to the site of recombination (*i.e.* P3) and one primer lying between the left and arms (*i.e.* P4). We were able to rescue all viruses (Fig. 4.7B-D), but we were only able to purify eight of these viruses, as we were not able to obtain pure deletion mutants of A22R, D4R, I2L, G5R or L4R. (vv- $\Delta$ N1L is not shown as it was engineered, rescued and purified by Dr. J.A. McCart's lab).

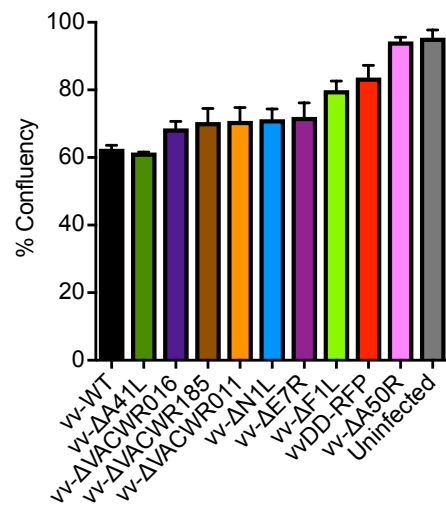
#### **4.3.3 VV DNA ligase deletion mutant vv- $\Delta$ A50R is attenuated in normal cells, but more potent than vvDD in multiple tumour cell lines**

We screened the single deletion mutants *in vitro* by infecting normal human fibroblast cells (*i.e.* GM38 cells) with the deletion mutants as well as with vvDD-RFP (Fig. 4.8A,B) and vvWT (*i.e.* rWT-F13L+). We found that vv- $\Delta$ A50R was the only deletion mutant that was as attenuated as vvDD-RFP (Fig. 4.8A,B) in normal human fibroblast cells. While vv- $\Delta$ A50R was attenuated in normal cells, it was more potent than vvDD and the other deletion mutants in two out of the three human tumour cell lines, namely U20S (osteosarcoma) and A549 (lung adenocarcinoma). In addition, vv- $\Delta$ A50R was more potent than vvDD in two out of the three murine cell lines, namely CT26 (colon carcinoma) and MC38 (colon carcinoma) (Fig 4.9A). In U20S cells, vv- $\Delta$ A50R also formed larger plaques than vvDD and vvWT (Fig. 4.9B,C). The human cell line where vv- $\Delta$ A50R was attenuated was NCI-H226. vv- $\Delta$ N1L is one deletion mutant that was more potent in NCI-H226 than vvDD and was the same or more potent than vvDD in all of the other tumour cell lines except EMT6 which was resistant overall (Fig. 4.9B,C).

**A.**



**B.**

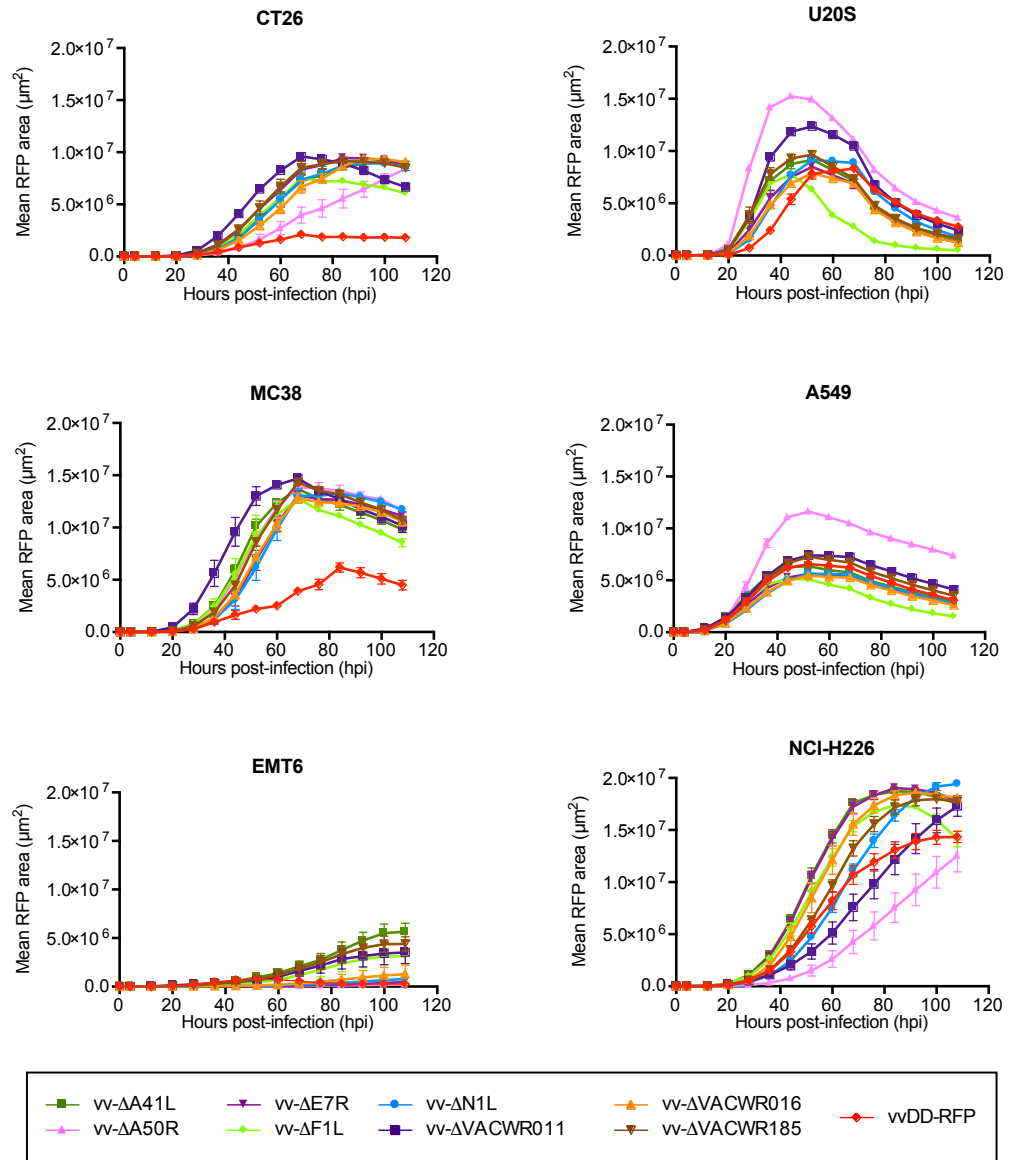




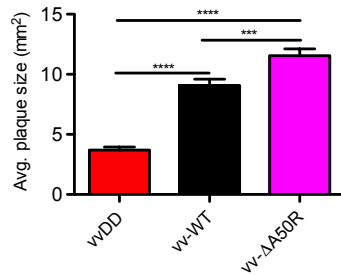
**Figure 4.8: *In vitro* screen of VV deletion mutants in normal cells**

(A) GM38 cells were seeded in 96-well plates and, 6 days post-seeding, were infected with the indicated viruses (20 PFUs/well; MOI of  $\sim 0.002$ ;  $n=3$ ) and imaged live. Plotted on the graph is the mean RFP area per well (*i.e.* 4 fields/well). (B) Cells were treated as in (A). Plotted on the graph is the percent confluency of cells at 108 hours post infection. Data presented as means  $\pm$  SEM

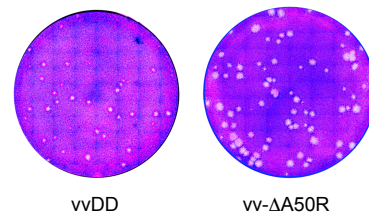
**A.**



**B.**



**C.**



**Figure 4.9: *In vitro* screen of VV deletion mutants in tumour cells**

(A) Confluent wells of the indicated murine (*i.e.* CT26, MC38, EMT6) and human (*i.e.* U20S, A549, NCI-H226) tumour cell lines were infected with the indicated viruses (20 PFUs/well; MOI of  $\sim 0.001$ -0.002;  $n=3$ ) and imaged live. Plotted on the graph is the mean RFP area per well (*i.e.* 4 fields/well). (B) U20S cells were infected with the indicated viruses and overlaid with carboxymethyl cellulose containing media. 3 days post-infection, cells were fixed with formaldehyde and stained with crystal violet. The mean plaque size for each virus is plotted on the graph ( $n=50$ -70). (C) Cells were treated as in (B). Scanned images of vvDD-RFP and vv- $\Delta$ A50R are shown. Data presented as means  $\pm$  SEM

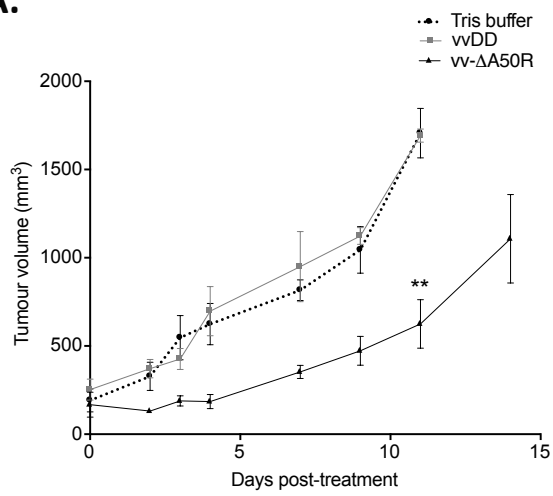
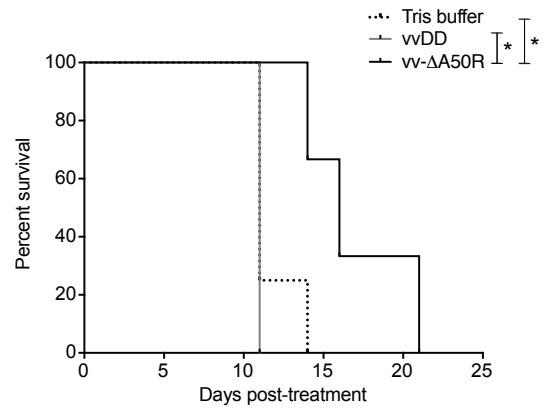
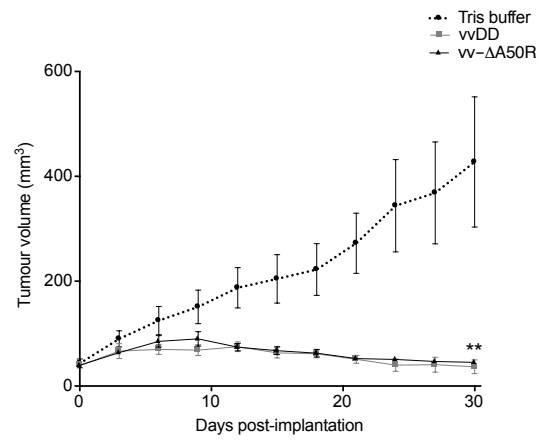
These results demonstrate that vv-ΔA50R would be a good candidate for further investigation, but also vv-ΔN1L, especially given that it has previously been established to be attenuated *in vivo* and exploits an immune-based tumour-specific mechanism.<sup>314</sup>

#### **4.3.4 Treatment with vv-ΔA50R is safe and improves outcomes of tumour-bearing immunocompetent mice**

We pursued further *in vivo* investigations with vv-ΔA50R in MC38 tumour-bearing mice, as the enhancement of spread was the most profound for this cell line out of the murine cell lines we tested *in vitro* (Fig. 4.9A). Mice with subcutaneous tumours on their right flanks treated by intratumoural injection with vv-ΔA50R had significantly delayed tumour progression compared to mice treated with vvDD ( $p \leq 0.01$ ) or vehicle control ( $p \leq 0.01$ ) (Fig. 4.10A). Mice treated with vv-ΔA50R also had significantly prolonged survival compared to mice treated with vvDD ( $p = 0.03$ ) or vehicle control ( $p = 0.04$ ) (Fig. 4.10B). A difficulty with this model is that it was highly prone to ulceration and mice had to be excluded from the study due to ulceration.

We also tested vv-ΔA50R in a human xenograft model with subcutaneous A549 tumours. There was a significant difference in tumour progression for mice treated by intratumoural injection with vv-ΔA50R or vvDD relative to vehicle control ( $p \leq 0.01$ ), but there was no significant difference between vv-ΔA50R and vvDD (Fig. 4.10C). Treatment with vv-ΔA50R appeared to be equally as safe as vvDD at the dose administered as no signs of toxicity were observed.

Larger studies are needed to draw definitive conclusions, but the results showing delayed tumour progression and prolonged survival in MC38 tumour-bearing mice are promising. No therapeutic advantage was achieved in the immunocompromised A549

**A.****B.****C.**

**Figure 4.10: *In vivo* testing of vv-ΔA50R**

(A) C57BL/6 mice bearing subcutaneous MC38 tumours in the right flanks were treated with Tris buffer (n = 4), vvDD (n = 3), or vv-ΔA50R (n = 3) by intratumoural injection on days 13, 16 and 20 post tumour cell implantation ( $1 \times 10^7$  PFUs/dose). The mean tumour volume of each group is plotted until the first mouse in each group reaches endpoint (*i.e.*  $\geq 1500 \text{ mm}^3$ ). (B) Kaplan-Meier survival plot of mice treated as in (A). (C) CD-1 Nude mice bearing subcutaneous A549 tumours in the right flanks were treated by intratumoural injection when the tumours became palpable (*i.e.*  $30\text{-}70 \text{ mm}^3$ ) with 3 doses of virus ( $1 \times 10^7$  PFUs/dose) or Tris buffer 72 h apart (n=5/group). \*p < 0.05, by Log-rank Mantel-Cox test; \*\*p ≤ 0.01, by one-way ANOVA and Tukey's post-test. Data presented as means ± SEM.

tumour-bearing mice treated with vv-ΔA50R relative to vvDD, but vv-ΔA50R was equally as safe as vvDD leaving the door open for treatment with a higher dose and potential therapeutic gains.

#### 4.4 Discussion

We conducted functional genomic screens to identify VV genes that could be deleted without limiting potency in tumour cells. We used a custom poxvirus siRNA library that contained 173 out of the 223 (78%) putative VV WR genes. The library was designed to encode not only siRNAs targeting VV WR genes, but also genes of two strains of monkeypox virus (*i.e.* MPXV-Congo and MPXV-USA) and one strain of variola virus (*i.e.* VARV-CHN48). One of the constraints in constructing the library was synthesizing siRNA that would not target multiple VV genes as well as host genes. With this library, we identified 35 genes that when knocked down with siRNA had no impact on VV replication in both NCI-H226 and U20S cells. We selected 13 out of the 35 genes for which to make deletion mutants. Out of the eight single deletion mutants that we could successfully purify, vv-ΔA50R was the only deletion mutant that we found to be as attenuated as vvDD in normal GM38 fibroblast cells *in vitro*; and, in addition, more potent than vvDD in multiple human and murine tumour cell lines. Furthermore, it was shown to provide a therapeutic advantage over vvDD in a pilot study in MC38 tumour-bearing mice and was demonstrated to have the same safety profile as vvDD in an immunocompromised mouse model.

A50R, as previously noted, encodes a DNA ligase. It has been shown that cellular DNA ligase I is recruited to virus factories and is able to compensate for the loss of A50R in proliferating cells, but not in resting cells.<sup>338</sup> DNA ligase I has been reported to

be elevated in human cancers. Sun *et al.*<sup>341</sup> found that DNA ligase I was elevated in human breast, lung, colon, ovarian, sarcoma and neuroblastoma tumour tissue specimens compared to benign tissue specimens where it was undetectable. In the same study, it was also observed that DNA ligase I levels were very high in human tumours grown in nude mice and appeared to be constitutively expressed throughout the course of tumour development.<sup>341</sup> These findings are consistent with our results where vv-ΔA50R was attenuated in resting normal human fibroblast cells (*i.e.* GM38s), but not in multiple cancer cell lines. They could also explain why vv-ΔA50R was attenuated in NCI-H226 cells as these are slowly dividing tumour cells compared to many other tumour cell lines listed on the National Cancer Institute website.<sup>311</sup> NCI-H226 has a reported doubling time of 61 h,<sup>311</sup> whereas A549 and U20S have reported doubling times of 23 h and 29 h,<sup>311,342</sup> respectively. Based on the screening results, we anticipated that vv-ΔA50R would not be attenuated in NCI-H226 cells; however, in the screens, A50R was only knocked-down. There was likely not a complete abrogation of the expression of viral ligase and cellular ligase was not targeted.

It is intriguing that vv-ΔA50R overall formed larger plaques than vvWT in U20S cells. In addition to having a role as a DNA ligase,<sup>343</sup> A50R has also been shown to recruit cellular topoisomerase II (TOP2) to virus factories.<sup>344</sup> TOP2 catalyzes double-strand DNA cleavage reactions. VV bearing A50R mutations have been shown to be resistant to TOP2 inhibitors (*i.e.* etoposide, mitoxantrone),<sup>345,346</sup> which act by increasing the formation of TOP2:DNA covalent complexes resulting in DNA lesions.<sup>347</sup> Data presented by Lin *et al.*<sup>344</sup> suggest that in the presence of etoposide or mitoxantrone, mutations in A50R limit the amount of topoisomerase at virus factories



leading to a reduction of cleavage complexes on VV DNA. Perhaps, an abrogation of A50R enhances vv-ΔA50R replication over WT in U20S cells by reducing the cleavage complexes and, ultimately, DNA lesions.

We anticipated discovering other deletion mutants besides vv-ΔA50R to be attenuated in normal cells, but not in tumour cells. It is possible that some of the other deletion mutants that we generated are attenuated *in vivo* similar to vv-ΔN1L and vv-ΔF1L that were not attenuated *in vitro*, but are *in vivo*.<sup>312,313,315,319</sup> We attempted to conduct a high-throughput RNAi screen with GM38 cells, so that we could filter out potential deletion mutants that might not be attenuated in normal cells prior to engineering them. However, the difficulty we found in conducting this screen is that quiescent GM38 cells generated a high degree of autofluorescence in the red channel that made it difficult to detect the expression of dsRed above background in fixed cells. We have also found that quiescent GM38 cells generate high background in the green channel as well, so using a VV expressing GFP would likely not have provided a solution. An alternative would be to conduct a screen using cell death as a readout.

The five deletion mutants, namely vv-ΔA22R, vv-ΔD4R, vv-ΔG5R, vv-ΔI2L and vv-ΔL4R, that we were able to rescue, but unable to purify may have also been attenuated in normal cells. With the exception of D4R, these genes had been deemed essential when they were deleted in a TK-negative background, as mutants with these genes deleted were severely impaired or could not be isolated. Based on the screening results with wild-type VV and our previous screens with vvDD, we speculated that they might not be essential in a wild-type VV background in tumour cells, but quite likely in normal cells and elected to proceed with generating deletion mutants. Perhaps the reason we

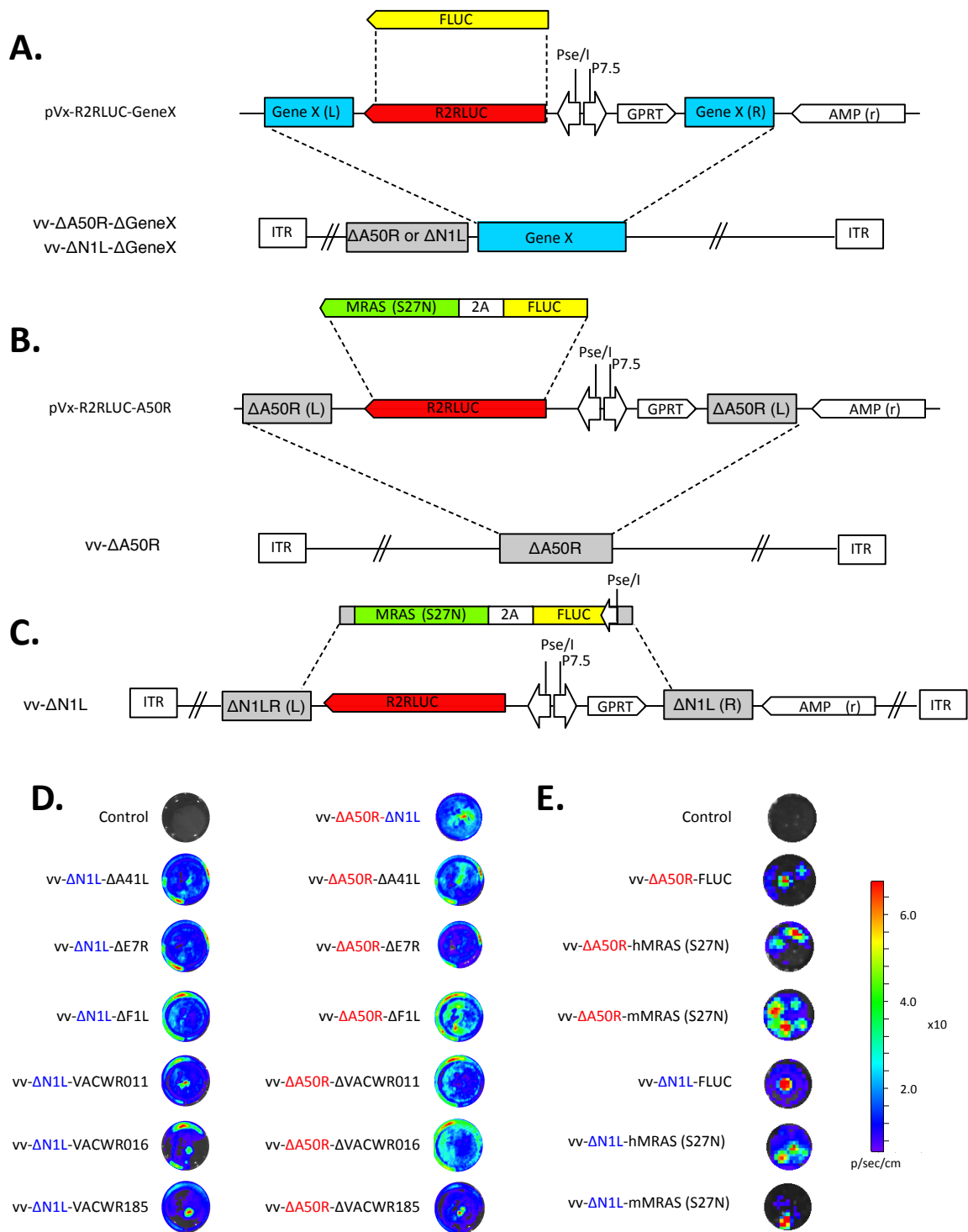
were not able to purify these mutants is that these genes are indeed indispensable; therefore, any plaque we picked would necessarily have some degree of wild-type virus present to compensate for the deletion. The explanation for why these genes scored as neutral in the screens with wild-type VV is that there was likely some level of expression of the gene thus enabling the replication of VV. In the vvDD screens, it could be that replication of vvDD was already attenuated and knocking down one of these genes in addition to TK and VGF was the final limiting factor. An additional measure we could have taken to facilitate the purification of these viruses was to use sonication. Generally, sonication is used to purify VVs as VV is known to form aggregates.<sup>348</sup> We did not use sonication; as in our hands, we were able to successfully purify 13 other VVs without it.

Our aim in conducting the screens was to find alternative means of tumour selectivity to make safe yet more potent oncolytic VVs. While the deletion of A50R could be classified as a cell proliferation-based mechanism similar to  $\Delta$ TK and/or  $\Delta$ VGF, we did find that vv- $\Delta$ A50R did provide a therapeutic advantage over vvDD in a small pilot study with the ectopic MC38 mouse model. As stated previously, a larger study is needed to confirm these results, especially in an orthotopic MC38 model not prone to ulceration. Despite the positive results with the MC38 mouse model, we did not observe any additional therapeutic benefit for vv- $\Delta$ A50R over vvDD in the xenograft A549 model; however, we did demonstrate that it was as safe as vvDD at the dose that we administered in this immunocompromised mouse model. The therapeutic advantage of vv- $\Delta$ A50R over vvDD is that it may be possible to treat with a much higher dose of virus, while still maintaining safety. It also remains to be determined the impact of

combining the deletion of A50R with the deletions of other genes and the gains these could provide.

Among the differences between cancer and normal cells that could potentially be exploited to enhance tumour specificity<sup>37</sup> such as differences in receptor expression, nucleotide synthesis, protein synthesis, cell proliferation, apoptosis and antiviral defences, the latter stands as distinct from the others. This path could prove the most beneficial particularly for treating slowly growing tumours. Future work will entail testing vv-ΔN1L as this was shown to be potent in the slowly growing NCI-H226 tumour cell line.

This work is a first step in designing the next generation of oncolytic VVs. The next step is to generate the best virus backbone by making combination deletion mutants pairing attenuating deletions with non-attenuating deletions, which we are currently working on. As vv-ΔA50R was attenuated in normal human fibroblasts, yet potent in multiple tumour cell lines *in vitro*, we are making double-deletion mutants of ΔA50R with the other candidate genes. We are also constructing combination mutants with ΔN1L as it was potent in multiple tumour cell lines, but is attenuating *in vivo*.<sup>313,315,349</sup> In addition, we are engineering single deletion mutants of vv-ΔA50R and vv-ΔN1L also expressing an MRAS dominant negative (S27N) from the A50R or N1L locus as we had previously found the expression of the MRAS dominant negative to enhance the spread of vvDD when expressed from the TK locus (see Chapter 3); we want to determine if there might be an even greater enhancement of spread in either or both of these backgrounds. We have been able to successfully rescue all viruses (Fig. 4.11), but further rounds of purification are required before they can be tested *in vitro*



#### Figure 4.11: Engineering and rescue of combination mutants

(A) Linear diagrams of pVx-R2RLUC-GeneX shuttle vector and the double deletion mutants, vv-ΔA50R-GeneX or vv-ΔN1L-GeneX. Gene X (L) and Gene X (R) consist of left and right segments of the candidate genes, (*e.g.* A50R, A41L, E7R) respectively. To generate double deletion mutants, a FLUC cassette was cloned in the place of the R2RLUC cassette in shuttle vectors already containing the left and right arms of the candidate genes. CV-1 cells were then infected with vv-ΔA50R-GeneX or vv-ΔN1L-GeneX, followed by transfection with the pVx-FLUC-GeneX shuttle vectors. (B) Linear diagrams of pVx-R2RLUC-A50R shuttle vector and vv-ΔA50R. To make vv-ΔA50R-MRAS (S27N), the cassette comprised of FLUC-2A-MRAS (S27N) was cloned in the place of the R2RLUC cassette in the shuttle vector. CV-1 cells were then infected with vv-ΔA50R followed by transfection with pVx-MRAS(S27N)-2A-FLUC-A50R. (C) Linear diagrams of vv-ΔN1L and the FLUC-2A-MRAS (S27N) cassette with 80 bp regions of homology with N1L on each end. To generate vv-ΔN1L-MRAS (S27N), CV-1 cells were infected with vv-ΔN1L followed by transfection with the PCR amplicon containing the MRAS(S27N)-2A-FLUC cassette and N1L homology regions. (D) Bioluminescent images of wells from 6-well plates following rescue after a minimum of 3 rounds of purification of the indicated viruses. (E) Bioluminescent images of wells from 96-well plates following rescue after a minimum of 3 rounds of purification of the indicated viruses R2RLUC: Red fluorescent protein (mCherry) and renilla luciferase genes with the intervening the foot-and-mouth disease virus 2A sequence motif; Pse/I: synthetic early/late promoter; P7.5: 7.5 promoter; AMP (r): ampicillin resistance gene; GPRT: xanthine-guanine phosphoribosyl transferase gene (*i.e.* drug selectable marker); ITR: inverted terminal repeat; FLUC: firefly luciferase; 2A: porcine teschovirus-1 virus 2A peptide sequence; hMRAS: human MRAS; mMRAS: murine MRAS.

and *in vivo*. We anticipate that testing the combination mutants might provide the most exciting results.

## CONCLUDING REMARKS

The key objective of my first project was to identify host target genes that could be manipulated to enhance oncolytic vaccinia virotherapy using functional genomic screening. I pursued studies with one target identified in the genome-wide RNAi screens, namely MRAS. A vvDD encoding a dominant negative mutant of MRAS [*i.e.* vvDD-mMRAS (S27N)] enhanced efficacy *in vivo* compared to vvDD alone. This study demonstrates the utility of functional genomic screening for identifying host targets and also serves as a proof-of-principle for pursuing further research on other targets identified in the primary RNAi screens. Preliminary studies showed additional promising gene targets that could be further tested on their own or in combinations. As stated in the discussion at the end of Chapter 3, it is unlikely that any one single approach to enhancing therapy will be a silver bullet. Employing functional genomic screening to identify host factors that can be manipulated to enhance spread is only one of many strategies, but it has proved fruitful. Constructing VV vectors that enhance spread could work well in combination with other approaches such as checkpoint inhibitors.

The main objective of the second project that I worked on in parallel to the first one was to increase the therapeutic index of oncolytic VV by identifying alternative means of tumour specificity to the current means, specifically the deletions of thymidine kinase and/or vaccinia growth factor. Functional genomic screening revealed other genes that could potentially be deleted without diminishing potency in tumour cells. We, ultimately, discovered that a VV with a deletion of the DNA ligase gene (*i.e.* vv- $\Delta$ A50R) resulted in tumour-specific replication *in vitro* and enhanced efficacy *in vivo* in

MC38 tumour-bearing mice over vvDD. I also generated other single deletion mutants based on the screening results with the ultimate goal of generating double and combination mutants. The double deletion and combination mutants that I am generating with A50R and N1L could prove the most interesting. One of my greatest interests is to combine the results of both projects and test the combination mutants vv-ΔA50R-MRAS (S27N) and vv-ΔN1L-MRAS (S27N).

In conclusion, there are multiple approaches to enhancing oncolytic virotherapies including vaccinia-based therapies. In the course of completing this project, there have been significant developments in the field of oncolytic virotherapy, and the present work brings an important contribution. In addition to the development of modified VVs with improved efficacy, it opens exciting opportunities for further studies possibly beneficial to cancer patients.



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## **CONTRIBUTIONS OF COLLABORATORS**

Dr. J. Andrea McCart (Toronto General Research Institute, Toronto) provided pVX-R2RLUC and the following viruses: vvDD-eGFP, vvDD-R2RLUC, vv- $\Delta$ N1L, rWT-F13L+, vvWT-RFP and VSC20.

Dr. J.C. Bell (Ottawa Hospital Research Institute, Ottawa) provided JX-594 B5R (P189S).

Dr. Jeffrey Medin (University Health Network, Toronto) provided the pDY lentivirus expression vector.

Dr. Stephen Baird arrayed the human and vaccinia virus siRNA libraries onto microtiter plates as well as assisted in developing analysis scripts, imaging plates and normalizing plasmid DNA used for the shRNA screen. Charles Lefebvre and Dr. David Stojdl assisted in seeding cells for the genome-wide screens.

Dr. Jason Fernandes assisted in the generation of CRISPR-Cas9 cell lines by conducting the FACS step using the MoFlo Cell Sorter at the Ottawa Hospital Research Institute Flow Cytometry Facility (Stemcore Laboratories) (see 2.2.6).

Katelynn Rowe and Melanie Labelle assisted in implanting cells for the 4T1 mouse study (see 2.2.13). Dr. Stephanie Swift and Melanie Labelle assisted with handling mice in the A549 mouse study (see 2.3.6). Katelynn Rowe, Melanie Labelle, Dr. Justyna Kmieck, and Dr. Stephanie Swift assisted in measuring tumours at various times.

## APPENDICES

### Appendix A: Supplementary Materials and Methods

#### I. Guide pair oligonucleotide sequences for generating CRISPR/Cas9n knockout cell lines (see 2.2.6):

h-gRNA#1: Forward guide oligonucleotides for annealing and cloning into pX462 (additional bases used for cloning purposes are in bold):<sup>248</sup>

5'-**CACCGT**ACGGAGATTGACAATCAAT-3'  
5'-**AAACATTG**ATTGTCAATCTCCGTAC-3'

h-gRNA#1: Reverse guide oligonucleotides for annealing and cloning into pX462 (additional bases used for cloning purposes are in bold):<sup>248</sup>

5'-**CACCGT**CAATGGTGGGGTCATAGTC-3'  
5'-**AAACGACTATG**ACCCCACCATTGAC-3'

m-gRNA#1: Forward guide oligonucleotides for annealing and cloning into pX462 (additional bases used for cloning purposes are in bold):<sup>248</sup>

5'-**CACCGT**GACAATCAGTGGGCCATCT-3'  
5'-**AAACAGATGG**CCCCACTGATTGTCAC-3'

m-gRNA#1: Reverse guide oligonucleotides for annealing and cloning into pX462 (additional bases used for cloning purposes are in bold):<sup>248</sup>

5'-**CACCGT**CAATGGTGGGGTCGTAGTC-3'  
5'-**AAACGACTACG**ACCCCACCATTGAC-3'

## II. Primers used for the construction of vvDD-MRAS (S27N) (see 2.2.11):

### 1. Primers for amplifying human and mouse MRAS from cDNA:

The forward and reverse primers consist of the following elements:

Forward primer:

ACTG + **SbfI** (restriction enzyme sequence) + **base** + **2A sequence** + sequence to amplify MRAS gene (The MRAS sequence starts with the second codon as the start codon is removed following the 2A sequence. The additional base to is to keep the MRAS sequence in frame)

Reverse primer:

ACTG + **BamHI** (restriction enzyme sequence)+ sequence to amplify MRAS gene

Human MRAS

Forward primer:

ACTG**CCTGCAGG****A**GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACC  
**CCGGTCCT**GCAACCAGCGCCGTCCCCAG

Reverse primer:

ATCG**GGATCC**TCACAAGATCACACATTGCAGTTTG

Mouse MRAS

Forward primer:

ACTG**CCTGCAGG****A**GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACC  
**CCGGTCCT**GCGACCAGCGCTGTTCCAAG

Reverse primer:

ATCG**GGATCC**TCACAAGATGACACACTGCAGTTTG

### 2. Mutagenesis of FLUC stop codon to SbfI restriction enzyme site

Mutagenesis primer (The SbfI site is highlighted in yellow):

AAGAAGGGCGGAAAGATCGCCGTG**CCTGCAGG**ACTGGGATCCAAGCTTCAATTGTG

### 3. Mutagenesis of MRAS (27S → N)

Mutagenesis primers:

Human MRAS (codon for asparagine indicated in red)

5'-GTGGGGGATGGGGGTGTGGGCAAA**AAT**GCCCTCACCATCCAGTTTTTCCAG-3'

Mouse MRAS (codon for asparagine indicated in red)

5'-GTGGGAGATGGTGGTGTGGGCAAG**AAT**GCGCTCACTATTCAGTTTTTCCAG-3'

#### 4. Primers for subcloning FLUC-2A-MRAS and FLUC into pVx-R2RLUC

##### LEGEND

Sall restriction site = **GTCGAC**

NotI restriction site= **GCGGCCGC**

15bp sequence used for In-Fusion cloning =           

**Yellow** = Start of FLUC

**Pink** = End of MRAS gene (human)

**Green** = End of MRAS gene (mouse)

**Red** = End of FLUC gene

Forward primer for FLUC and FLUC-2A-MRAS cassettes:

5'-TAAGCTCGAA**GTCGAC****ATGGAAGACGCCAAAAACATAAAGAAAGG**-3'

Reverse primers:

Human MRAS:

5'-TCTAGAGTC**GCGGCCGC****TCACAAGATCACACATTGCAGTTTG**-3'

Mouse MRAS:

5'-TCTAGAGTC**GCGGCCGC****TCACAAGATGACACACTGCAGTTTG**-3'

FLUC:

5'-TCTAGAGTC**GCGGCCGC****TTACACGGCGATCTTTCCGCCCTTC**-3'

### III. Primers used for the construction of VV single deletion mutants (see 2.3.2)

#### 1. Sequences used to amplify VV genes:

Restriction enzyme (RE) cut sites:

Esp3I: **TGAGACG**

KasI = **GGCGCC**

Paul = **GCGCGC**

SpeI = **ACTAGT**

The following sequences were appended to each arm (The 15 bp sequence used for In-Fusion cloning is underlined):

#### LEFT ARM

Forward primer (RE = Esp3I):

5'-AACTTTTGGCGAAAA**TGAGACG**-3'

Reverse primer (RE = SpeI):

5'-TAATAGGCCT**ACTAGT**-3'

#### RIGHT ARM

Forward primer (RE = Paul):

5'-AGGGTTAATT**GCGCGC**-3'

Reverse primer (RE = KasI):

5'-CTATGGAAAAACGCC**GGCGCC**-3'

#### A22R

##### LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**GCAGAGAATGTTTGGGATATCCAG

Reverse primer:

5'-TAATAGGCCT**ACTAGT**CCTTCTAGGCTGACGTTCTAG

##### RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**CCTCGCCTGTCATGTCTGGTAATTC

Reverse primer:

5'-CTATGGAAAAACGCC**GGCGCC**CATATCCTCTGAGGAAATGCGTTC

#### A41L

##### LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**ACGTGGTTGACCATTCTTGGTG



Reverse primer:

5'-TAATAGGCCT**ACTAGT**CCGTATACGCGAAGTTTGCTAG

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**TCCTCCGTTTGATACAGATGCAC

Reverse primer:

5'-CTATGGAAAAACGCC**GCGGCC**GTAATCAACGATGGCGGCATC

### **A50R**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**GACGTCGCTTCGCGAATTTAG

Reverse primer:

5'-TAATAGGCCT**ACTAGT**GTTAAGTACGTACCGAGGACC

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**GGAGTATACGAACCGGAAAGAG

Reverse primer:

5'- CTATGGAAAAACGCC**GCGGCC**CCATTTGCGGTATGGGACTTGG

### **D4R**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**GACTGTATCACACGCGCCATATAC

Reverse primer:

5'-TAATAGGCCT**ACTAGT**ATTCCAGGGTATAACCCCGTC

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**CTGCTGCAGCATATAACTAAACACG

Reverse primer:

5'- CTATGGAAAAACGCC**GCGGCC**CCCTCATCCCTAAGACTTTTGAATAG

### **E7R**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**CCTTGGATAAAAGCATCCATCTAACC

Reverse primer:

5'-TAATAGGCCT**ACTAGT**GTTGCGTTTCTGGAGAACTTTTCTC

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**GCTAGAACAACTCTCTACCCTAAAC

Reverse primer:

5'- CTATGGAAAAACGCC**GGCGCC**CTCCAAATTGCAGTTTCTGGTATCC

### **F1L**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**CATTACAGATCTGATGACCGACG

Reverse primer:

5'-TAATAGGCCT**ACTAGT**CCGAATTGGGTAGACGATGTATG

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**CTAGTCTTTGTGGGCAACTGTC

Reverse primer:

5'- CTATGGAAAAACGCC**GGCGCC**GGAGGCGGTGTAATAGACGAAG

### **G5R**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**CAAGACGTTAGAGACAAGAGACG

Reverse primer:

5'-TAATAGGCCT**ACTAGT**TAGCATAGTATCCTGATCGGTAC

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**CGGCGTTAGTGAATGGATGTG

Reverse primer:

5'- CTATGGAAAAACGCC**GGCGCC**CCTTGCGTGGTTCTAACGCATAC

### **I2L**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**GGATTCCCTCCATAATTGCACAATC

Reverse primer:

5'-TAATAGGCCT**ACTAGT**GTGATGAGAAAACAGTCACATCAAC

RIGHT ARM

Forward primer:

5'-AGGGTTAATT**GCGCGC**CACCAAATATAGCGGCGTACAAC

Reverse primer:

5'- CTATGGAAAAACGCC**GGCGCC**CGACTGGAGAATCATCCATTCTC

### **L4R**

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA**TGAGACG**GTTTGTAAACATCGGTACGGGTATTC

Reverse primer:

5'-TAATAGGCCTACTAGTAGGCATAACTTCATTGTTAGGATCC

RIGHT ARM

Forward primer:

5'-AGGGTTAATTGCGCGCGGTTATCTTCCGTCAAGGATCATC

Reverse primer:

5'-CTATGGAAAAACGCCGCGGCCGTGGGCTCTATAAACACAGG

**VACWR011** (Orientation of gene: L)

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA TGAGACGGACGCACCGGTTTCTCTTCG

Reverse primer:

5'-TAATAGGCCTACTAGTGTGGTTATTGATTGGATAACCGATG

RIGHT ARM

Forward primer:

5'-AGGGTTAATTGCGCGCCATATAGTCCCTTCCGGCGATAC

Reverse primer:

5'-CTATGGAAAAACGCCGCGGCCCTATTACACCGGCTGAGTATGC

**VACWR016** (Orientation of gene: L)

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA TGAGACGCTCGAGTGCAACATGTTAGATCG

Reverse primer:

5'-TAATAGGCCTACTAGTGGTGTGGTAGAACATGGATTTGAC

RIGHT ARM

Forward primer:

5'-AGGGTTAATTGCGCGCCTAGTAGAGCCATCGCTATATCC

Reverse primer:

5'-CTATGGAAAAACGCCGCGGCCACGGAGGTGTAAAATCTGGAAC TG

**VACWR185** (Orientation of gene: R)

LEFT ARM

Forward primer:

5'-AACTTTTGGCGAAAA TGAGACGGTTACTGCTGTGGACGTACATGG

Reverse primer:

5'-TAATAGGCCTACTAGTGCATTTCTGGTCTGGGACTCC

RIGHT ARM

Forward primer:

5'-AGGGTTAATTGCGCGCCTTCAGGCTGGTGAATCAGTC

Reverse primer:

5'-CTATGGAAAAACGCCGCGGCCCTGCAAACACAGCACAACAGC

## *2. Additional primers for verifying purity of deletion mutants*

Reverse primer (anneals to a site within the R2RLUC cassette):

5'-TTCGTGCTGAAGAACGAGCAG-3'

### **A41L**

Forward primer (anneals to a site upstream of the LEFT arm):

5'-TCCCAAACCTGCATTTCGTGTTACAC-3'

Reverse primer (anneals to a site between the LEFT and RIGHT arms):

5'-CAGTTGGTGTATACACTAATCGCG-3'

### **A50R**

Forward primer (anneals to a site upstream of the LEFT arm):

5'-TGA TAT CCG CGA ACG ATA TTT GTG-3'

Reverse primer (anneals to site between the LEFT and RIGHT arms):

5'-ACGCCTTATTGACAGAATCACACG-3'

### **VACWR011**

Forward primer (anneals to a site upstream of LEFT arm):

5'-GTTATGTTTCTAAAACGGGTACGAC-3'

Reverse primer (anneals to a site between the LEFT and RIGHT arms):

5'-CTTACTGAACTATACGTAAGTGC-3'

#### IV. Primers used for the construction of VV combination deletion mutants (see 2.3.7)

1. Primers used to amplify the FLUC cassette from pVx-FLUC and the FLUC-2A-MRAS (S27N) cassette from pVx-FLUC-2A-MRAS (S27N) for cloning into pVx-R2RLUC-A50R:

##### LEGEND

Restriction sites

Sall = **GTCGAC**

NotI = **GCGGCCGC**

15 bp sequence (for In-Fusion cloning) = underlined

**Yellow** = Start of FLUC

**Pink** = End of FLUC

**Red** = End of MRAS

Forward primer

5'-TAAGCTCGAA**GTCGAC****ATGGAAG**-3'

Reverse primer

5'-TCTAGAGTC**GCGGCCGC****TTAC**-3'

Reverse primer (mouse and human)

5'-TCTAGAGTC**GCGGCCGC****TC**-3'

2. Primers used to amplify the FLUC cassette from pVx-FLUC and the FLUC-2A-MRAS (S27N)

cassette from pVX-FLUC-2A-MRAS (S27N) with 80bp homology ends to N1L:

##### LEGEND

**Start of Pse/I promoter**

**LEFT ARM** (N1L)

**End of mouse FLUC-2A-MRAS** cassette

**End of human FLUC-2A-MRAS** cassette

**END of FLUC** cassette

**RIGHT ARM** (N1L)

**Primer to amplify N1L left arm (for mouse MRAS):**

5'**ATAGATCAATCATTAGATCATCAAAATATGTTTCAATCATCCTAAAGAGTATGGTGAATG**  
**ACTCTTCCCATCTAATTTCTCTCACAAGATGACACACTGCAG**-3'

**Primer N1L left arm (for human MRAS):**

5' ATAGATCAATCATTAGATCATCAAAATATGTTTCAATCATCCTAAAGAGTATGGTGAATG  
ACTCTTCCCATCTAATTTCT GCTCACAAGATCACACATTGCAG-3'

**Primer to amplify N1L left arm (for FLUC only cassette):**

5' ATAGATCAATCATTAGATCATCAAAATATGTTTCAATCATCCTAAAGAGTATGGTGAATG  
ACTCTTCCCATCTAATTTCT TTACACGGCGATCTTCCGC-3'

**Primer to amplify N1L right arm (for mouse or human MRAS and FLUC only)**

5' ATGAGGACTCTACTTATTAGATATATTCTTTGGAGAAATGACAACGATCAAACCTATTAT  
AATGATGATTTTAAAAAGCT AAGCGGGTGGGTTTGGAATTAG-3'

## Appendix B: JoVE article (published)

I wrote this article in its entirety with minor edits by Dr. David Stojdl. Dr. D.J. Mahoney, Dr. S.D. Baird, C.A. Lefebvre, and I contributed to Figure 4.



Journal of Visualized Experiments

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### Video Article

## Genome-wide RNAi Screening to Identify Host Factors That Modulate Oncolytic Virus Therapy

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URL: <https://www.jove.com/video/56913>

DOI: [doi:10.3791/56913](https://doi.org/10.3791/56913)

Keywords: Cancer Research, Issue 134, Oncolytic, virus, RNAi, screen, high-throughput, cancer, vaccinia, rhabdovirus, cell, host, functional genomics

Date Published: 4/3/2018

Citation: Allan, K.J., Mahoney, D.J., Baird, S.D., Lefebvre, C.A., Stojdl, D.F. Genome-wide RNAi Screening to Identify Host Factors That Modulate Oncolytic Virus Therapy. *J. Vis. Exp.* (134), e56913, doi:10.3791/56913 (2018).

### Abstract

High-throughput genome-wide RNAi (RNA interference) screening technology has been widely used for discovering host factors that impact virus replication. Here we present the application of this technology to uncovering host targets that specifically modulate the replication of Maraba virus, an oncolytic rhabdovirus, and vaccinia virus with the goal of enhancing therapy. While the protocol has been tested for use with oncolytic Maraba virus and oncolytic vaccinia virus, this approach is applicable to other oncolytic viruses and can also be utilized for identifying host targets that modulate virus replication in mammalian cells in general. This protocol describes the development and validation of an assay for high-throughput RNAi screening in mammalian cells, the key considerations and preparation steps important for conducting a primary high-throughput RNAi screen, and a step-by-step guide for conducting a primary high-throughput RNAi screen; in addition, it broadly outlines the methods for conducting secondary screen validation and tertiary validation studies. The benefit of high-throughput RNAi screening is that it allows one to catalogue, in an extensive and unbiased fashion, host factors that modulate any aspect of virus replication for which one can develop an *in vitro* assay such as infectivity, burst size, and cytotoxicity. It has the power to uncover biotherapeutic targets unforeseen based on current knowledge.

### Video Link

The video component of this article can be found at <https://www.jove.com/video/56913/>

### Introduction

High-throughput genome-wide RNAi screening has proven to be an invaluable tool for revealing biological insights in diverse areas of study including virus biology. Over the past decade or so, numerous groups have undertaken cell-based large-scale RNAi screening to extensively identify host genes that modulate virus replication (Reviewed in<sup>1,2,3,4,5,6,7</sup>). Interestingly, a large number of these screens have been conducted in cancer cells and several with viruses that are current oncolytic virus candidates including vaccinia virus<sup>8,9,10</sup>, Myxoma virus<sup>1</sup>, herpes simplex virus<sup>12</sup>, vesicular stomatitis virus<sup>13,14</sup>, and Maraba virus<sup>15</sup>. While the focus of the majority of these studies was on identifying host factors that influence some aspect of virus replication and not on identifying biotherapeutic targets for enhancing oncolytic virus therapy, valuable data could be mined from these data sets in this regard. It is clear that the powerful potential to identify host targets that can be manipulated to enhance oncolytic virus therapy has not been fully exploited.

A plethora of oncolytic virus platforms are currently being tested in preclinical and clinical studies including herpes simplex virus, reovirus and vaccinia virus, which have had demonstrable success in late-stage clinical trials. For the majority of these platforms, efforts have been directed toward altering the virus genomes to enhance therapy. For instance, to increase tumor specificity, specific virus genes have been deleted or mutated to significantly restrict viral replication in normal cells, but not in tumor cells. In some cases, transgenes have been added like GM-CSF (granulocyte macrophage colony-stimulating factor) to potentiate the immune response or NIS (sodium iodide symporter) to enable *in vivo* imaging and cellular radiiodide uptake for therapeutic purposes. However, there has been very little work focused on manipulating host/tumor genes and exploring the impact of host/tumor genes on oncolytic virus replication. With the advent of high-throughput screening technology, it is now possible to probe host-virus interactions on a genome scale and to decipher opportunities to manipulate the host genome to enhance oncolytic virus therapy (Reviewed in<sup>16,17,18</sup>).

We reported the first study demonstrating proof-of-concept for using high-throughput genetic screening to identify host targets that could be manipulated to enhance oncolytic virus therapy. To discover host genes that modulate Maraba virus oncolysis, an oncolytic rhabdovirus currently being tested in phase I and II trials, we conducted genome-wide RNAi screens across three different tumor cell lines in duplicate with an siRNA (short interfering RNA) library targeting 18,120 genes<sup>15</sup>. Pathway analysis of hits identified in the screens revealed enrichment of members of

the ER (endoplasmic reticulum) stress response pathways. We selected 10 hits within these pathways for secondary validation using siRNA with different targeting sequences from those of the primary screen. As part of tertiary validation, we conducted a rescue experiment with IRE1 $\alpha$  (inositol-requiring enzyme 1  $\alpha$ ) to confirm that the hit was on target. *In vitro* and *in vivo* testing using a small molecule inhibitor of IRE1 $\alpha$  resulted in dramatically enhanced oncolytic efficacy.

Workenhe *et al.*<sup>12</sup> also conducted a genome-wide RNAi screen using a pooled lentiviral shRNA (short hairpin RNA) library targeting 16,056 genes to identify host factors restricting the human herpes simplex virus type 1 (HSV-1) mutant KM100-mediated oncolysis of breast cancer cells. In the primary screen, they identified 343 genes the knockdown of which lead to enhanced cytotoxicity of KM100-infected cells over mock-infected cells; they selected 24 of these genes for secondary validation. Out of the 24 genes, 8 genes were confirmed in the secondary screen with one of them being SRSF2 (serine/arginine-rich splicing factor 2) which was subsequently confirmed using a genetic rescue experiment during tertiary validation. The group went on to identify a DNA topoisomerase inhibitor chemotherapeutic that reduced the phosphorylation of SRSF2 and showed that the combination treatment of HSV-1 KM100 with this inhibitor lead to extended survival of TUBO tumor-bearing mice.

In the aforementioned studies, a similar workflow was followed. Each began with a primary genome-wide RNAi screen followed by a secondary screen on a select number of hits identified in the primary screen. This was followed by tertiary validation studies, which included confirming that the hit was on-target by performing genetic rescue experiments *in vitro* with ultimate validation performed by manipulating the target gene product *in vivo*. In this article, we first provide a general protocol for the development and validation of a high-throughput siRNA-screening assay, which involves determining the optimal transfection conditions, amount of virus, and length of infection. We also offer a detailed protocol for conducting a primary a high-throughput siRNA screen as well as an overall description of the method for performing secondary screen validation and tertiary validation.

## Protocol

### 1. Development and validation of a high-throughput siRNA screening assay

NOTE: For all experiments, use Hepes-buffered growth media appropriate for each cell line. See **Figure 1** for an overview of the workflow from assay optimization to tertiary validation.

#### 1. Determination of the optimal transfection conditions

1. Select a tumor cell line or ideally multiple tumor cells lines with which to optimize transfection conditions (e.g. 786-O, NCI-H226, SF268, U373, OVCAR-8, U2OS; see Discussion for more on the selection of cell lines).
2. Select a transfection reagent or several to test. It may be necessary to test several transfection reagents, as some may be more toxic or efficient than others in a given cell line. Also, keep in mind that some may impact on virus infection or may generate high background signal upon imaging.
3. Decide on positive [e.g. siRNA targeting PLK-1 (Polo Like Kinase 1) mRNA, which induces cell death] and negative transfection controls (e.g. non-targeting siRNA).
4. Follow the reverse transfection protocol for the particular transfection reagent, but vary the amount of transfection reagent (e.g. 0.05, 0.1, 0.15 and 0.2  $\mu$ L/well) and cell seeding densities (e.g. 625, 1250, 2500 cells/well). Include replicates of the following conditions: untreated cells, mock transfected cells (*i.e.* cells plus transfection reagent), and cells transfected with positive and negative transfection control siRNA (see **Figure 2A** for a sample plate layout).
  1. In general, prepare 80 nM dilutions of each siRNA for a final concentration in the well of 10 nM (depending on the library, a higher concentration of siRNA may be required). Unless otherwise specified, dilute the siRNA with the transfection reagent diluent. Prepare dilutions of the transfection reagent.
  2. Pipet 5  $\mu$ L of siRNA per well followed by 5  $\mu$ L of diluted transfection reagent. To facilitate this step, distribute along a column of a U-bottom 96-well plate, the siRNA mixes and the siRNA diluent alone (for untreated and mock transfected cells). Distribute along a second column, the diluted transfection reagent and transfection reagent diluent alone (for untreated cells).
    1. Using an electronic pipet, pipet 5  $\mu$ L/well of the contents of the first column containing siRNA or siRNA diluent into the corresponding wells of the 384-well plate followed by 5  $\mu$ L/well of the diluted transfection reagent or transfection reagent diluent from the second column.
3. Centrifuge the plates at 1026 x g for 1 min at room temperature, and incubate for the time prescribed for the transfection reagent (e.g. 5-20 min).
4. While the plates are incubating, prepare cell suspensions of different densities. Pipet 30  $\mu$ L of cell suspension per well. Before placing the plates in the incubator, let the plates sit for 45-60 min at room temperature to allow for the uniform settling of cells<sup>19</sup>. Note: If the incubation period prescribed for the transfection reagent with siRNA is short, prepare the cell suspensions before the incubation.
5. Incubate for 48 to 72 h depending on the desired length of knockdown chosen for the screen.
5. Prepare a fixing and staining solution consisting of formaldehyde, Hoechst 33342 and PBS (phosphate-buffered saline) for a final concentration in each well of 4% formaldehyde and 5  $\mu$ g/mL of Hoechst 33342. As needed, try a higher (e.g. 7.5  $\mu$ g/mL) or lower concentration (e.g. 2  $\mu$ g/mL) of Hoechst if the signal is too weak or too strong, respectively.
6. Dispense 30  $\mu$ L of the fixing and staining solution directly into all wells. Incubate the plates for 30 min at 37 °C. Store the plates at 4 °C. If using a non-confocal microscope, wash the plates with PBS using a plate washer; in most cases, washing is unnecessary when using a confocal microscope.
7. Develop a script to quantify the number of cells per well.

NOTE: There are several software suites available for this purpose and, while each may have different ways of generating scripts, they all generally have a means of identifying nuclei, cells and regions of interest as well as calculating intensities and morphological properties. When developing a screening script, it is important to consider the time required to run the script per plate and to perhaps include only essential measurements to minimize processing time. For example, to quantify cell number, one might calculate the



Hoechst area per well above a specified intensity; and to quantify virus spread, one might calculate the GFP (green fluorescent protein) area per well above a specified intensity. The "pared down" script will need to be compared to the more elaborate script to ensure no essential information is lost.

8. Analyze the results to determine the optimal transfection conditions, that is the transfection reagent amount and the cell seeding density that results in significant cell death (0-30% cell survival) and is minimally toxic to the cells (e.g. 90-100% cell survival). Note: It may take longer to observe the cell death phenotype in cell lines with long doubling times. See **Figure 2B** and **2C** for representative results. Also, ensure that the cells transfected with non-targeting siRNA are approximately 90-100% confluent at the time of fixation as later on one will want to infect cells that are of this confluency.

## 2. Determination of the optimal amount of virus and length of infection

1. Perform the same reverse transfection steps as described above (see 1.1.4.1 to 1.1.4.4); however, use only the optimal transfection conditions (see 1.1.8) (e.g. for 786-O cells: 1500 cells/well and 0.05  $\mu$ L/well of RNAiMAX) and, in addition, include extra wells to be infected with virus [e.g. wells with untreated cells, mock transfected cells, cells transfected with non-targeting siRNA, and cells transfected with positive virus controls if ones are known (i.e. siRNA targeting host genes that will inhibit or enhance replication)].
  1. See **Figure 3A** for a sample plate layout. Incubate for 48-72 h.
2. Infect the additional virus wells with an oncolytic virus expressing a fluorescent reporter protein (e.g. GFP) with a range of multiplicities of infection (MOIs) (e.g. 0.001 to 10; The range chosen will be depend on the level of resistance or susceptibility of the cell line.). Include uninfected wells as well. Pipet 40  $\mu$ L of each virus dilution per well for a final volume of 80  $\mu$ L. Centrifuge each plate at 400 x g for 30 min at room temperature.
3. Following infection, image the cells live with a high-content, automated microscope at regular intervals (e.g. every 4-8 h). See **Figure 3B** and **3C**.
4. Develop a script to quantify virus spread (e.g. GFP area per well) (see 1.1.7). Analyze the images and select a time-point and an MOI that allow for the detection of increases and decreases in spread. Ensure the time-point and MOI fall within a linear range to facilitate detection of small changes in spread. Estimate the Z-factor (or Z') for the given time-point and MOI.  
NOTE: Estimated Z-factor =  $1 - 3(\sigma_p + \sigma_n)/|\mu_p - \mu_n|$ , where  $\sigma_p$  is the sample standard deviation of the positive virus controls and  $\sigma_n$  is the sample standard deviation of the negative virus controls; and  $\mu_p$  is the sample mean of the positive virus controls and  $\mu_n$  the sample mean of the negative controls. An estimated Z-factor of 0.5 - 1.0 is considered to be an excellent assay and suitable for screening<sup>20,21</sup>.
5. Confirm that the negative virus control siRNA has no impact on virus replication by comparing it to the mock transfected and untreated wells. It is often necessary to test multiple negative controls as some negative siRNA controls can impact on virus replication.

## 3. Final validation of the high-throughput screening assay

1. Repeat steps 1.2.1 and 1.2.2 above, but only this time infect cells with the optimal MOI. Instead of live imaging, fix and stain the plate (as described in 1.1.5 and 1.1.6) at the optimal time-point (see 1.2.4).
2. Image the plate. Analyze the results using the same script that will be used for the screen (see 1.1.7 and 1.2.4). Confirm the transfection conditions (i.e. good knockdown in wells transfected with positive transfection control and minimal toxicity in wells transfected with negative transfection control). Estimate the Z-factor to determine the robustness of the assay (see 1.2.4).

## 4. Further testing and considerations for conducting high-throughput screening

1. Test the durability of the microtiter plates as some plates may crack during centrifugation, especially when the lids are kept on and multiple plates are centrifuged at once. To reduce cracking, centrifuge without the lids (provided the plates have seals), or decrease the number of plates centrifuged per bucket.
2. Test the stability of the transfection reagent mix over time. Once the transfection reagent is added to the plates, there may be a significant delay before the addition of cells; therefore, it is important to know the period of time for which the transfection reagent mix will remain effective. For the screen, it may be necessary to prepare cells in advance of adding the transfection reagent mix to the plates.
3. Prepare for using the liquid dispenser. Determine the volumes required to dispense transfection reagent, cells, virus, and fix taking into account the volume that the tubing holds, the volume lost to pre-dispensing (if the unit has this feature), and the residual volume required in the bottles used for dispensing.
  1. Set and test the programs that will be used for dispensing transfection reagent, cells, and virus. To minimize the loss of transfection reagent, limit, if possible, the number of pre-dispenses. Establish a protocol for calibrating the liquid dispenser and testing its accuracy and precision.
4. Prepare for the bulk dispensing of transfection reagent, cells and virus
  1. First determine the number of plates to be processed in one batch considering the number of plates of cells that can feasibly be trypsinized at once, the number of microtiter plates that can be centrifuged at once, the length of time required to process one batch of plates with the liquid dispenser, and also the time required for imaging. Note: Generally, processing approximately 30 plates at a time is quite feasible and processing 3 batches of plates is manageable in one day.
  2. Empirically verify the volume of transfection reagent required to process one batch of plates. Test that this volume is adequate by dispensing 5  $\mu$ L of distilled water multiple times across one plate the equivalent number of times that will be required to process one batch.
  3. To ensure the even distribution of cells, estimate the time necessary to dispense cells across one batch of plates. Prepare a bottle containing the volume of cells required for a batch, and dispense the cells into multiple columns of a plate at a time, at regular intervals over the same course of time it will take to dispense across a batch of plates. Test how often it will be necessary to mix the cells to maintain an even distribution of cells.
  4. To ensure the even distribution of virus, repeat the same procedure as above (see 1.4.4.3), but this time dispensing virus onto confluent wells. If the virus is not evenly distributed, consider preparing the virus in conical tubes and vortexing each tube prior to dispensing.

5. Track the growth of cells to determine the timeline for growing enough cells for the screen. Also, determine the average number of cells that can be harvested per plate of cells growing in log phase in order to be able to estimate the number required for the screen.
5. **Final preparation steps for high-throughput screening**
  1. Up to one month prior to commencing screening, determine all of the materials necessary for the screen. Order any required reagents (see Materials). Ensure the required amount of virus is on hand (Preferably, use the same stock that was used during optimization.).
  2. Up to two weeks prior to commencing screening, thaw and grow up the cells required for the screen. Ensure the availability of the centrifuge and square centrifuge buckets.
  3. During the week prior to commencing screening, prepare media bottles for the reverse transfection and infection, a stock of 70% ethanol, 2X transfection reagent diluent (e.g. 2X Opti-MEM), if necessary (e.g. if the siRNA library is diluted in RNase-free water, one will want to use 2X to transfect cells), and aliquots of virus (one per batch of plates). Verify the condition of the cells.
    1. Prepare a stock solution of Hoechst 33342 stain (e.g. 5 mg/mL). Prepare a balance plate if one will be required for centrifuging.
    2. Ensure the plate washer is in good working order if it will be used. Ensure the liquid dispenser is properly calibrated and dispensing accurately and precisely. Also, ensure the programs are properly set on the liquid dispenser.

## 2. Conducting the primary high throughput screen

NOTE: Prior to commencing the RNAi screen, microtiter plates (e.g. 384-well plates) need to be arrayed with the siRNA library and screening controls. The following steps are best conducted by two persons with one person (i.e. Person A) being primarily responsible for dispensing the transfection reagent and cells across the plates, and the second person (i.e. Person B) being responsible for centrifuging the plates immediately prior to transfection and for preparing the cells. Included in parentheses are some specifics for processing one batch of 33 plates (i.e. half of the library) with the 786-O cell line.

1. **Preparation for reverse transfection**
  1. Person A: Place enough media, trypsin and PBS necessary to process one batch of plates in a 37 °C water bath. Optional: Trypsinize a plate of cells, and count the number of cells per plate in order to provide a real-time estimate of the number of plates required per batch.
  2. Obtain a batch of plates (e.g. 33 plates) from the freezer and wait 20-30 min to allow the plates to defrost. While the plates are defrosting, continue with the following steps.
    1. Person A: Pipet into 50 mL conical tubes the amount of transfection reagent diluent necessary for processing one batch of plates (e.g. 2 x 37.125 mL) and place them in a 37 °C water bath. Fill two bottles with 70% ethanol (e.g. 250-500 mL bottles). Fill two 50 mL conical tubes with PBS and one with distilled water.
    2. Person A: Immerse the liquid dispenser tubing into a bottle of 70% ethanol. If all of the tips will not be used for dispensing the transfection reagent (e.g. if the outer wells of the plate are not being used), detach the unnecessary tubing first. Place a piece of masking tape around the tubing to mark the point below which the tubing can be considered sterile. Prime with approximately 25 mL. Pour additional ethanol into the bottle to replace the lost volume. Let the tubing sit in ethanol for a minimum of 5 min.
    3. Person B: Prepare to trypsinize cells. Spray the tissue culture (TC) hood with 70% ethanol. Spray and place the necessary pipets, tips, bottles and microfuge tubes for preparing cells in the hood (see 2.2.1.1 and 2.2.1.2). Centrifuge the sealed plates in sets at 1026 x g for 2 min at room temperature. Remove the lids from the plates in a TC hood, if previously determined that the plates might crack during centrifugation when the lids are kept on (see 1.4.1).
  3. Person A: Remove the seals from the plates. Before, during or after the seals have been removed (depending on the length of incubation required), pipet the required amount of transfection reagent (e.g. 375 µL/tube for a final concentration of 0.05 µl of RNAiMAX/well) into the conical tubes containing the pre-warmed transfection reagent diluent (e.g. 37.125 mL/tube).
  4. Mix by pipetting up and down 10 times. Incubate at room temperature for the time prescribed for the transfection reagent being used (e.g. 0-10 min).
2. **Reverse transfection**
  1. Have Person B complete the following tasks.
    1. Begin trypsinizing the cells (e.g. 3 plates of 786-O cells). Aspirate the media from each plate. Rinse with 9 mL of PBS. Pipet 3 mL of trypsin per plate. Incubate at 37 °C for approximately 5 min. Resuspend the cells with 22 mL of media. Pipet up and down 10 times.
    2. Combine the cell suspension from each tissue culture plate into a sterile bottle. Mix the cell suspension by inverting. Pipet 1 mL aliquots of the cell suspension into two separate microfuge tubes. Remove a sample from each microfuge tube and count the cells. Calculate the volume of cell suspension required for the density required (e.g. 1500 cells/well), and prepare enough diluted cell suspension (e.g. 500 mL) to dispense across one batch of plates.
  2. Have Person A complete the following tasks in parallel to Person B.
    1. Empty the liquid dispenser tubing of ethanol. Prime the tubing with approximately 25 mL of PBS, and then empty the tubing. Be careful when handling the tubing to ensure that the section of tubing below the masking tape that will be immersed in transfection reagent and later in the cell suspension is kept sterile. Set apart plates that can safely be done with one conical tube of transfection reagent solution.
    2. Pipet the transfection reagent solutions up and down 10 times. Prime the tubing with the transfection reagent solution. To minimize the loss of transfection reagent, prime with just enough solution to barely clear the tips. Select the correct program on the liquid dispenser and dispense 5 µl of transfection reagent per well.  
CAUTION: Closely monitor the level of the transfection reagent so that it is replenished before it runs out. The completion of the plates that are set apart should provide a cue to add more transfection reagent solution.

3. Centrifuge the plates at 1026 x g for 1 min at room temperature. This time the lids will be on, so it may be necessary to centrifuge less plates at a time to prevent cracking (e.g. 2 plates per bucket). Incubate the plates at room temperature for the time prescribed for the transfection reagent being used (e.g. 10-20 min).
4. During the incubation, empty the tubing of the transfection reagent solution, and place it into a full 50 mL conical tube containing PBS. Rinse the tubing with approximately 25 mL of PBS by priming and emptying.
  1. If more of the tips are going to be used for dispensing cells than were used for dispensing transfection reagent, re-attach the unused tubing, and re-sterilize all the tubing with 70% ethanol before rinsing the tubing with PBS (see 2.1.2.2).
5. Mix the bottle containing the cell suspension once it has been prepared. Place the tubing in the cell suspension, and prime enough to see that each tip is dispensing properly. Dispense 30 µL per well of the cell suspension across all the plates. If necessary, mix the cell suspension at regular intervals to avoid settling.  
CAUTION: Sometimes droplets of suspension containing media may stick to the sides of the tips. When this is observed, aspirate as soon as possible or wipe with lint-free tissue doused in 70% ethanol.
6. Empty the tubing of the cell suspension. Rinse the tubing with approximately 25 mL of PBS, followed by approximately 50 mL of distilled water.
3. Allow the plates to sit for 45-60 min from the time of cell seeding at room temperature before returning the plates to the incubator. Incubate the plates in an undisturbed incubator for the desired length of gene silencing (e.g. 48 h) (see 1.1.4.5).
3. **Infection**
  1. To save time, the day prior to infecting the cells, pipet into sterile bottles the precise amount of media required to infect each batch of plates including media for uninfected wells (e.g. 2 x 350 mL and 2 x 50 mL for 33 plates). In addition, test the liquid dispenser for precision and accuracy, and re-calibrate if necessary.
  2. Warm the media at 37 °C. Fill two bottles with 70% ethanol, two 50 mL conical tubes with PBS and one with distilled water. Verify that the centrifuge is at room temperature.
  3. Sterilize the tubing with 70% ethanol, and rinse with PBS as previously described (see 2.1.2.2 and 2.2.2.1). While the tubing is sitting in the ethanol, pipet the precise volume of virus required into the bottle containing the media previously prepared for infection (see 2.3.1). Rinse the tubing with PBS by priming with 25 mL and then emptying.
  4. Remove the plates from the incubator and place them into the TC hood. Prime the tubing with media. Dispense 40 µL of media into the uninfected control wells. Empty the tubing of media and rinse with 25 mL PBS. (see 2.2.2.5 for CAUTION)
  5. After mixing the virus, place the tubing into the bottle containing the virus. Dispense 40 µL of virus across the plates. Centrifuge the plates for 30 min at 400 x g at room temperature. Return the plates to the incubator. Incubate the plates for the previously determined length of time (e.g. 24 h) (see 1.2.4).
4. **Fixing and staining cells**
  1. To save time, the day prior to fixing, prepare a bottle of fixing and staining solution containing formaldehyde and PBS (e.g. 179 mL of 37% formaldehyde and 270 mL of PBS for a final concentration of 4% formaldehyde) for each batch of plates, but omit the Hoechst stain. Store in the flammable cabinet away from light.
  2. Rinse the tubing with 70% ethanol and PBS as previously described (see 2.1.2.2 and 2.2.2.1). Add the required volume of Hoechst (e.g. 2.5 mL of a 5 mg/mL of Hoechst for a final concentration in the well of 7.5 µg/mL) to the fixing and staining solution and mix.
  3. Dispense 30 µL of fixing and staining solution across all the plates. Place the plates in the incubator for 30 min. Following incubation, apply the seals to the plates, and store the plates at 4°C protected from light. Wash the plates if necessary (see 1.1.6).
5. **Imaging and image analysis**
  1. Image the plates with an automated, high-content microscope. Use the previously determined settings, but first test the settings to ensure no adjustments need to be made.
  2. Quantify the GFP (or other fluorescent reporter) area and Hoechst area per well using the previously developed script (see 1.3.2 ). Test the script with a handful of plates first to determine if any slight modifications need to be made before batch analyzing all of the plates.
  3. Identify hits. Note: The robust Z-score/MAD (median absolute deviation) method is a method that is often used towards this end. Typically, scores of >2 or <-2 are set as cut-offs. This method is best used when the siRNA samples are randomly distributed across the plates and not, for example, grouped by function as the samples are used as *de facto* negative controls. Filter out cytotoxic siRNAs as these would be expected to non-specifically decrease virus spread (see Discussion for further details on filtering out cytotoxic hits).

### 3. Secondary screen validation of hits with the TRC (The RNAi Consortium) shRNA library

NOTE: See Discussion for details on selecting hits for secondary validation and possible screening technologies. If siRNA technology is selected for secondary validation, the same assay development and validation protocol can be used as was used for the primary screen (see 1).

1. Obtain a custom TRC shRNA library targeting the candidate genes of interest as glycerol stocks.
2. Prepare transfection quality plasmid DNA from the glycerol stocks. Note: A detailed protocol (*i.e.* "shRNA/sgRNA/ORF Glycerol and Plasmid DNA Preparation") can be found here: <http://portals.broadinstitute.org/gpp/public/resources/protocols>.
3. Produce lentiviruses expressing shRNA targeting the genes of interest with the DNA plasmids. Note: A detailed protocol for lentivirus production in 96-well plates [*i.e.* "shRNA/sgRNA/ORF High Throughput Viral Production (96 well)"] can be found here: <http://portals.broadinstitute.org/gpp/public/resources/protocols>.
4. Infect cells with the lentiviruses. Note: A detailed protocol for lentiviral infection in 96-well plates (*i.e.* "Lentiviral Infection") is available online at <http://portals.broadinstitute.org/gpp/public/dir/download?dirpath=protocols/production&filename=TRC%20viral%20infection%20200909.pdf>.

5. Infect cells with oncolytic virus either following puromycin selection (see 3.4 for the selection protocol) of successfully transduced cells or immediately following transduction (without puromycin selection). To determine the optimal amount of virus and the length of incubation with virus, use the same method used for the primary screen.

## 4. Tertiary validation

NOTE: The purpose of tertiary validation is to primarily confirm that the hit is on target, tumor specific, and enhances efficacy *in vivo*. One can also test whether it modulates spread across a spectrum of tumor cell lines

1. **Confirmation that knockdown of the candidate gene is on-target**
  1. First confirm that there is a correlation between the phenotype and gene silencing. Use the same assay that was developed for the screen or modify it for a 6-well format to assess the enhancement or inhibition of spread in a larger format. Conduct a time-course with cells transfected with siRNA targeting the gene(s) of interest plus and minus virus.
  2. Image the wells containing virus-infected cells, and collect the cell lysates from the uninfected wells at regular time intervals. Determine whether there is a correlation between gene silencing and enhancement or inhibition of spread. Assess gene silencing by Quantitative Polymerase Chain Reaction (qPCR) and/or Western blotting.
  3. Perform a genetic rescue experiment to confirm the hit is on-target. Note: In a typical rescue experiment, the candidate gene is knocked-down with RNAi while at the same time cells are transfected transiently with RNAi resistant cDNA (e.g. ortholog of the gene) expressing the candidate gene to determine whether the phenotype of the gene is restored or "rescued." Stable cell lines overexpressing RNAi resistant cDNA of the candidate gene of interest can also be employed.
  4. Alternatively, instead of a genetic rescue experiment, use multiple orthogonal assays to confirm that the hit is on-target [e.g. determine whether the same phenotype is obtained upon knockdown of the target gene with multiple siRNAs, upon knockdown of the target gene in multiple stable cell lines expressing shRNA against the target gene, and upon knockout with CRISPR (Clustered regularly interspaced short palindromic repeat)-Cas9 technology in multiple cell lines.].
2. **Confirmation that the hit is tumor-specific and modulates spread across a range of tumor cell lines**
  1. Conduct a similar assay as in 4.1.1, but with normal cells as well as with other tumor cell lines.
3. **Confirmation that manipulation of the candidate gene enhances efficacy *in vivo***

NOTE: Described below are three possible methods for manipulating the gene target *in vivo*.

  1. Find a drug to manipulate the gene target. If there is a drug such as a small molecule inhibitor that targets the same gene, administer it with the oncolytic virus *in vivo*. It is important to determine the optimal timing for administering the drug.
  2. Engineer the virus to inhibit or overexpress the gene target depending on whether one desires to inhibit or enhance the gene target. To inhibit the gene, engineer the oncolytic virus to express a dominant negative of the gene or to express shRNA targeting the gene. To enhance the expression of the gene target, clone the oncolytic virus with a transgene overexpressing the gene.
  3. Engineer cell lines with the gene knocked-down or knocked-out using shRNA or CRISPR-Cas9 technology, respectively. Implant the engineered cell line and the wild-type cell line *in vivo* and subsequently infect with the oncolytic virus. Note: This method serves as a proof-of-principle and can help determine whether investing additional time and resources to develop a drug to manipulate the gene *in vivo*, for example, would be worthwhile.

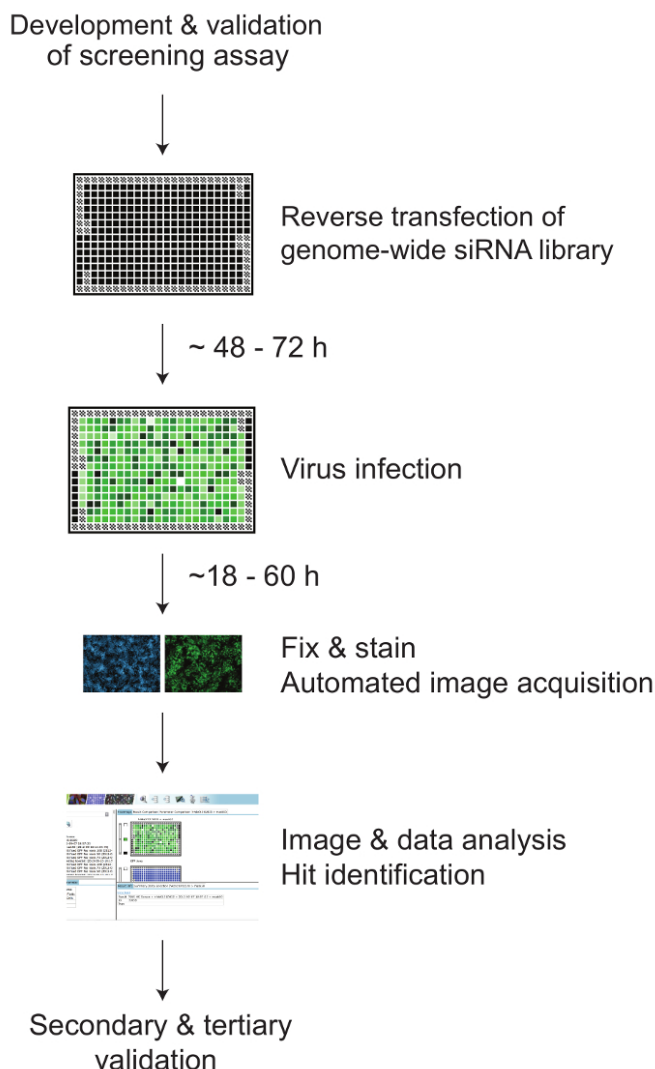
## Representative Results

An overview of the workflow for identifying host targets to enhance oncolytic virus therapy is presented in **Figure 1**.

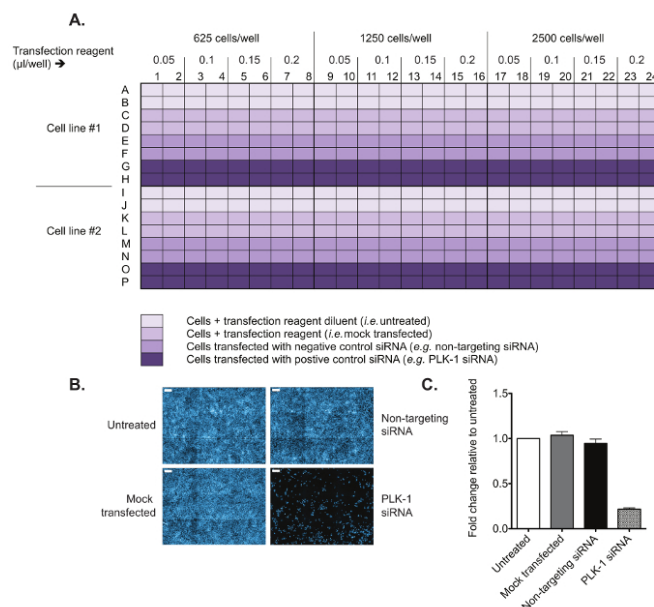
As illustrated in **Figure 1**, the critical first step to conducting a high-throughput RNAi screen is assay development. **Figure 2A** provides a sample plate layout for the transfection optimization assay. **Figure 2B** consists of representative images of expected results, and **Figure 2C** is a representative plot of the expected results. Following siRNA knockdown of PLK-1, a gene that induces cell death and can be used as a positive control to monitor siRNA knockdown efficiency, one would expect the cell survival to be between 0-30%, unless the cell line has a long doubling time in which case it will take longer to observe the cell death phenotype. The non-targeting siRNA should also be minimally toxic to the cells with a similar relative survival to that of untreated cells.

Another key element of assay development is to determine the MOI at which to infect the cells and the length of time for infection. **Figure 3A** provides a sample plate layout for determining the amount of virus and the length of incubation with virus. **Figure 3B** depicts the results of a live time-course of 786-O cells infected with vDD-eGFP (oncolytic vaccinia virus expressing enhanced GFP with the Thymidine Kinase and Vaccinia Growth Factor genes deleted) at various MOIs. Representative images of cells infected with vDD-eGFP at an MOI of 0.05 are shown in **Figure 3C**. Based on the results plotted in **Figure 3B**, one might select an MOI of 0.05 and a length of infection of 21 h as this would allow for the detection of increases and decreases in spread as this time-point is within a linear range, and the estimated Z-factor at this time-point is 0.6 for cohorts that increase and for cohorts that decrease spread. As part of validating this assay, one will want to fix the cells at this MOI and time-point, and calculate the Z-factor.

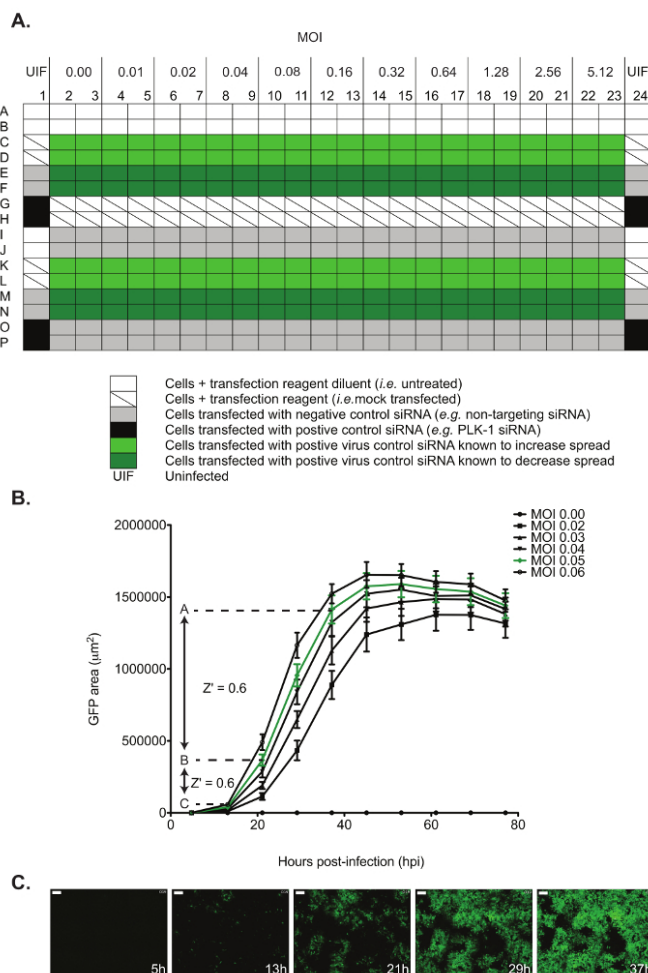
Following the protocol described, we conducted genome-wide RNAi screens across three different tumor cell lines (e.g. OVCAR-8, U373, NCI-H226) in duplicate with a siRNA library targeting 18,120 genes<sup>15</sup>. Using the MAD method, we identified 1008 hits common to at least 2 out of the 3 cancer cell lines (**Figure 4A**). Pathway analysis of hits identified in the screens revealed enrichment of members of the UPR (unfolded protein response) and ERAD (Endoplasmic-reticulum-associated protein degradation) pathways (**Figure 4A**). We selected 10 hits within these pathways for secondary validation using siRNA with different targeting sequences from those of the primary screen (**Figure 4B**). As part of tertiary validation, we conducted a genetic rescue experiment with IRE1α and ATF6α (activating transcription factor 6 alpha) to confirm that these hits were on target (**Figure 4C**).



**Figure 1: Schematic of workflow for identifying host targets to enhance oncolytic virus therapy.** The first step is to develop and validate an assay for high-throughput RNAi screening, which includes optimizing transfection conditions for introducing siRNA into cells, determining the optimal amount of virus (*i.e.* MOI) to infect the cells with and the length of incubation with virus. The second step is to conduct a high-throughput genome-wide screen. An siRNA library targeting genes across the entire genome is arrayed on microtiter plates. Cells are then reverse transfected with the siRNA library. Following a 48-72 h incubation period to allow for gene silencing, cells are infected with the optimal amount of virus. After the optimal length of incubation with virus, cells are fixed and stained, and subsequently imaged with a high-content microscope. Subsequent image and data analysis will help to identify genes that significantly modulate virus replication, which are referred to as "hits." A secondary validation screen is performed on select hits from the primary screen generally using siRNA or shRNA with different seed regions. Tertiary validation experiments are performed to confirm that the hits are on-target, tumor specific and enhance efficacy *in vivo*. [Please click here to view a larger version of this figure.](#)

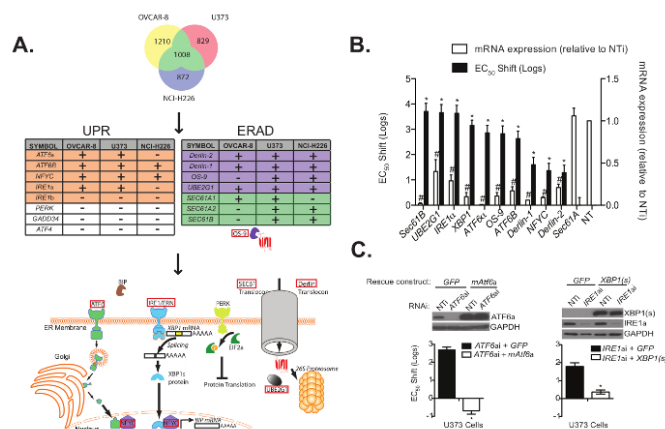


**Figure 2: Optimization of transfection conditions for high-throughput RNAi screening assay.** (A) A sample plate layout for optimizing transfection conditions with two different cell lines. (B) Representative images of 786-O cells (1500 cells/well) stained with Hoechst 33342 following 72 h incubation with transfection reagent diluent alone (*i.e.* untreated), with transfection reagent and diluent (*i.e.* mock transfected), with non-targeting siRNA and with siRNA targeting PLK-1. (C) Representative results from transfection optimization experiment showing 22% survival of cells transfected with PLK-1 siRNA (n=24) and 94% survival of cells transfected with non-targeting siRNA (n=8). Error bars =  $\pm$  SD, standard deviation; scale bars = 200  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 3: Determination of the amount of virus and length of incubation with virus for high-throughput RNAi screening assay.** (A) A sample plate layout for optimizing the amount of virus and length of incubation with virus. Columns 1 and 24 contain transfection controls and are left uninfected. Columns 2 to 23 contain untreated cells, mock transfected cells and cells transfected with positive and negative virus controls all infected with a range of MOIs starting with no virus in columns 2 and 3. (B) 786-O cells were reverse transfected with non-targeting siRNA and incubated for 48 h. They were subsequently infected with vvDD-GFP at the MOIs indicated and imaged at 8 h intervals starting at 5 hours post-infection (hpi). The mean GFP area per well ( $\pm$  SD) was calculated for each time-point ( $n=12$ ) and plotted on the graph. The Z-factor calculated between points A and B is 0.6 and the Z-factor calculated between points B and C is 0.6. (C) Representative live images of a well infected with an MOI of 0.05 at the time-points indicated. Magnification, 10x; 9 fields/well; scale bars = 200  $\mu\text{m}$ . [Please click here to view a larger version of this figure.](#)





**Figure 4: Genome-wide Screen Identifies ER Stress Response Blockade as a Potent Sensitizer of Rhabdovirus-Mediated Oncolysis. (A)** Venn diagram outlining the number of overlapping hits of high-throughput RNAi screens in 3 tumor cell lines, and a table (+, synthetic lethal; -, no interaction) and schematic diagram (hits outlined in red) illustrating key hits within the UPR and ERAD pathways. **(B)** EC<sub>50</sub> shifts (black bars, left y axis) were determined for U373 cells treated with Maraba virus (48 h) following treatment with siRNA (72 h) targeting a series of UPR/ERAD hits from the screen. Relative mRNA expression for each gene following siRNA knockdown (72 h) is depicted in white (right y axis). **(C)** Cell viability assays were conducted 48 h after Maraba virus infection, in U373 cells ectopically expressing mouse ATF6a (or control GFP) ± siRNA targeting human ATF6a (or non-targeting [NT] control; left panel) or human XBP1(s) (or control) ± siRNA targeting human IRE1a (or control; right panel). Western blots demonstrating gene silencing and ectopic gene expression are shown. EC<sub>50</sub> shifts = the shift in the dose of virus required to kill 50% of the cells; Error bars = ±SD; XBP1(s) = X-box binding protein 1 (spliced); In (B), one-way ANOVAs were performed followed by a Bonferroni multiple comparison's post hoc test to derive p values; In (C), student's t-tests were performed to derive p values; \*p < 0.05; #p < 0.05. (This figure has been modified from Mahoney *et al.*, 2011<sup>15</sup>). [Please click here to view a larger version of this figure.](#)

## Discussion

Here we present a protocol for employing high-throughput RNAi screening to identify host targets that can be manipulated to enhance oncolytic virus therapy. This has been tested successfully with oncolytic Maraba virus and oncolytic vaccinia virus, but, as noted, it can be adapted for use with other oncolytic viruses or other viruses generally to identify host genes that modulate virus replication. The screening protocol is also designed to identify genes that increase or decrease spread, but it can be readily modified for other readouts. Our screens with Maraba virus, for instance, were designed to identify genes modulating oncolysis utilizing a simple resazurin-based vital dye assay to score cell viability (see **Figure 4**)<sup>15</sup>. In these screens, following incubation with virus, instead of fixing cells with formaldehyde and staining with Hoechst, we dispensed resazurin dye into each well (final concentration of 20 µg/mL) and, after a 6 h incubation, we measured absorbance (573 nm) with a plate reader. We could have also used cell number based on Hoechst staining as a readout. While using a surrogate reporter to monitor virus replication was unnecessary in this screen, a virus with a reporter protein, particular a fluorescent protein does facilitate screen optimization as one can, for example, monitor virus replication live; furthermore, a virus with a reporter protein is less cumbersome and costly than using antibodies to stain for viral antigens, but it is possible to screen without one. In addition, one could make even further modifications to the assay to investigate host factors that impact on infectivity, burst size, and even more detailed sub-phenotypes like immunological cell death. In each case, one would follow a similar assay development protocol, screening strategy and secondary and tertiary validation methods.

In the protocol, we suggest optimizing transfection conditions ideally with multiple tumor cell lines. There are at least two reasons for optimizing with multiple cell lines. One reason is that not every cell line is amenable to high-throughput screening as some may be difficult to transfect or may not adhere well, but a key reason is to enable screening with multiple cell lines in order to avoid cell intrinsic bias. The choice of cell lines is largely dependent on one's objectives. One may elect to focus on a particular cancer type or select disparate cancer types to cover a broader spectrum. Alternatively, one may wish to focus on tumor cell lines that are resistant to identify host factors that can be manipulated to make the tumor cell lines less resistant to oncolytic virus infection.

In addition to the selection of cells lines, the selection of positive and negative virus controls is an important consideration for any screen. In some instances, virus genes that are required for or restrict replication may not be known or, if they are known, they may be cell-specific. Consequently, one can still determine the approximate robustness and dynamic range of the assay by using surrogate controls. For example, one could use uninfected cells or cells infected with a low MOI as a surrogate positive control for identifying genes that decrease spread upon knockdown. For identifying genes that enhance spread upon knockdown, one could infect cells with a dilution series of virus, and use wells with the maximum signal as a surrogate positive control.

Selection of hits for secondary screen validation and the type of technology to employ is another matter that requires careful deliberation. Candidates are generally selected for secondary screen validation based on the magnitude of the hit, on whether they significantly modulate spread in multiple cancer cell lines (if primary screens were conducted in multiple cell lines) and on biological interest. Bioinformatics tools such as DAVID<sup>22,23</sup>, PANTHER<sup>24</sup>, STRING<sup>25</sup> and PINDb<sup>26</sup> can help to isolate pathways and hits of biological interest. Mercer *et al.*<sup>8</sup> describe the use



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## Appendix C: *Cancer Cell* article (published)

I developed and validated the high-throughput RNAi screening assays as well as conducted the genome-wide screens in U373 and Ovarcar8 cells that formed the basis of this work.

Cancer Cell  
Article

Cell  
PRESS

# Virus-Tumor Interactome Screen Reveals ER Stress Response Can Reprogram Resistant Cancers for Oncolytic Virus-Triggered Caspase-2 Cell Death

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DOI 10.1016/j.ccr.2011.09.005

## SUMMARY

To identify therapeutic opportunities for oncolytic viral therapy, we conducted genome-wide RNAi screens to search for host factors that modulate rhabdoviral oncolysis. Our screens uncovered the endoplasmic reticulum (ER) stress response pathways as important modulators of rhabdovirus-mediated cytotoxicity. Further investigation revealed an unconventional mechanism whereby ER stress response inhibition preconditioned cancer cells, which sensitized them to caspase-2-dependent apoptosis induced by a subsequent rhabdovirus infection. Importantly, this mechanism was tumor cell specific, selectively increasing potency of the oncolytic virus by up to 10,000-fold. In vivo studies using a small molecule inhibitor of IRE1 $\alpha$  showed dramatically improved oncolytic efficacy in resistant tumor models. Our study demonstrates proof of concept for using functional genomics to improve biotherapeutic agents for cancer.

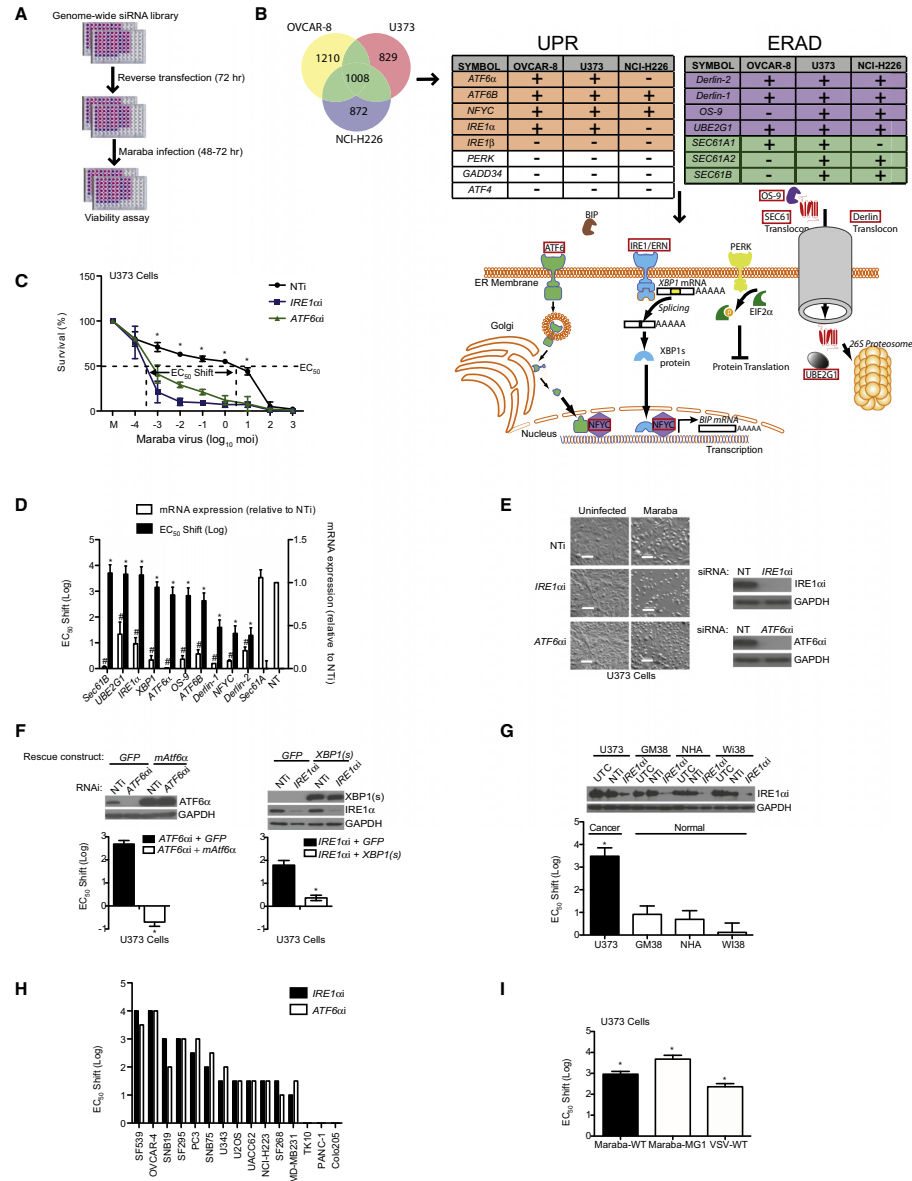
## INTRODUCTION

Despite major advances in our understanding of cancer over the last 50 years, it remains one of the most important health challenges worldwide. Innovative approaches are needed to complement current therapeutic strategies, and oncolytic viruses represent one such promising tool in the fight against cancer (Vähä-Koskela et al., 2007). In oncolytic virus therapy (OVT), replicating viruses selectively target tumors based on the genetic abnormalities of their malignant cells, including aberrant cell division, innate immune defects, and tumor-specific gene expression (Sinkovics and Horvath, 2008). In addition to inducing direct tumor cell lysis, a productive virus infection

leads to recruitment of the patient's immune system to attack the tumor (Parato et al., 2009), and modulation of the tumor vasculature to starve and kill cancer cells en masse (Breitbach et al., 2007). In aggregate, the high tumor selectivity, multimodal mechanisms for tumor destruction, and lack of genotoxicity with OVT offer great potential for increasing efficacy while eliminating the side and late effects that plague current therapeutic strategies. Over the past decade a growing number of natural and genetically modified viruses have been shown to cure disease in a diverse set of rodent tumor models (Sinkovics and Horvath, 2008; Vähä-Koskela et al., 2007). Importantly, three such virus platforms are now entering late-phase clinical trials following impressive Phase II data (Kaufman and Bines,

## Significance

Although therapeutic approaches using tumor-killing viruses (oncolytic viruses) are showing great promise in human trials, with response rates in Phase II trials ranging from 30% to 70%, clearly not all patients are benefiting. In this study we screened the human genome looking for potent opportunities to sensitize cancer cells to oncolytic virus therapy. Our work reveals that tumor cells can be tricked into turning on a dormant suicide pathway by causing a mild stress to their protein-folding machinery. These "reprogrammed" tumor cells are triggered to die only when they are subsequently infected by an oncolytic virus. This "one-two punch" is cancer selective, can be induced genetically or chemically, and regresses tumors that are resistant to either therapy alone.



**Figure 1. Genome-wide Screen Identifies ER Stress Response Blockade as a Potent Sensitizer to Rhabdovirus-Mediated Oncolysis**  
(A) Schematic representation of the screen.  
(B) Venn diagram outlining the number of overlapping hits, and a table (+, synthetic lethal; -, no interaction) and schematic diagram (hits outlined in red) illustrating key hits within the UPR and ERAD pathways.

2010; Lal et al., 2009; D.K. Jennerex Biotherapeutics, personal communication).

Host/virus interactions at the cellular and organismal level govern the outcome of any virus encounter, including OVT. Thus far, most efforts to improve OVT have focused on genetically modifying the virus. However, the outcome of any host/virus interaction is governed by contributions from both genomes. We sought to understand the host's contribution to the oncolytic virus effect by using functional genomic screening to elucidate the molecular mechanisms that control a productive rhabdoviral infection and contribute to rhabdovirus-mediated tumor cell killing. By understanding these mechanisms in detail, we envisioned that we could engineer maximally effective therapeutic viruses, and/or design potent drug/virus combinations that exploit unforeseen biological interfaces between oncolytic viruses, the host and cancer.

## RESULTS

### Blockade of the Endoplasmic Reticulum (ER) Stress Response Sensitizes Cancer Cells toward Viral Oncolysis

Wild-type and engineered strains of rhabdoviruses, such as vesicular stomatitis virus (VSV) and more recently Maraba virus, have been shown to be potent oncolytic agents in several rodent models of cancer (Stojdl et al., 2000b, 2003; Balachandran and Barber, 2000; Brun et al., 2010). To search for host factors that modulate rhabdovirus-mediated oncolysis, we performed a synthetic lethal RNAi screen of the human genome across three tumor-derived cell lines (Figure 1A). We used an arrayed library of siRNA pools to target ~18,200 genes in OVCAR-8 (ovarian carcinoma), U373 (glioblastoma), or NCI-H226 (non-small cell lung carcinoma) cells. Transfected cells were either mock infected or infected with wild-type Maraba virus as a representative oncolytic rhabdovirus. Following infection we incubated the cells for 48–72 hr, after which we scored cell viability using resazurin vital dye. To identify primary “hits,” we analyzed data from two independent screens for each cell line using the median absolute deviation method (Chung et al., 2008). Subtracting those genes scoring positively in the siRNA alone screens defined 1008 synthetic lethal hits common to at least 2 out of 3 cancer lines from the primary screen (Figure 1B, and see Table S1 available online). Subsequent bioinformatics analysis revealed an enrich-

ment of hits within the ER stress response pathways (Figure 1B and Table S2), including members of two of the three known signaling cascades that comprise the unfolded protein response (UPR). Key hits therein included the transcription factors ATF6 $\alpha$  and ATF6B, the endoribonucleases/protein kinase IRE1 $\alpha$ , and a transcriptional coactivator common to both pathways, NFYC. Together, the ATF6 and IRE1 pathways serve to rescue the ER from an overload of unfolded proteins by increasing chaperone production and ER lipid biogenesis (Todd et al., 2008). Our screen also identified several members of the SEC61 and the HRD ligase protein translocation complexes (e.g., Derlin-1; Figure 1B). These proteins are critical for ER-associated degradation (ERAD), which helps rescue an unfolded protein burden by removing misfolded polypeptides from the ER and shuttling them to the 26S proteasome (Vembar and Brodsky, 2008).

The UPR and ERAD hits were particularly interesting because ER stress has been reported to be a defining feature of the tumor cell state, and components of these pathways are currently being pursued as cancer-specific targets for stand-alone treatment (Hetzel, 2009; Wang et al., 2009). Thus, we performed secondary validation on ten hits within these pathways, using siRNA with targeting sequences distinct from those employed in the primary screens. To quantify sensitization, we infected RNAi-treated cells with increasing doses of Maraba virus and generated survival curves (Figure 1C). We then modeled the curves using nonlinear regression and determined the shift in the dose of virus required to kill 50% of the cells (EC<sub>50</sub> shift). In all cases in which RNAi induced significant gene knockdown (nine of ten targets), U373 cells were significantly sensitized to virus-mediated killing (ranging from 1.4 to 3.7 log shifts in EC<sub>50</sub>; Figure 1D, and representative images in Figure 1E). Although XBP1 did not emerge from our primary screen, given its critical function in mediating IRE1 $\alpha$ -dependent transcription (Glimcher, 2010) we reevaluated it using RNAi duplexes distinct from those used in the primary screen. Indeed, these analyses demonstrated that, similar to IRE1 $\alpha$  and our other UPR hits, XBP1 depletion dramatically sensitized U373 cells to Maraba virus killing (EC<sub>50</sub> shift, 3.2 logs, Figure 1D). To rule out off-target effects, we performed rescue experiments for ATF6 $\alpha$  and IRE1 $\alpha$ , which were chosen because they are not only representative ER stress response hits from the primary screen but also strong candidate druggable targets. Cells stably expressing murine ATF6 $\alpha$  (mATF6 $\alpha$ ) were completely refractory to the

(C) U373 cells were treated with siRNA for 72 hr prior to Maraba virus infection, and viability assays were performed after 48 hr. The “EC<sub>50</sub> shift” for cells silenced for IRE1 $\alpha$  is depicted. M, mock infected.

(D) EC<sub>50</sub> shifts (black bars, left y axis) were determined for U373 cells treated with Maraba virus (48 hr) following treatment with siRNA (72 hr) targeting a series of UPR/ERAD hits from the screen. Relative mRNA expression for each gene following siRNA knockdown (72 hr) is depicted in white (right y axis).

(E) Phase-contrast images of U373 cells treated first with siRNA (72 hr), followed by Maraba virus infection (moi 5; 24 hr). Scale bar, 100  $\mu$ m.

(F) Cell viability assays were conducted 48 hr after Maraba virus infection, in U373 cells ectopically expressing mouse ATF6 $\alpha$  (or control green fluorescent protein [GFP])  $\pm$  siRNA targeting human ATF6 $\alpha$  (or nontargeting [NT] control; left panel) or human XBP1(s) (or control)  $\pm$  siRNA targeting human IRE1 $\alpha$  (or control; right panel). Western blots demonstrating gene silencing and ectopic gene expression are shown.

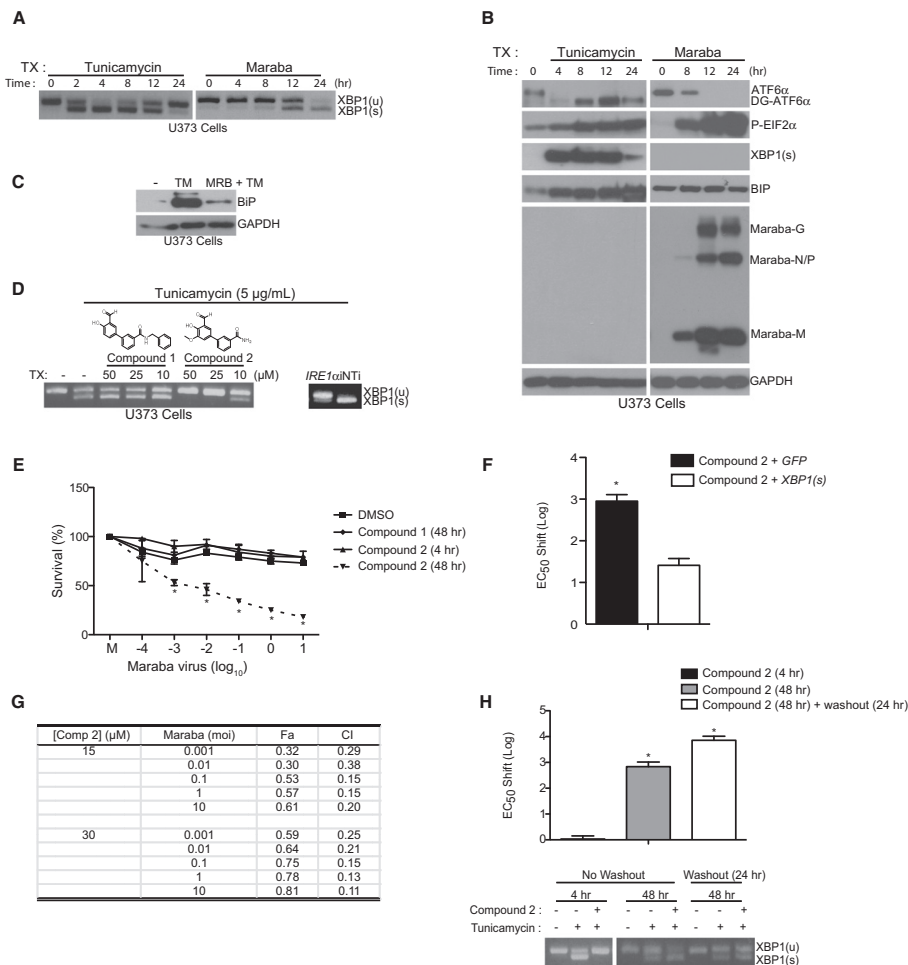
(G) Representative tumor and normal cell lines were treated with siRNA targeting IRE1 $\alpha$  for 72 hr followed by Maraba virus. Cell viability assays were performed 48 hr later and EC<sub>50</sub> shifts determined.

(H) Cell viability assays were performed on a panel of cancer-derived cell lines 48 hr after Maraba virus infection, which followed siRNA-mediated knockdown of IRE1 $\alpha$  or ATF6 $\alpha$  (72 hr).

(I) Cell viability was measured, and EC<sub>50</sub> shifts were determined after 48 hr infection with wild-type (WT) or attenuate (MG1) Maraba virus (Brun et al., 2010) or VSV-WT, which followed 72 hr siRNA treatment.

For all experiments, \*p < 0.05; data shown as  $\pm$ SD.

See also Tables S1 and S2.



**Figure 2. Synthetic Lethal Interaction between ER Stress Response Blockade and Rhabdovirus Infection Requires a Preconditioning Process**

(A) U373 cells were treated with the ER stressor TM (5 μg/ml) or Maraba virus (moi 5). Total RNA was collected and RT-PCR for *XBP1* splicing performed. (B) Cells were treated as in (A). Total cell lysates were collected and immunoblot analyses conducted (DG-ATF6α, deglycosylated ATF6α, due to the inhibitory effect of TM on glycosylation). (C) U373 cells were untreated (–) or treated with TM (5 μg/ml; 24 hr) or Maraba virus (moi 5) for 6 hr prior to TM for 24 hr (MRB + TM). Total cell lysates were collected and immunoblots performed. (D) U373 cells were treated with putative IRE1α small molecule inhibitors (2 hr) prior to TM treatment (4 hr). Total RNA was collected and RT-PCR performed. RNAi targeting *IRE1α* (72 hr) was used as a control (right panel). (E) U373 cells were treated with compound 2 (50 μM) or controls for 4 or 48 hr prior to Maraba virus infection. Cell viability assays were performed 48 hr later. M, mock infected. (F) U373 cells ectopically expressing XBP1(s) (or control) were treated with compound 2 (50 μM) for 48 hr prior to Maraba virus infection. Cell viability assays were performed 48 hr later. (G) U373 cells were treated with compound 2 (48 hr) followed by Maraba virus (48 hr). Cell viability assays were conducted and combination index analyses performed.

synthetic lethal phenotype associated with Maraba virus infection and *hATF6 $\alpha$*  knockdown (Figure 1F), whereas the sensitization phenotype induced by *IRE1 $\alpha$*  knockdown was largely reversed in cells expressing constitutively active XBP1(s) (the product of the endonuclease activity of IRE1 $\alpha$ ; Figure 1F). Taken together, these experiments validate our primary screening result that Maraba virus-mediated oncolysis is greatly enhanced by knocking down components of the UPR and ERAD pathways.

To evaluate therapeutic index, we silenced *IRE1 $\alpha$*  in a series of primary human cell lines (GM38 skin fibroblasts, normal human astrocytes [NHAs], and Wi38 lung fibroblasts) prior to rhabdovirus infection. In contrast to the pronounced sensitization observed in U373 glioblastoma cells, *IRE1 $\alpha$*  knockdown had limited to no effect on Maraba virus-mediated killing of the normal cell lines (Figure 1G). We next examined the scope of the synthetic lethal phenotype in a representative subset of the NCI 60 tumor cell panel (Brun et al., 2010). RNAi-mediated knockdown of *IRE1 $\alpha$*  or *ATF6 $\alpha$*  significantly sensitized the majority of cancer cell lines tested to Maraba virus-mediated killing (Figure 1H). Moreover, oncolysis by the prototypic oncolytic rhabdovirus VSV (Stojdl et al., 2000b) and an engineered clinical candidate strain of Maraba virus (Maraba-MG1) (Brun et al., 2010) was similarly enhanced by UPR inhibition (Figure 1I). Collectively, these data suggest that the enhancement of rhabdovirus-mediated oncolysis conferred by inhibiting components of the ER stress response is tumor cell specific and may have widespread utility across a diverse range of tumor types.

#### Synthetic Lethal Interaction between ER Stress Response Blockade and Rhabdovirus Infection Requires a Preconditioning Process

VSV infection has recently been shown to cause ER stress (Liu et al., 2009), and thus, at first glance the most parsimonious mechanism to explain our synthetic lethal phenotype is an inadequate host cell response to virus-induced ER stress resulting in accelerated cell death. Indeed, Maraba virus infection caused noticeable ER stress in cancer cells, characterized by the activation of the upstream UPR sensors IRE1 $\alpha$  (measured by *XBP1* mRNA splicing; Figure 2A), ATF6 $\alpha$  (measured by its cleavage; Figure 2B), and PERK (measured by EIF2 $\alpha$  phosphorylation; Figure 2B). However, as part of co-opting the host cell's synthetic machinery for the generation of viral progeny, rhabdoviruses are known to potentially inhibit host gene expression by blocking mRNA transcription (Black and Lyles, 1992), nuclear to cytoplasmic mRNA shuttling and protein translation (Brun et al., 2010; Stojdl et al., 2000a, 2003). Therefore, not surprisingly, despite having strongly activated the UPR, a Maraba virus infection did not lead to elevated levels of the representative downstream UPR effector proteins XBP1(s) and BIP (Figure 2B). Moreover, the expression of BIP in response to the ER poison tunicamycin (TM) was dramatically blunted by a preceding infection with Maraba virus, further demonstrating that this rhabdo-

virus can actively block UPR gene expression in the face of ER stress (Figure 2C). Taken together, these results indicate that although a Maraba virus infection generates an ER stress that triggers the UPR-signaling cascade, it concomitantly blocks UPR-mediated gene expression, thereby rendering the response functionally inert. As such, an inadequate response to virus-induced ER stress is not responsible for the observed synthetic lethal interaction between rhabdovirus and UPR/ERAD knockdown because the UPR is already functionally inhibited upon rhabdovirus infection independently of external manipulation.

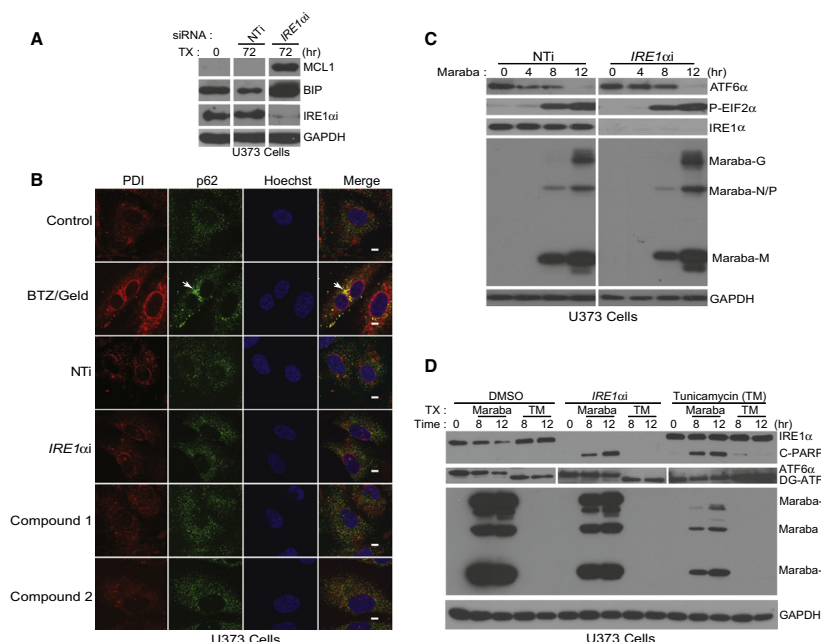
Because siRNA knockdown of UPR/ERAD targets was performed for up to 72 hr before virus was added to the cultures, we postulated that sustained inhibition during the siRNA knockdown period might be responsible for the sensitization observed. To test this idea, we synthesized a number of salicylaldehyde-based compounds that had been reported to inhibit IRE1 $\alpha$  (Mannkind-Corporation, 2008) along with several variants of the original structure. We first measured their ability to inhibit *XBP1* splicing by IRE1 $\alpha$  in a cell-based assay and found that several were effective in the micromolar range (representative subset depicted in Figure 2D). We then evaluated the most potent of these, designated compound 2, and found that it greatly enhanced rhabdoviral oncolysis in U373 cells when dosed for 48 hr (compound administered 48 hr and again 24 hr prior to virus infection, i.e., sustained inhibition), but not 4 hr (i.e., acute inhibition; Figure 2E). Importantly, drug-mediated sensitization significantly correlated with IRE1 $\alpha$  inhibition (Figure S1), and ectopically expressed XBP1(s) significantly reduced the sensitization phenotype observed (Figure 2F). These data strongly suggest that IRE1 $\alpha$  is indeed the functional target of compound 2, and are in agreement with a recent report demonstrating that this class of salicylaldehyde-based compounds directly and selectively inhibits the endonuclease activity of IRE1 $\alpha$  (Volkman et al., 2011). To determine whether the interaction between Maraba virus and IRE1 $\alpha$  inhibition was synergistic or additive, we performed combination index experiments (Chou et al., 1994), which demonstrated that compound 2 interacted synergistically (CI < 1.0) with Maraba virus across a range of doses and multiplicity of infections (mois) (Figure 2G). In contrast we did not observe an interaction between the negative control compound 1 and Maraba virus at any dose across the same range (Figure 2E; data not shown). Finally, we sought to determine if the sensitization phenotype actually required IRE1 $\alpha$  inhibition at the time of virus infection. For these experiments we treated U373 cells with compound 2 for 48 hr before washing the cells and adding new media (i.e., "washout") for another 24 hr prior to Maraba virus infection. Remarkably, the degree of sensitization was unaltered (Figure 2H, top panel) even after the drug was removed and IRE1 $\alpha$  activity was restored (Figure 2H, bottom panel). In fact during the course of this experiment, we noticed that compound 2 was no longer inhibiting

(H) Top panel shows that U373 cells were treated with compound 2 (30  $\mu$ M) for either 4 hr, 48 hr (dosed at t = -48 hr and -24 hr), or 48 hr, followed by a 24 hr "drug washout" period, and infected with Maraba virus. Cell viability assays were performed 48 hr post-virus infection and EC<sub>50</sub> shifts determined. Bottom panel illustrates that U373 cells were treated with compound 2 (30  $\mu$ M) (as described for top panel) prior to being treated with TM (5  $\mu$ g/ml) for 4 hr. Total RNA was collected and RT-PCR performed for *XBP1* splicing.

For all experiments, \*p < 0.05; data shown as  $\pm$ SD. Fa, fractional effect; CI, combination index.

See also Figure S1.





**Figure 3. UPR Inhibition Leads to ER Preconditioning in Cancer Cells, which Sensitizes Them to Rhabdoviral Oncolysis**

(A) U373 cells were treated with siRNA (72 hr), total cell lysates were collected, and immunoblots performed. (B) U373 cells were treated as indicated with chemical ER stressors (bortezomib [25 nM]/Geldanamycin [120 nM]; compound 1 [30  $\mu$ M], compound 2 [30  $\mu$ M]) for 48 hr or treated with nontargeting or *IRE1 $\alpha$*  targeting siRNA for 72 hr and then fixed and imaged for the protein aggregation marker p62. PDI is a marker for the ER, and Hoechst stains the nucleus. White arrow points to large foci of gross protein aggregation in positive control cells. Scale bars, 3  $\mu$ m. (C) Cells were treated with siRNA (72 hr), followed by infection with Maraba virus (moi 5). Total cell lysates were collected and immunoblots performed. (D) Cells were treated as in (C) or treated with TM (5  $\mu$ g/ml) for 24 hr followed by 24 hr "washout," after which cells were infected with Maraba virus (moi 5) or treated with TM (5  $\mu$ g/ml). Total cell lysates were collected at the indicated time points and immunoblots performed. See also Figure S2.

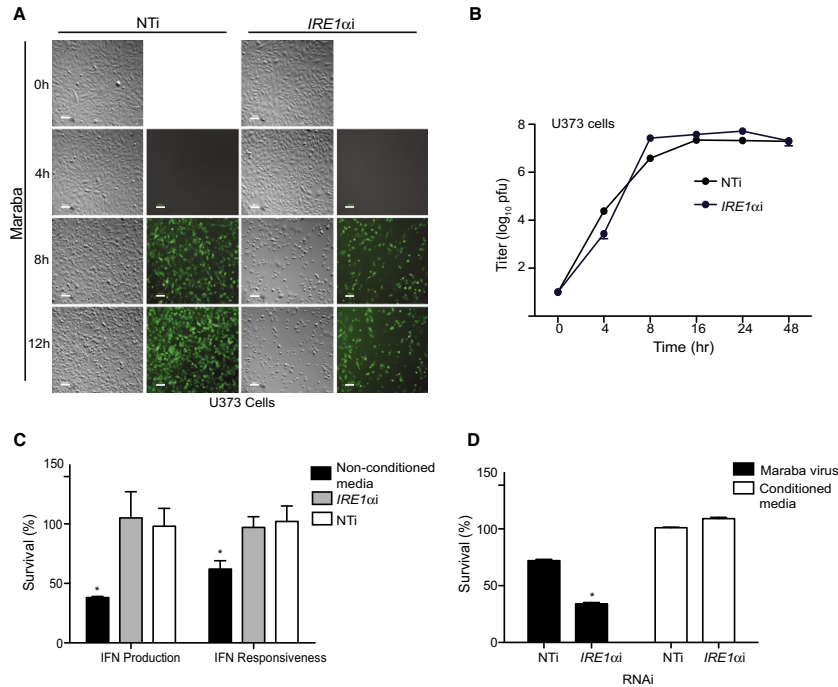
*IRE1 $\alpha$*  activity 24 hr after it was added to the media, even without a washout. Collectively, these data demonstrate that the sensitization mediated by ER stress blockade and the triggering of cell death by virus infection are temporally separable events. This observation points toward a preconditioning process that durably alters the tumor cell's response to a subsequent rhabdovirus infection.

#### ER Preload Rewires Cancer Cells for Caspase-2-Dependent Apoptosis

Many cancer cells accumulate unfolded proteins as a function of their malignant nature (Moenner et al., 2007). Therefore, we postulated that inhibiting a component of the ER stress response might gradually lead to an unfolded protein burden in the ER of cancer cells, which would predispose them to ER stress-mediated apoptosis in response to a subsequent rhabdovirus infection. Indeed, we found that cells treated with RNAi targeting *IRE1 $\alpha$*  for 72 hr had increased levels of both BIP and MCL1 protein (Figure 3A), which are each known to be transcriptionally

induced following ER stress independently of *IRE1 $\alpha$* . These data demonstrate that sustained inhibition of *IRE1 $\alpha$*  in tumor cells leads to a sufficiently large ER protein burden as to trigger a UPR, and this was not observed in normal cells (Figure S2A). However, immunostaining for ubiquitin or p62 revealed no gross protein aggregation following *IRE1 $\alpha$*  knockdown or compound 2 treatment (Figure 3B; Figure S2B). We also did not detect any evidence of an active ER stress response at this time of virus infection because early markers of UPR activation (*EIF2 $\alpha$*  phosphorylation and *ATF6 $\alpha$*  cleavage) were indistinguishable between control and *IRE1 $\alpha$*  siRNA pretreated tumor cells (Figure 3C, 0 hr). Moreover, loss of *IRE1 $\alpha$*  had no effect on the magnitude or kinetics of UPR activation following Maraba virus infection (Figure 3C), suggesting that the capacity of the tumor cell to respond to an unfolded protein burden was not diminished at the time of virus infection. Collectively, these data suggest that the protein load induced upon genetic knockdown or chemical inhibition of *IRE1 $\alpha$*  was transient, having likely been sufficiently managed by the other arms of the UPR it evoked.





**Figure 4. ER Preconditioning Has No Bearing on Rhabdovirus Infectivity, Productivity, or the Ability to Secrete or Respond to Interferons, and Does Not Lead to "Bystander Killing"**

(A) U373 cells were treated with siRNA (72 hr) prior to Maraba infection (moi 5), and phase-contrast and fluorescent microscopy images were captured following infection. Scale bars, 100  $\mu$ m.

(B) U373 cells were treated as in (A), and single-step growth analyses conducted.

(C) Production: Ovar-4 cells treated with siRNA targeting IRE1 $\alpha$  (or nontargeting (NTi)) RNAi for 72 hr were infected with VSV- $\Delta$ 51 (Stojdl et al., 2003) for 18 hr, at which time the conditioned media were collected and virus neutralized. Conditioned media (gray and white bars) or nonconditioned media (complete DMEM as a control; black bars) were then added to Vero cells for 24 hr prior to Maraba virus infection. After 48 hr infection, viability assays were performed. Responsiveness: conditioned media were generated from PC3 cells infected with VSV- $\Delta$ 51 for 18 hr. Conditioned media were then added to OVCAR-4 cells that had been treated with siRNA targeting IRE1 $\alpha$  or NTi (72 hr) (gray and white bars), or nonconditioned media (black bars) were added to untreated OVCAR-4 cells as a control. After 24 hr, cells were infected with Maraba virus for 48 hr and viability assays performed.

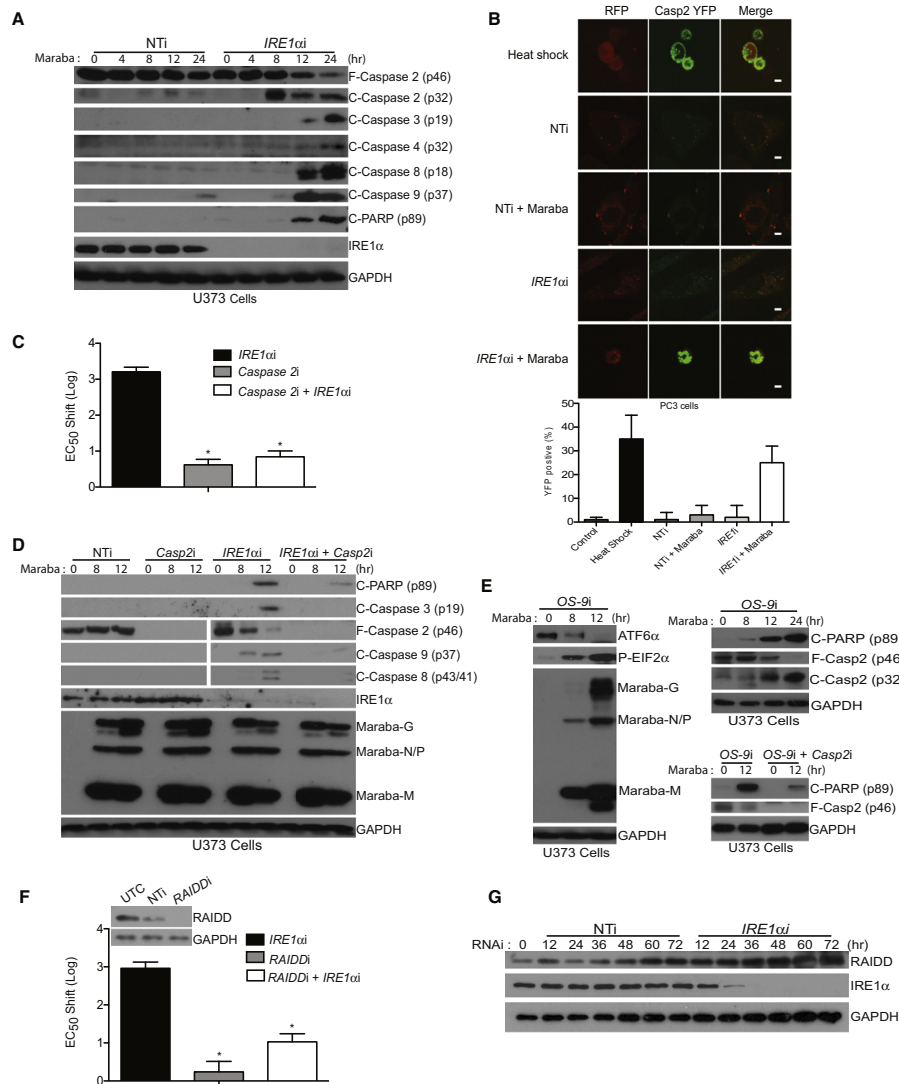
(D) Black bars (control experiments) indicate U373 cells that were treated with non (NT)- or IRE1 $\alpha$ -targeting siRNA prior to infection with Maraba virus (moi 10). White bars (bystander experiments) show U373 cells that were infected with Maraba virus (moi 10), and after 24 hr infection, the conditioned media were collected and transferred onto U373 cells that had been previously treated with NT- or IRE1 $\alpha$ -targeting RNAi. Cell viability was assessed after 72 hr.

For all experiments, \*p < 0.05; data shown as  $\pm$ SD.

As a complement to these studies, we induced ER preload by treating cells with either RNAi targeting IRE1 $\alpha$  or pulsing them with a dose of the strong ER stress inducer TM, and evaluated how the cells responded to a further ER insult by treating them with a second dose of TM. In these experiments we found that the preloaded cells were able to respond to the second ER stressor with similar kinetics and dynamics as compared to naive cells (Figure 3D, ATF6 $\alpha$  blots, lanes 9–10 versus 4–5). Importantly, this further ER stress did not trigger cell death (Figure 3D, PARP blot, lanes 9–10 and 14–15 versus 7–8 and 12–13). Taken together, these data demonstrate that IRE1 $\alpha$  inhibition does not sensitize cancer cells toward rhabdovirus-mediated killing as a

result of diminished capacity to deal with virus-induced ER stress.

Thus, we postulated that it is a response to ER stress prior to virus infection rather than ER stress per se that is the sensitizing agent. We first asked whether ER preload affected any aspect of the virus life cycle in cancer cells. We noted that UPR inhibition had no bearing on viral protein expression following a Maraba virus infection (Figures 3C and 3D), and further experiments confirmed that knocking down IRE1 $\alpha$  did not alter the infectivity (Figure 4A) or productivity (Figure 4B) of Maraba virus in cancer cells. These data demonstrate that transient ER stress does not affect the life cycle of Maraba virus. It is well established that



**Figure 5. ER Preconditioning Rewires Cancer Cells for Caspase-2- and RAIDD-Dependent Apoptosis in Response to Rhabdovirus Infection**  
(A) U373 cells were treated with siRNA (72 hr), followed by infection with Maraba virus (moi 5). Total cell lysates were collected and immunoblots performed. (B) In the top panel, PC3 cells were treated for 24 hr with siRNA as indicated and then transfected with Casp2-CARD VC, Casp2-CARD VN, and pmKATE2 (RFP transfection control). Twenty-four hours later, cells were either left untreated or infected with Maraba virus (moi = 5). Positive control cells were heated for 1.5 hr at 44°C (heat shock). Cells were assessed 8 hr after virus treatment for the percentage of cells that were Venus positive (green). Scale bars, 3 μm. Bottom panel is a histogram (top panel) with quantified caspase-2 activation denoted as the percentage of transfected cells that showed YFP complementation. A minimum of 200 cells per well from 24 replicate wells was counted. (C) Cells were treated with siRNA (72 hr), followed by infection with Maraba virus across a 6-log dose range. After 48 hr, cell viability was measured, and EC<sub>50</sub> curves were plotted.

rhabdoviruses, including Maraba virus, are exquisitely sensitive to the human innate immune system via type I interferon signaling (Müller et al., 1994). Interferons are protective cytokines that are processed in the ER/Golgi system before being secreted to warn neighboring cells of an impending virus encounter. These cytokines signal through the type I interferon receptor (IFNRI), which is also processed in the ER/Golgi system. Previous work has shown that ER stress can inhibit proper IFNRI processing in the ER/Golgi system, which leads to IFNRI depletion and enhanced susceptibility to a rhabdovirus infection (Liu et al., 2009). Thus, we asked whether transient ER stress could alter a cancer cell's ability to secrete interferon in response to a rhabdovirus infection, or to respond to interferon secreted by neighboring cells. Using an interferon bioassay, we found that silencing *IRE1α* in OVCAR-4 cells (which have an intact interferon system) had no bearing on their ability to secrete or respond to interferon cytokines (Figure 4C). These data demonstrate that UPR inhibition does not induce an interferon defect in cancer cells in our assay system. Finally, we asked whether transient ER stress could sensitize cancer cells toward death induced by other cytokines secreted upon Maraba virus infection (e.g., TNFα), the so-called "bystander effect." We did not detect any cytotoxicity when conditioned media from virus-infected U373 cells were transferred to U373 cells that had been previously treated with either non- or *IRE1α*-targeting RNAi (Figure 4D).

Thus, we hypothesized that a transient ER stress might rewire the cancer cell's signaling networks toward increased cell death upon subsequent oncolytic virus challenge. Indeed, *IRE1α* knockdown greatly enhanced the kinetics of apoptosis during Maraba virus infection, as measured by the cleavage of PARP and members of the caspase cascade (Figure 5A). Notably, caspase-2 was strongly activated in tumor cells (but not normal cells) early after virus infection only when *IRE1α* was knocked down (Figure 5A; Figure S3A). Bimolecular fluorescent complementation analyses of caspase-2 activation by induced proximity (Bouchier-Hayes et al., 2009) confirmed that caspase-2 was strongly activated in response to combined *IRE1α* inhibition and Maraba virus infection (Figure 5B; Figure S3B). Caspase-2 is an initiator caspase that has been implicated in several stress-mediated apoptotic cascades, such as those emanating from DNA damage (Vakifahmetoglu-Norberg and Zhivotovsky, 2010) as well as ER stress (Cheung et al., 2006). To examine the relevance of caspase-2 activation, we knocked it down simultaneously with *IRE1α* and measured apoptosis following Maraba virus infection. In these experiments caspase-2 knockdown almost completely rescued the synthetic lethal interaction between *IRE1α* knockdown and virus infection (Figures 5C and 5D). Qualitatively similar caspase-2 rescue data were obtained when cells were rendered null for the ERAD component OS-9 (Figure 5E). Consistent with the observation that caspase-2

was activated early following a virus infection in cells treated with RNAi targeting *IRE1α*, caspase-2 knockdown inhibited the activation of the activator caspases 8 and 9 as well as the downstream effector caspase 3 following a virus infection in cells treated with *IRE1α* siRNA. Collectively, these data suggest that a transient ER stress preconditions cancer cells to commit caspase-2-dependent apoptosis in response to a subsequent Maraba virus infection.

We next asked whether caspase-2 itself senses ER preload in response to UPR inhibition, or whether the link from ER preload to caspase-2 lies upstream. By western blotting, immunostaining, and bimolecular fluorescence complementation (BiFC) analyses, we have been unable to detect any changes in caspase-2 levels, localization, activation, or phosphorylation status during the preload period (data not shown). This suggests that whereas caspase-2 is central to sensitization, it itself is not directly responsive to ER stress during the preload period. However, when we examined the known caspase-2-interacting proteins PIDD and RAIDD, we found that whereas PIDD was dispensable for sensitization (Figure S3C), RAIDD knockdown largely rescued *IRE1α* knockdown-induced sensitization (Figure 5F). Moreover, RAIDD protein levels markedly increased throughout an ER preload period generated by RNAi targeting *IRE1α* (Figure 5G) and by compound 2 (Figure S3D), beginning at about the time cells become sensitized. Taken together, these data demonstrate that ER preload-mediated rewiring for caspase-2-dependent apoptosis upon viral infection is dependent on the adaptor protein RAIDD. Given these findings, we asked whether other stressors that have been shown to induce caspase-2- and RAIDD-mediated apoptosis are similarly enhanced by ER preload. Indeed, we found that both genetic and chemical inhibition of the UPR and/or ERAD dramatically sensitized tumor cells to killing by the DNA-damaging agent doxorubicin, a phenotype that was dependent on both RAIDD and PIDD (Figures S3E–S3H). These data appear to indicate that ER preload alters cell signaling upstream of caspase-2 in such a way as to generically sensitize to a number of caspase-2 activation complexes initiated from disparate triggers (proposed model depicted in Figure 6).

#### UPR Inhibition Potentiates Oncolytic Therapy in Primary Patient Samples and a Preclinical Model of Cancer

To evaluate the clinical relevance of this therapeutic strategy, we examined whether compound 2 pretreatment could sensitize freshly derived patient tumor samples to Maraba virus-mediated oncolysis. Cell cultures isolated from three patients with primary glioblastoma multiforme were treated with compound 2 for 48 hr prior to Maraba virus infection, and 48 hr later, viability assays demonstrated that each was significantly sensitized toward death (Figure 7A).

We next sought to evaluate the efficacy in several animal models of cancer. To begin, we undertook maximum tolerable

(D) Cells were treated with siRNA (72 hr), followed by infection with Maraba virus (moi 5). Total cell lysates were collected and immunoblots performed.

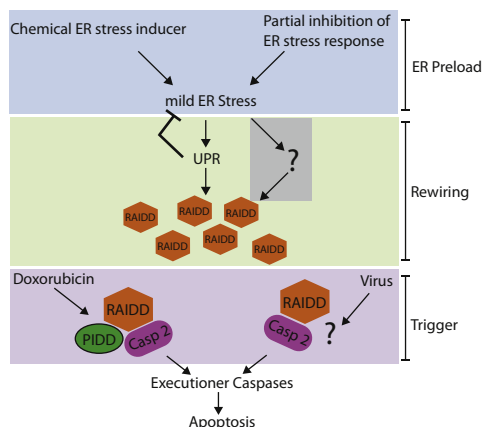
(E) Cells were treated with siRNA targeting OS-9 (±caspase-2) for 72 hr followed by Maraba virus for 48 hr, and western blots were performed.

(F) Cells were treated with siRNA (72 hr), followed by infection with Maraba virus across a 6-log dose range. After 48 hr, cell viability was measured, and EC<sub>50</sub> curves were plotted.

(G) Cells were treated with siRNA (72 hr), followed by infection with Maraba virus (moi 5). Total cell lysates were collected and immunoblots performed.

For all experiments, \*p < 0.05; data shown as ±SD.

See also Figure S3.



**Figure 6. ER Preload Rewires Cancer Cells for Caspase-2 and RAIDD-Dependent Viral Oncolysis**

Partial UPR (or ERAD) inhibition or treatment with a chemical ER stress inducer (e.g., TM) leads to a mild ER stress in cancer cells (ER Preload). As a result, a corrective UPR is activated, and RAIDD protein levels are increased (Rewiring). In response to a subsequent virus infection (or DNA-damaging agent), caspase-2 is activated eccentrically due to the increased surface area with which to seed induced proximity (Trigger), and apoptosis ensues.

dose (MTD) and pharmacokinetic (PK) studies of compound 2 in CD-1 nude mice. These experiments showed that a single dose of up to 1000 mg/kg of compound 2 was tolerated, had a half-life of >6 hr, and had properties consistent with efficient bio-distribution to the extravascular tissues (Figure S4). To validate our combination therapy approach, we chose a chemoresistant (Boettcher et al., 2010; Hamilton et al., 1983) orthotopic OVCAR-4 xenograft model (Louie et al., 1985) that is also refractory to OVT. OVCAR-4 cells stably expressing firefly luciferase were injected intraperitoneally (i.p.) into CD-1 nude mice. We monitored tumor growth using *in vivo* optical imaging, and initiated treatment during the growth phase. To induce ER preconditioning, we treated animals with compound 2 for 3 days prior to the first virus injection. Consistent with our findings in cell culture, combination therapy dramatically reduced tumor burden in animal models, an effect that was sustained for >30 days with negligible tumor regrowth (Figures 7B and 7C). In contrast, rapid regrowth occurred after an early period of tumor regression using either virus or compound 2 alone.

As a complement to these experiments in human xenografts, we sought to test this treatment regimen in an immune-competent rodent tumor model. *In vitro* testing determined that the EMT6 breast cancer line, which is particularly resistant to stand-alone rhabdovirus therapy, was significantly sensitized to oncolytic virus killing when pretreated with compound 2 (Figure 7D). Using these cells to generate a tumor model, we confirmed that neither drug nor virus had an appreciable effect on tumor growth as single agents; however, combination therapy significantly reduced tumor burden (Figure 7E). Extending compound 2 treatment to more than 12 days in combination with

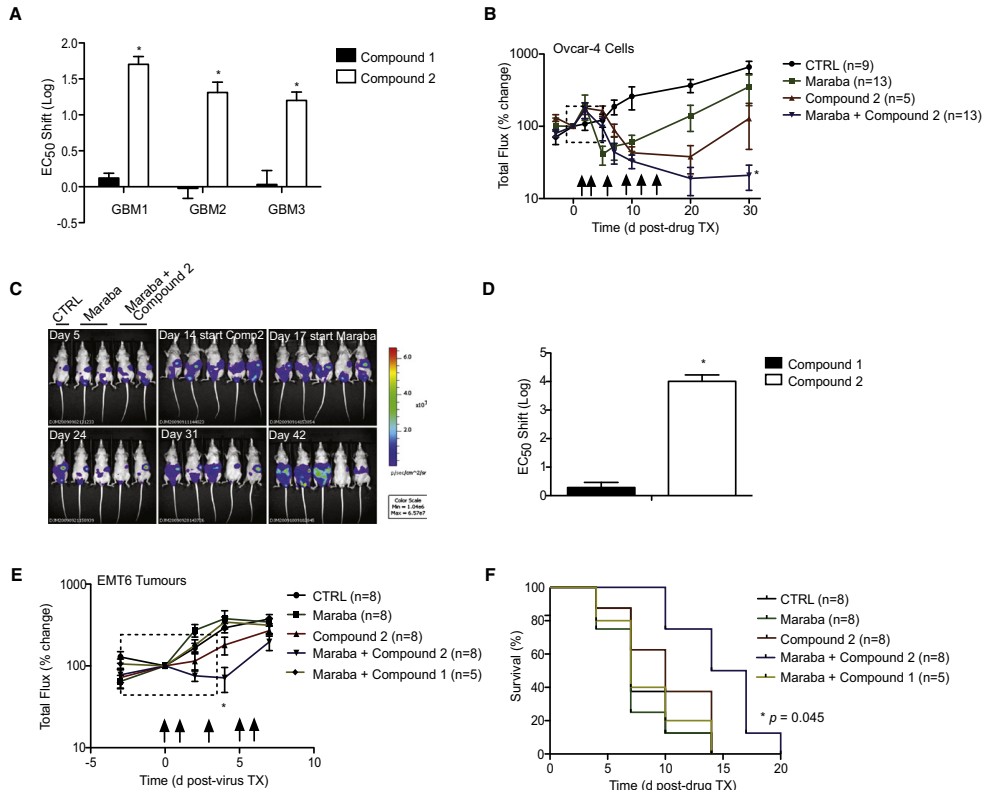
virus treatment continued to increase efficacy (Figure 7F). Taken together, these data demonstrate proof of concept that modulating the ER stress responses can be exploited to enhance OVT *in vivo*. We anticipate that the ongoing development of more clinically relevant nanomolar compounds and/or simultaneous targeting of multiple components of the ER stress response may provide even greater boosts in efficacy.

## DISCUSSION

Using a genome-wide RNAi-screening approach, we have discovered that inhibiting the ER stress response preconditions cancer cells to undergo caspase-2-dependent apoptosis when subsequently challenged with an oncolytic rhabdovirus. This exploitation of the host/virus interaction increased the potency of oncolytic Maraba virus cytotoxicity by up to 10,000-fold throughout a diverse range of tumor-derived cell lines, without increasing toxicity in normal cells. To validate ER preload as a clinically relevant therapeutic strategy, we show that UPR inhibition can sensitize primary patient glioblastoma tumors to Maraba infection. Moreover, we demonstrate that this combination strategy can improve the effectiveness of oncolytic Maraba virus in both an immunocompetent mouse model and an immunodeficient human xenograft model of cancer. This provides an additional mechanism by which oncolytic viruses can selectively target tumor cells and serves to further increase the therapeutic index of this class of experimental cancer therapy. We are currently working to identify more potent IRE1 $\alpha$  inhibitors and exploring the use of multiple UPR/ERAD inhibitors to further enhance the effectiveness of this combination strategy.

We also show that the sensitization of tumor cells to Maraba virus is dependent on a cellular response to a transient ER stress, but not a concurrent one. This response leads to the predisposition toward a caspase-2-mediated apoptotic death pathway. We are currently pursuing both the mechanism by which ER preload is able to prime a caspase-2 response in tumor cells, as well as the means by which rhabdoviruses and other stressors can subsequently trigger this response to kill cells. Currently, it has been established that caspase-2 activation can occur through both PIDD-dependent and independent mechanisms (Tinel and Tschopp, 2004) (Manzl et al., 2009). Our data suggest that the PIDDosome is not involved in virus-mediated activation of caspase-2 following ER preload but is involved in its activation by doxorubicin (Figure S3). This seems to indicate that ER stress alters cell signaling upstream of PIDD and that ER preload can generically sensitize to a number of caspase-2 activation complexes initiated from disparate triggers. Consistent with this model, we have found that RAIDD is required for sensitization and that its protein content is markedly elevated in response to ER preload. It may be that RAIDD is upregulated during UPR and/or ERAD responses and that this would help prepare the cell for the decision to commit apoptosis should the UPR/ERAD not be sufficient to resolve the damage. Experiments are currently ongoing to identify more of the components of this potent synthetic lethal signaling pathway.

Our study establishes a successful strategy for the rational design of “chemical-biological combination therapies” using functional genomic screening for host/virus interactions.



**Figure 7. Inhibiting IRE1 $\alpha$  Enhances Oncolytic Therapy in Patient Tumor Samples and Murine Cancer Models**

(A) Cells isolated from three patients with glioblastoma multiforme were treated with compound 1 or 2 (15  $\mu$ M) or DMSO control for 48 hr, followed by Maraba virus across a 6-log dose range. Cell viability assays were performed 48 hr later and EC<sub>50</sub> shifts determined (plotted relative to DMSO control). (B) Luciferase-tagged OVCAR-4 cells (5e<sup>6</sup>) were delivered i.p. into CD-1 nude mice. At day 14, mice were treated twice daily with compound 2 (250 mg/kg; i.p. delivery) or vehicle for 6 consecutive days (drug TX window is outlined by the dotted lines). Maraba-MG1 treatment (1e<sup>7</sup> PFU; IV injections) was initiated 48–72 hr later (virus injections depicted by arrows). Tumors were regularly evaluated using IVIS bioluminescent imaging. The graph depicts relative change in luminescent signal, which corresponds to tumor size. (C) Representative bioluminescent images from (B). (D) EMT6 cells were treated with compound 1 or 2 (50  $\mu$ M) or DMSO control for 48 hr prior to Maraba virus infection across a 6-log dose range. Viability assays were conducted 48 hr later and EC<sub>50</sub> shifts determined (plotted relative to DMSO). (E) Luciferase-tagged EMT6 cells (1e<sup>5</sup>) were implanted into the breast fat pads of Balb/c mice. Compound 1 or 2 or PBS treatment was initiated at day 7 (250 mg/kg; i.p.; twice daily for 6 days; treatment window depicted by the dotted box). Maraba-DM injections (1e<sup>7</sup> PFU; IV) commenced on day 10 (black arrows). Bioluminescent data are plotted (as above). (F) Kaplan-Meier curve depicting mouse survival in an EMT6 model. The experiment was done as in (E), except that compound 1 and 2 treatments were extended for an additional 6 days. For all experiments, \*p < 0.05; data shown as  $\pm$ SD. See also Figure S4.

Although other chemovirus combination therapies have been reported, with enhancements to oncolytic efficacy (Ottolino-Perry et al., 2010), our study demonstrates the utility of genome-scale systematic analyses of host/virus interactions to identify unpredicted synergies. This study adds to the breadth of opportunities

for combinations beyond those predicted by our current molecular understanding of host/tumor/virus interactions. For example whereas it is known that rhabdoviruses induce UPR (Liu et al., 2009) and that UPRs are largely transcriptional, vesiculoviruses such as VSV and Maraba virus are known to block host

transcriptional responses, and we show here that UPR is effectively nullified during a normal virus infection of mammalian cells. Therefore, it would not be predicted that further inhibition of this pathway would be a fruitful avenue for a combination therapy. Yet, our screens identified a host pathway that robustly modulates rhabdovirus cytotoxicity through an unconventional mechanism of action where the cellular response to an ER stress, and not the stress itself, is the critical component of oncolytic synergy.

Finally, it is noteworthy that rhabdoviruses are currently in clinical development (D.F.S., unpublished data), whereas UPR (Hetzel, 2009) and ERAD (Wang et al., 2009) inhibitors are being actively pursued for cancer treatment. Our data suggest that these agents will act synergistically when used in combination, and support their evaluation in the clinical setting.

## EXPERIMENTAL PROCEDURES

### RNAi Screening

An arrayed library of siRNA pools (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA) was used to target ~18,200 human genes in OVCAR-8 (ovarian carcinoma), U373 (glioblastoma), or NCI-H226 (nonsmall cell lung carcinoma) cells. Tumor cells were seeded in 384-well plates (OVCAR-8, 1250 cells/well; U373, 625 cells/well; NCI-H226, 625 cells/well). Each plate had additional control wells with a nontargeting control siRNA (Dharmacon nontargeting Pool #2) to measure the effect of siRNA transfection on infection, and siRNA targeting *pik-1* (Dharmacon) was used to monitor knockdown efficiency. Quadruplicate plate sets were reverse transfected with siRNA (10 nM) using RNAimax (Invitrogen, USA) and incubated for 72 hr. From these, duplicate sets of plates were either mock infected or infected with wild-type Maraba virus (moi: OVCAR-8, 0.1; U373, 0.05; NCI-H226, 0.1). Infections were incubated for 48 hr (OVCAR-8) or 72 hr (U373 and NCI-H226), after which resazurin dye (20 µg/ml) was added to each well, incubated for 6 hr, and assayed for absorbance (573 nm) to score cell viability.

### In Vitro Cytotoxicity Assays with RNAi and Determination of "EC<sub>50</sub> shifts"

Cells were seeded onto 96-well plates to a confluence of ~50%. The following day, siRNA transfections were performed, and 72 hr later, the cells were infected at log 10 dilutions with wild-type Maraba virus (except for Figure 1G, which used the indicated viruses). After 48–72 hr of infection (depending on the cell line), Resazurin sodium salt (Sigma-Aldrich) was added to a final concentration of 20 µg/ml. After a 6 hr incubation, the absorbance was read at a wavelength of 573 nm. To determine the EC<sub>50</sub>, kill curves were modeled using nonlinear regression analysis (GraphPad Prism Software) and EC<sub>50</sub> values determined. The EC<sub>50</sub> shift was calculated by subtracting the EC<sub>50</sub> of UPR targeted from nontargeted cell lines, and is represented as log 10 values.

### XPB1-Splicing Assays

For qualitative analyses, total RNA was extracted from cells using a standard RNeasy spin column kit, as described by the manufacturer (QIAGEN). RNA was reverse transcribed to cDNA using SuperScript II RT (Invitrogen) following the manufacturer's guidelines. Standard PCR was performed using the following primers: XPB1-F, 5'-cct tgt agt tga gaa cca gg-3'; XPB1-R, 5'-ggg gct tgg tat ata tgt gg-3'. The PCR products were run out on a 3% agarose gel and visualized with UV imager. Images were scanned, and densitometry was performed using Scion Image software. For quantitative analyses, total RNA was extracted (as above), and real-time quantification was performed with Taqman One-Step RT-PCR Master mix reagents (Applied Biosystems) using the following primers and probes: forward primer 5'-AAGCCAAGGGGAATGAAGT-3', reverse primer 5'-CCAGATGCCCAA CAGGATA-3'; XPB1(u) probe, 5'-56-FAM-AGCACTCAGACTACGTGCACCT-3IABKFQ-3', XPB1(s) probe 5'-56-JOE-CTGAGTCCGACGAGGTGCAGG-3IABKFQ-3'.

### Protein Aggregate Formation

For protein aggregation imaging, cells were cultured in 384-well optical imaging plates (Aurora Biotechnologies, Carlsbad, CA, USA) and subjected to either chemical or genetic ER stressor as indicated for 48–72 hr, respectively. Cells were fixed with 4% formaldehyde, stained, and imaged in PBS at room temperature. Images were collected using a confocal microscope (FluoView 1000D; Olympus Inc.) equipped with a 60× water immersion 1.2 NA objective and processed using the Fiji distribution of ImageJ (<http://fiji.sc/wiki/index.php/Fiji>). P62 was detected using rabbit polyclonal antisera (Cell Signaling Technology; #5114) and anti-rabbit Alexa 488 (Invitrogen). Ubiquitin was detected using rabbit polyclonal antisera (Cell Signaling Technology; #3933) and anti-rabbit Alexa 488. The ER marker PDI was stained using the mouse monoclonal antibody RL77 (Thermo, USA). Cells were stained with Hoechst 33342 to visualize nuclei. All images were acquired at 100 µs/pixel at 512 × 512 pixel resolution with 3 pass Kalman filter processing.

### Caspase-2 BiFC

To measure caspase-2 activation by induced proximity, we employed a caspase-2 BiFC system (Bouchier-Hayes et al., 2009). Cells were reverse transfected in 384-well format with siRNA targeting *IRE1α* or a nontargeting control siRNA. Twenty-four hours later, cells were forward transfected with a mixture of 10 ng of Casp2 CARD VN, 10 ng Casp2 CARD VC, and 5 ng pmKATE2 (RFP; Evrogen, Moscow) using FuGENE HD (Roche, USA). After 24 hr, cells were either mock infected or infected with Maraba virus at an moi of 5. Alternatively, an additional plate of cells was exposed to 44°C heat shock for 90 min and returned to the incubation as a positive control for caspase-2 activation. Following 8 hr of virus infection (or post-heat shock), cells were fixed in 4% formaldehyde and stained with Hoechst 33342 to visualize nuclei. Plates were imaged using an OPERA high-throughput confocal microscope (PerkinElmer, USA) using a 40× water immersion objective. Image analysis and quantification were performed using Acapella software (PerkinElmer).

### Small Molecule Screening

U373 cells were plated in 6-well format to a confluence of ~75%. The following day, candidate small molecules were dissolved in DMSO and added directly to the cell culture media at a range of concentrations. After 2 hr, TM (5 µg/ml) was added, and total RNA was collected 4 hr later. RNA extraction and RT-PCR for *XPB1* splicing were performed as described above.

### In Vitro Cytotoxicity Assays with Small Molecules

Cells were seeded onto 96-well plates to a confluence of ~50%. The following day, siRNA transfections were performed, or small molecule IRE1α inhibition was initiated. For the small molecules, DMSO was used as a vehicle with a drug concentration of 20–50 µM. Drug treatment occurred for either 4 hr ("acute" treatment), or was reapplied at 24 hr and left for 48 hr total ("sustained" treatment). Following knockdown or chemical inhibition, the cells were infected at log dilutions with the indicated rhabdoviruses. After 48–72 hr of infection (depending on the cell line), Resazurin sodium salt was added to a final concentration of 20 µg/ml. After a 6 hr incubation, the absorbance was read at a wavelength of 573 nm.

### Ovarian Xenograft Model

Human ovarian carcinoma-derived OVCAR-4 cells, adapted for bioluminescent imaging, were injected into 6- to 8-week-old athymic CD-1 nude mice (i.p. injection, 5 × 10<sup>5</sup> cells per mouse). Untreated animals developed measurable abdominal tumors (assessed by IVIS imaging) by 4–7 days, became icteric by 3–4 months, and had to be euthanized shortly thereafter due to systemic disease, as characterized by enlarged cancerous liver and spleen, pale kidneys, and cancerous lymph nodes on the abdominal mesentery. For efficacy experiments, compound 2 (250 mg/kg; or vehicle [10% Tween 80]) was administered twice daily (i.p. injections), beginning on day 14 and ending on day 19. Maraba-MG1 (1 × 10<sup>5</sup> pfu per cell) or PBS was injected IV (tail vein) on days 16, 17, 19, 23, 25, and 27. Animals were monitored daily for weight loss, morbidity, hind leg paralysis, and respiratory distress. Tumor images were captured twice weekly with a Xenogen 200 IVIS system (Caliper LS, USA), and total luminescent flux was analyzed on computerized software (Xenogen). All animal experiments were approved by the University of Ottawa



Animal Research Ethics Board and carried out in accordance with guidelines of the National Institutes of Health and the Canadian Council on Animal Care.

#### Glioblastoma Patient Samples

Clinical samples were used in accordance with research ethics board approval from the Institutional Ethics Board at the Montreal Neurological Institute and Hospital (Montreal, Canada). Written informed consent was obtained for all patient samples in this study.

#### Statistical Analyses

For all statistical analyses except survival curves, one- and two-way ANOVAs were performed, followed by a Bonferroni multiple comparison's post hoc test to derive *p* values (GraphPad Prism Software). For survival curves, Mantel-Cox log rank analysis was used to compare plots (GraphPad Prism). For all tests, statistical significance was defined as *p* < 0.05.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at doi:10.1016/j.ccr.2011.09.005.

#### ACKNOWLEDGMENTS

We kindly thank A.E. MacKenzie and R. Screaton for critically reading the manuscript, D. Green for his caspase-2 BIFC plasmid system, E. Brown for anti-VSV antibody, J.S. Diallo for help with the combination index analyses, and K. Petrecca for primary patient samples. D.F.S. is supported by the National Cancer Institute of Canada (NCIC), the Ontario Institute for Cancer Research (OICR), the Terry Fox Foundation, the Canadian Foundation for Innovation (CFI), Ontario Research Fund, Ottawa Regional Cancer Foundation, the Angels of Hope Foundation, the CHEO Foundation, and the Honey Badger Innovation Project.

Received: April 23, 2011

Revised: August 2, 2011

Accepted: September 13, 2011

Published: October 17, 2011

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## High-throughput screening to enhance oncolytic virus immunotherapy

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**Abstract:** High-throughput screens can rapidly scan and capture large amounts of information across multiple biological parameters. Although many screens have been designed to uncover potential new therapeutic targets capable of crippling viruses that cause disease, there have been relatively few directed at improving the efficacy of viruses that are used to treat disease. Oncolytic viruses (OVs) are biotherapeutic agents with an inherent specificity for treating malignant disease. Certain OV platforms – including those based on herpes simplex virus, reovirus, and vaccinia virus – have shown success against solid tumors in advanced clinical trials. Yet, many of these OVs have only undergone minimal engineering to solidify tumor specificity, with few extra modifications to manipulate additional factors. Several aspects of the interaction between an OV and a tumor-bearing host have clear value as targets to improve therapeutic outcomes. At the virus level, these include delivery to the tumor, infectivity, productivity, oncolysis, bystander killing, spread, and persistence. At the host level, these include engaging the immune system and manipulating the tumor microenvironment. Here, we review the chemical- and genome-based high-throughput screens that have been performed to manipulate such parameters during OV infection and analyze their impact on therapeutic efficacy. We further explore emerging themes that represent key areas of focus for future research.

**Keywords:** oncolytic, virus, screen, high-throughput, cancer, chemical, genomic, immunotherapy

### Introduction

High-throughput screens can rapidly and efficiently capture large amounts of data across multiple biological parameters. Depending on the screen design, the ability to collect unbiased information can identify novel ways of impacting pathways of interest that may be overlooked by rationally designed approaches.

Screening technologies have considerable power to systematically probe novel targets, across multiple stages of virus infection. Genome-wide RNA interference (RNAi) screens have focused on short interfering RNA (siRNA) and short hairpin RNA (shRNA) technologies.<sup>1</sup> Genome-editing screens have employed clustered, regularly interspaced, short palindromic repeats (CRISPR)–Cas9 technology.<sup>2</sup> Pharmaceutical screens have also explored a variety of chemical compound libraries, including bioactive and small molecules, chemotherapeutic compounds, and clinical drug panels.

Typically, a primary screen is performed to find a number of hits above a certain statistical threshold, followed by a smaller secondary screen to validate and replicate these hits (Figure 1). Finally, independent assays are performed to confirm activity and therapeutic relevance (Figure 1). Such approaches have not only yielded biological

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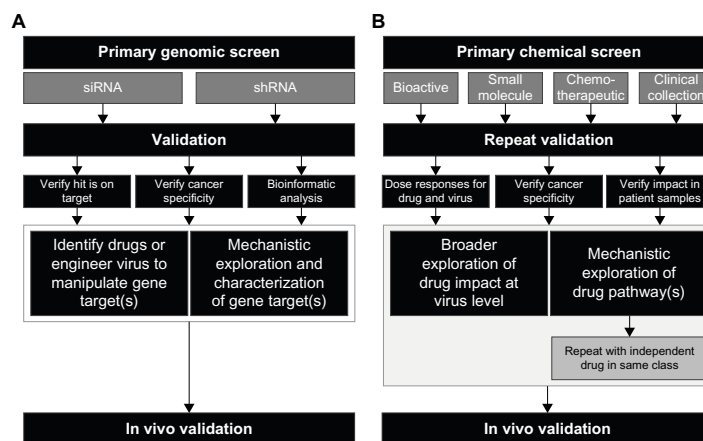
<http://dx.doi.org/10.2147/OV.S56217>

Oncolytic Virotherapy 2016:5 15–25



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**Figure 1** Flow charts showing typical experimental approaches for high-throughput screening.

**Notes:** General overview of the experimental approaches taken for (A) RNAi or (B) chemical library screens designed to explore the contributions of different host or OV parameters during oncolytic virus immunotherapy.

**Abbreviations:** OV, oncolytic virus; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, short interfering RNA.

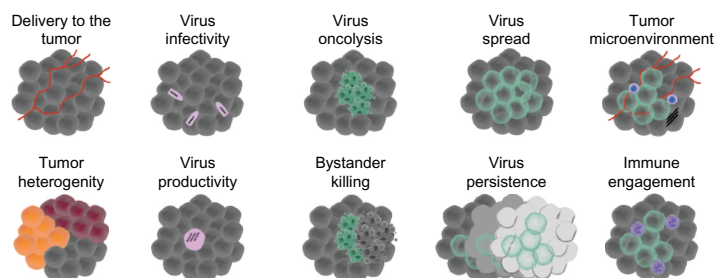
insights in diverse fields that include cancer biology, developmental biology, cellular metabolism, and virology but have also discovered biological and biotherapeutic targets for disease. However, the vast potential of these powerful new technologies to uncover targets in the oncolytic virus immunotherapy (OVIT) field has yet to be fully realized.

A huge network of highly complex biological interactions occur between an oncolytic virus (OV) and an infected host. OVs have multimodal capabilities, including the direct lysis of infected cells, the establishment of an inflammatory environment, and the amplification of immunostimulatory signals as viral replication proceeds.<sup>3–5</sup> OVs can also infect endothelial cells, which can lead to the collapse of tumor vasculature.<sup>6</sup> Perhaps, most importantly, OVs can activate antitumor immune responses that can control, regress, and prevent the relapse of tumors.<sup>7,8</sup> This occurs, at least in part, through the immunogenic release of tumor-associated antigens (TAAs) following oncolysis of infected tumor cells.<sup>9–11</sup> Such TAAs are processed and presented by dendritic cells (DCs) to drive T-cell engagement against a broad repertoire of tumor targets in a phenomenon known as “antigen spreading.”<sup>12,13</sup> This capacity to generate TAA-specific T-cell responses can also be artificially enhanced during OVIT by encoding one or multiple TAAs into the viral vector. For example, rhabdoviruses expressing the melanoma TAA, human dopachrome tautomerase, can drive large curative CD8<sup>+</sup> T-cell responses against

subcutaneous B16.F10 tumors in vivo.<sup>14,15</sup> In addition to directly stimulating CD8<sup>+</sup> T-cell responses, OVs can selectively minimize immunosuppressive cell populations within the tumor microenvironment through the depletion of regulatory T-cells (Tregs)<sup>16</sup> or myeloid-derived suppressor cells (MDSCs).<sup>17,18</sup>

Several key areas of the interaction between an OV and an infected host represent potential biological targets that can be manipulated to impact therapeutic outcomes. From a virological perspective, these parameters may include delivery to the tumor site, infectivity, oncolysis, bystander killing, replication/productivity/burst size, spread, and persistence (Figure 2). These distinct temporally regulated stages of the OV infection process can be assessed by performing screens at early, intermediate, or late time points following virus inoculation to uncover targets that are therapeutically relevant at multiple stages of infection, following single or multiple rounds of replication. From a host perspective, extra levels of complexity that can potentially be modified to enhance OVIT outcomes include immune engagement and manipulation of the tumor microenvironment (Figure 2).

Here, we review the application of high-throughput genomic and chemical screens to manipulate such parameters during OVIT to improve therapeutic outcomes. While relatively few screens have been performed, of particular interest are those that have explored viruses that are currently undergoing clinical testing, or are already clinically



**Figure 2** Ten key parameters affecting oncolytic virus therapeutic efficacy that can be probed by high-throughput in vitro or in vivo screens.

**Notes:** In the example illustration, tumor cells (gray) are targeted via blood vessels (red) by infectious rhabdoviral particles (pink) containing ssRNA (black) expressing enhanced green fluorescent protein (green). The tumor microenvironment depicted reflects the diverse range of cell types at the tumor site, including cancer cells, endothelia, resident immune cells (including MDSCs and Tregs), stroma, and fibroblasts. Tumor heterogeneity is depicted by three genetically distinct clonal populations (orange, red, gray).

**Abbreviations:** MDSCs, myeloid-derived suppressor cells; ssRNA, single strand RNA; Treg, regulatory T-cell.

approved, including herpes simplex virus (HSV), vesicular stomatitis virus (VSV), vaccinia virus (VV), and Maraba (MRB) virus.

### Modulating OV infectivity and productivity at the tumor site

There are currently two concepts that address the desirability of viral replication and persistence at the tumor site. One concept aims to maximize the replicative capacity and burst size of the OV to enhance oncolysis at the tumor site. This potentially enables a greater magnitude of endogenous TAA spreading since a larger number of heterogeneous tumor cells, presumably across a wider antigenic spectrum, are lysed in a highly inflammatory context. An alternative concept aims to minimize the persistence of the virus at the tumor site to ensure immune responses are focused on lysed tumor antigens rather than viral targets.

Several RNAi screen have identified host genes that can be knocked down to reduce viral infection.<sup>19–21</sup> Two of these screens assessed genes that had an early impact on VSV or VV infection by analyzing outcomes at 7–8 hours following inoculation,<sup>20,21</sup> while a third screen analyzed outcomes at 18 hours following VSV inoculation to capture genes involved at an intermediate stage of the infection cycle (Table 1).<sup>19</sup> Such genes could be knocked down during OVIT to minimize viral persistence at the tumour site, or, alternatively, could be encoded directly into an OV to achieve local overexpression at the tumour site with the aim of enhancing viral replication. For example, SMAD family member 2 (Smad2), which can be knocked down to inhibit VSV replication,<sup>19</sup> functions as a tumor suppressor,<sup>22</sup> and loss

of Smad2 and other Smad proteins correlates with advanced disease and poor prognosis for colorectal cancer.<sup>23</sup> Yet, overexpressing Smad2 in subcutaneously implanted Mv1Lu cells can inhibit cellular growth.<sup>24</sup>

Conversely, screens have also identified host genes that, when knocked down, can improve virus replication. Beard et al<sup>25</sup> conducted a screen in HeLa cells with a druggable siRNA human genome library targeting 6,719 genes during infection with recombinant VV-A5-EGFP, a VV strain expressing enhanced green fluorescent protein fused to the A5 core protein in the Western Reserve backbone (Table 1). The primary screen identified 153 proviral host factors (factors that inhibited replication following knockdown) and 149 antiviral factors (factors that increased replication upon knockdown). Seventy-two of these genes were selected for secondary validation using a deconvoluted set of four individual siRNAs targeting each gene. Tumor necrosis factor receptor-associated factor 2 was among their confirmed proviral hits. Further mechanistic exploration determined that this gene precipitated rapid entry of VV into the host cell.<sup>26</sup> The nuclear pore protein, nucleoporin 98, was also a confirmed proviral hit. Since VV replication and morphogenesis take place in the cytoplasm, such nuclear-localized hits were unexpected; yet, other RNAi screens have previously shown that nuclear pore proteins have an important role in VV replication.<sup>20,27</sup> Among the antiviral hits, only seven genes could be validated, but no further assessment was performed to decipher their role in VV replication.

Using a powerful in vivo screening approach, Varble et al<sup>28</sup> identified host gene targets capable of improving viral replication in the context of an in vivo infection with

Table 1 Overview of key high-throughput screens involving oncolytic viruses

| Screen type                         | Virus | Strain         | Approach | Biological parameter being analysed | Readout                          | Multiplicity of infection | Analysis time point (h) | Cell line(s) screened          | Number of genes targeted in I <sup>1</sup> screen | Number of hits identified in I <sup>1</sup> screen | Reference                    |
|-------------------------------------|-------|----------------|----------|-------------------------------------|----------------------------------|---------------------------|-------------------------|--------------------------------|---------------------------------------------------|----------------------------------------------------|------------------------------|
| <b>RNAi</b>                         |       |                |          |                                     |                                  |                           |                         |                                |                                                   |                                                    |                              |
| siRNA                               | MRB   | wt             | In vitro | Oncolysis                           | Amar blue assay                  | 0.05–0.1                  | 48–72                   | OVCAR-8, U373 and NCI-H226     | 18,120                                            | 1,008 <sup>†</sup>                                 | Mahoney et al <sup>14</sup>  |
| siRNA                               | VSV   | rVSV-EGFP      | In vitro | Replication                         | % GFP+ cells                     | 0.5                       | 18                      | HeLa                           | 22,909                                            | 233 <sup>↓</sup>                                   | Panda et al <sup>19</sup>    |
| siRNA                               | VV    | WR-EGFP        | In vitro | Infection                           | % GFP+ cells                     | 1                         | 8                       | HeLa MZ                        | 6,979                                             | 276 <sup>↓</sup>                                   | Mercier et al <sup>20</sup>  |
| siRNA                               | MYXV  | Lausanne-LacZ  | In vitro | Replication                         | β-Galactosidase activity         | 1                         | 48                      | MDA-MB-231                     | 21,585                                            | 1,048 <sup>†</sup>                                 | Telfer et al <sup>42</sup>   |
| siRNA                               | VV    | IHD-J/GFP      | In vitro | Replication                         | % GFP+ cells                     | 0.2                       | 18                      | HeLa                           | 21,566                                            | 540 <sup>↓</sup>                                   | Sivan et al <sup>27</sup>    |
| miRNA                               | SINV  | TE12Q          | In vivo  | Replication                         | Viral clone population frequency | 5×10 <sup>6</sup> pfu     | 48                      | N/A                            | 10,000                                            | 576 <sup>↓</sup>                                   | Varble et al <sup>28</sup>   |
| siRNA                               | VV    | WR AS-EGFP     | In vitro | Replication                         | GFP MFI                          | 0.05                      | 48                      | HeLa                           | 6,719                                             | 149 <sup>†</sup>                                   | Beard et al <sup>25</sup>    |
| siRNA                               | VSV   | rVSV-EGFP      | In vitro | Infection                           | % GFP+ cells and GFP MFI         | 1                         | 7                       | HeLa                           | 21,121                                            | 153 <sup>↓</sup>                                   | Lee et al <sup>21</sup>      |
| siRNA                               | HSV   | KM100-GFP      | In vitro | Oncolysis                           | MTT assay                        | 1                         | 48                      | H5578T                         | 16,056                                            | 343 <sup>†</sup>                                   | Wörkenhe et al <sup>16</sup> |
| siRNA                               | VV    | KIL-C7L/GFP    | In vitro | Permissivity                        | % GFP+ cells                     | 0.1                       | 18                      | HeLa                           | 21,566                                            | 30 <sup>†</sup>                                    | Sivan et al <sup>16</sup>    |
| <b>Chemical</b>                     |       |                |          |                                     |                                  |                           |                         |                                |                                                   |                                                    |                              |
| Small molecule                      | VSV   | Indiana VSVΔS1 | In vitro | Oncolysis                           | Amar blue assay                  | 0.03                      | 40                      | 4T1                            | 12,280                                            | 30 <sup>†</sup>                                    | Diallo et al <sup>14</sup>   |
| Bioactive molecule                  | HSV   | G47Δ-GFP       | In vitro | Replication                         | % GFP+ cells                     | 0.05                      | 48                      | PC-3                           | 2,640                                             | 15 <sup>†</sup>                                    | Passer et al <sup>21</sup>   |
| Small molecule and chemotherapeutic | MYXV  | MYXV-dsRed     | In vitro | Oncolysis                           | Amar blue assay                  | 1                         | 48                      | BT025 (patient BTICs)          | 73                                                | 11 <sup>†</sup>                                    | McKenzie et al <sup>14</sup> |
| NIH clinical collection             | Ad    | Delta24-RGD    | In vitro | Oncolysis                           | CellTiter-Glo assay              | 50                        | 120                     | GS79 and GS102 (patient gCSCs) | 446                                               | 10 <sup>†</sup>                                    | Pont et al <sup>13</sup>     |

**Notes:** Arrows indicate if knockdown of target genes or the application of drug libraries increased (<sup>†</sup>) or decreased (<sup>↓</sup>) the viral parameter being analyzed. \*Number remaining following the exclusion of toxic genes and/or the application of validation thresholds. \*Common genes identified across two of three cell lines. \*Virus delivered via the footpad.

**Abbreviations:** Ad, adenovirus; BTIC, brain tumor-initiating cell; dsRed, discosoma species red fluorescent protein; EGFP, enhanced green fluorescent protein; gCSC, glioblastoma cancer stem cell; GFP+, green fluorescent protein positive; h, hours; HSV, herpes simplex virus; LacZ, β-galactosidase; MFI, mean fluorescent intensity; miRNA, microRNA; MOI, multiplicity of infection; MRB, Maraba virus; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MYXV, Myxoma virus; RGD, Arg-Gly-Asp; RNAi, RNA interference; SINV, Sindbis virus; siRNA, short hairpin RNA; siRNA, short interfering RNA; VSV, vesicular stomatitis virus; VV, vaccinia virus; WR, Western Reserve; wt, wild type.

the OV candidate, Sindbis virus (SINV) (Table 1). A library of miR30-based microRNAs (miRNAs) was encoded into a large cohort of SINVs such that each individual virus encoded a unique miRNA targeting a single open-reading frame (ORF). After infecting naïve mice with this library, viral clones equipped with replication-enhancing miRNAs outpaced poorly replicating clones to become the dominant circulating viral populations. This process of natural selection in a host animal identified ~20 viral clones in the first of four parallel screens. Of those 20 clones, eight were shared between two parallel screens, three were shared between three parallel screens, and a single clone was shared between all four parallel screens. The viral clones that provided the greatest replicative advantage across the parallel screens targeted genes encoding zinc finger protein X or MAX gene-associated protein. Both proteins (or their close homologs) have previously been shown to have a role in cellular self-renewal, in addition to cell cycle and cell death pathways.<sup>29,30</sup> In this screen, functional analysis suggested that both genes had a role in maintaining the host antiviral response to SINV replication, with a reduction in interferon (IFN)-stimulated gene expression following knockdown.<sup>28</sup> From a therapeutic perspective, it is eminently conceivable to perform similar *in vivo* screens with RNAi library-encoding OVs in tumor-bearing animals to allow the natural selection and subsequent identification of high-value viral clones capable of replicating to high titers at tumor sites.

Finally, in a chemical screen, Passer et al<sup>31</sup> analyzed the impact of 2,640 bioactive compounds across three pharmacologically active libraries on the replication index of the oncolytic HSV-1, G47Δ (Table 1). From this library, 15 drugs were identified that amplified viral replication. Two drugs within the same class of ENT1 inhibitors, dipyrindamole and dilazep, were further explored. Both dipyrindamole and dilazep increased viral productivity across multiple tumor cell lines and in several independent human patient prostate tumor explants. Overall, this correlated with an increase in the killing capacity of the virus on tumor cells following combination therapy; yet, some tumor cell lines remained resistant to the additive activity of the drug and virus combination, despite expressing the target ENT1 protein by Western blot. The combination therapy also improved tumor control in xenograft mice bearing subcutaneous DU145 tumors. Interestingly, the ability of HSV vectors to achieve synergistic outcomes following ENT1 inhibition was fully dependent on the infected cell protein 6 (ICP6)-null status of the HSV vector used. ICP6 encodes for the large subunit of ribonucleotide reductase (RR<sub>L</sub>) in HSV-1,<sup>32</sup> which is

essential for viral DNA replication; the removal of ICP6 from oncolytic HSV-1 solidifies its cancer specificity by restricting infection to replicating cells. Indeed, it was subsequently demonstrated that ENT1 inhibition promoted the activity of host ribonucleotide reductase to enhance viral productivity in infected cells.<sup>31</sup>

## Enhancing oncolytic death

Oncolysis is a key step in the viroimmunotherapeutic process that not only directly kills malignant cells to ablate the tumor mass but also releases TAAs to drive inflammatory immune engagement against tumor targets. The level of oncolysis and the type of tumor cell death elicited are critical for establishing immunogenic responses.<sup>33</sup>

To our knowledge, our laboratory was the first to perform a high-throughput siRNA screen to analyze the impact of host gene manipulation on viral oncolysis to uncover new therapeutic targets (Table 1).<sup>34</sup> We carried out two independent genome-wide screens with a siRNA library targeting 18,120 genes across three distinct tumor cell lines to identify host genes that mediated MRB virus-induced oncolysis. We observed 1,008 synthetic lethal hits that were common to at least two of the three malignant cell lines. Following gene cluster analysis, we observed several hits in expected biological pathways, including metabolism, cellular mitosis, and adenylate cyclase activity. However, we also uncovered an unexpected and critical role for endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) during MRB infection. Following further mechanistic exploration, we subsequently demonstrated that specific inhibition of the inositol-requiring enzyme 1 alpha, an ER transmembrane protein kinase/endoribonuclease that acts as an UPR sensor, increased the apoptotic index following MRB infection in a tumor-specific manner. Such transient ER stress led to the accumulation of the caspase adaptor protein, RIP associated Ich-1/CED homologous protein with death domain, which prepared the cell to undergo caspase 2-dependent death following virus infection. Preconditioning tumor cells with a drug that inhibited ER stress sensitized patient glioblastoma cells to MRB oncolysis. Critically, pretreatment with this drug *in vivo* rendered resistant EMT6 syngeneic breast tumors sensitive to MRB oncolysis and extended survival in tumor-bearing animals. We observed that combining this drug with virus therapy did not simply achieve an additive effect but was in fact synthetic lethal. Essentially, the target cell was manipulated into allowing access to a cell death pathway that would otherwise have not been available to the virus. Strikingly, we found this to have a wider therapeutic application

since inhibiting ER stress in combination with doxorubicin, a DNA-damaging chemotherapeutic agent that depends on caspase-2 signaling,<sup>35</sup> also sensitized cancer cells to die.<sup>34</sup> Linking such diverse biological pathways would have been highly improbable using traditional scientific approaches but was made possible through the unbiased nature of high-throughput screening. This study provided the first proof-of-concept that functional screening could be used to identify OVIT targets. It also highlighted how such knowledge could be used to design combination therapies not only for OVIT but also for broader cancer therapies.

Workenhe et al<sup>36</sup> specifically tailored a genome-wide RNAi screen to identify host factors responsible for inhibiting HSV-1 KM100 oncolysis in a breast cancer cell line that was resistant to infection. KM100 is a HSV-1 mutant that lacks ICP0, making it hypersensitive to IFN.<sup>37</sup> Using a pooled lentiviral shRNA library consisting of 78,432 shRNAs targeting 16,056 genes, 343 genes were identified that could be knocked down to improve KM100 oncolysis (Table 1). After selecting 24 genes for secondary validation and pathway analysis, the authors focused on the serine/arginine-rich splicing factor 2 (*SRSF2*) gene, which increased KM100-mediated oncolysis following knockdown in the HS578T breast cancer cell line but not in nonmalignant primary fibroblasts. *SRSF2* belongs to a family of serine-arginine RNA-binding proteins that are critical for alternative and constitutive splicing of messenger RNA (mRNA) precursors, mRNA export, and translation<sup>38</sup> and have been implicated in mammary gland tumorigenesis.<sup>39,40</sup> Since *SRSF2* is phosphorylated by DNA topoisomerase I,<sup>41</sup> the authors explored the utility of combining KM100 with DNA topoisomerase I inhibitors. This combination synergistically enhanced the level of killing observed for in vitro breast cancer cell lines and increased survival in syngeneic TUBO tumor-bearing mice.

## Improving virus spread across the tumor

Achieving virus coverage across the entire tumor is a rare occurrence during OVIT but has been documented on occasion in some patients in clinical trials (personal communication). Virus spread across the tumor site depends on at least two critical factors: how much virus makes it to the tumor site following inoculation and how well the virus replicates to produce progeny that can subsequently spread across the local (and distant) tumor mass.

The initial virus load at the tumor is often dependent on the route of delivery, with direct intratumoral injections typically depositing a larger functional viral load (Stojdl, 2015 unpublished data). Conversely, intravenously administered

virus can accumulate at the tumor site but typically at lower levels than the input dose and with potentially lower levels of infectivity depending on the antibody serostatus of the treated host. Such route considerations represent physical constraints of therapy; yet, the ability of the virus to generate large numbers of infectious progeny that can spread across the tumor mass and the susceptibility of uninfected neighboring tumor cells to allow subsequent virus attachment and transduction are two key areas that can be leveraged by screening approaches.

Sivan et al<sup>27</sup> conducted a genome-wide RNAi screen in HeLa cells with recombinant VV IHD-J/GFP, a strain with a point mutation that increases the release of extracellular enveloped virus from the cell surface (Table 1). This virus strain was deliberately employed to facilitate the identification of host genes that were capable of impacting virus spread. Taking a novel approach, two primary screens were conducted in parallel using different commercial siRNA libraries targeting the entire host genome. One library contained a pool of four siRNAs per well targeting 18,120 genes, while a second library comprised three nonpooled siRNAs targeting 21,566 genes. Both libraries shared 17,693 overlapping gene targets; 576 genes were identified that could be silenced to decrease replication, while 530 genes could be silenced to increase replication. Approximately 30% or 50% of these genes, respectively, were hits across both independent libraries from the primary screen. Intriguingly, several of the validated genes that decreased replication upon knockdown were nuclear pore proteins, including nucleoporin 62, which diminished infectious virus yield by ~100-fold in one-step growth curves and negatively impacted morphogenesis. No secondary validation was performed on the genes that improved viral replication upon knockdown since these were not the focus of the study but at least 27 of them enhanced spread with a minimum of three different siRNA reagents.

Teferi et al<sup>42</sup> performed RNAi screens to identify host factors that impacted the replication of Myxoma virus (MYXV) using a recombinant Lausanne strain expressing LacZ from a late promoter (Table 1). A primary whole-genome screen was conducted using a siRNA library targeting 21,585 genes in MDA-MB-231 human breast cancer cells. This identified 1,048 antiviral genes and 540 proviral genes. After screening out toxic siRNAs and performing validation on hits from both the primary screen and an additional kinome screen, 711 genes were identified that could be knocked down to increase replication, while 333 genes could be knocked down to decrease virus replication. In follow-up studies, certain subsets of these genes were explored with a focus on glycolysis, mitogen-activated protein kinase signaling, DNA replication, and the cell cycle. Knockdown of genes that promoted

glycolysis (eg, phosphofructokinase) was detrimental to virus growth, whereas knockdown of genes that interfered with glycolysis (eg, fructose-1,6-bisphosphatase 1) favored virus growth. This is particularly interesting since cancer cells typically rely on the glycolytic pathway for energy metabolism, a phenomenon described as the “Warburg effect.”<sup>43</sup> This reliance on glycolytic gene function for MYXV replication may be at least partially responsible for the tumor tropism of the virus. This study highlights the utility of high-throughput screening for customizing OVIT by revealing genetic or metabolic signatures that could predict responses to therapy.

## Dealing with tumor heterogeneity

Tumors are formed from a mixed population of subclones, each harboring a distinct mutational variance. OVs have several inherent natural features that can at least partially mitigate tumor heterogeneity, including the ability to induce antigen spreading. Yet, additional therapeutic value may be achieved by pairing OVs with complementary interventions that unlock their capacity to target a wider genetic complement of tumor subclones.

To identify small molecules capable of modulating OV activity in resistant tumor cells, Diallo et al<sup>44</sup> screened a panel of 12,280 chemical compounds to identify small molecules capable of rendering the partially resistant 4T1 breast cancer cell line sensitive to oncolysis by the VSV mutant, VSVΔ51 (Table 1). This mutant harbors a single point mutation in the M protein that renders it unable to block nuclear export, thereby allowing the normal expression of host genes involved in the antiviral immune response, among others.<sup>45</sup> A lead viral sensitizer compound, Vse1, rendered four malignant cell lines across a spectrum of resistance susceptible to VSVΔ51 activity, including those derived from breast, colon, kidney, and brain tumors. Nonmalignant cells remained unaffected. Virus replication capacity was also improved across patient-derived colon, vulvar, and bone tumor explants in the presence of Vse1. Finally, in syngeneic subcutaneous CT26 tumor-bearing animals, Vse1 improved the ability of intratumorally administered VSVΔ51 to control tumor growth. Subsequent mechanistic analysis demonstrated that Vse1 was capable of suppressing 96% of the cellular transcripts typically stimulated by virus infection.<sup>44</sup> Importantly, when paired with the wild-type VSV virus, which has an intact M protein capable of blocking nuclear transport, Vse1 displayed no ability to improve viral replication. This suggested that the majority of Vse1 activity with VSVΔ51 may have been impacting the innate antiviral immune signaling response.

Sivan et al<sup>46</sup> screened a siRNA library across 21,566 host genes to identify modulators of viral permissivity (Table 1).

Using a VV mutant with deletions in K1L and C7L genes that block the infectious cycle prior to late-stage gene expression to prevent infection in most human cells, host genes were identified that allowed the virus to regain the capacity to replicate in the nonpermissive HeLa cell line. While only a small number of genes could be knocked down to improve permissivity beyond baseline, knockdown of sterile alpha motif domain-containing 9 (*SAMD9*) demonstrated a particularly noteworthy increase in infectivity from ~0% to 27%–47%, depending on the particular siRNA used. This represented an almost complete rescue since ~50% of cells were infected under the same conditions in the permissive BS-C-1 cell line. Further validation using CRISPR/Cas9 gene editing confirmed the role of *SAMD9* in viral permissivity and replication for this particular virus strain. Mechanistic studies previously demonstrated that *SAMD9* can be antagonized by poxvirus-encoded host range factors to prevent the formation of antiviral granules.<sup>47</sup>

Such screening approaches that focus on host genes capable of reversing resistance to infection have important therapeutic implications for heterogeneous tumor masses since manipulating such genes may achieve permissivity in otherwise resistant tumor subclones. While tumors represent genetically diverse populations, there are nevertheless several common cellular signaling pathways that sustain growth. These represent conserved targets that may offer therapeutic benefit across broad patient populations by allowing multiple stages of an entire signaling cascade to be targeted rather than focusing on a single gene or protein. As mentioned previously, our laboratory discovered that transient inhibition of components of the typically conserved ER stress response, in particular the activating transcription factor 6 and inositol-requiring enzyme 1 pathways, specifically sensitized tumor cells to caspase-2-dependent apoptosis induced by MRB virus infection.<sup>34</sup> Inhibition of these ER components increased the potency of MRB virus up to 10,000-fold across several malignant cell lines, with no concomitant increase of cytotoxicity in normal cells, and substantially improved antitumor efficacy in vivo. Indeed, ER stress and UPR activation are considered crucial for oncogenic transformation, development, and growth<sup>48</sup> and therefore represent prime targets for such approaches.

## Directed targeting of specific tumor populations

In addition to screening for genomic or chemical interventions that increase replication within the bulk tumor mass, an alternative approach has been to perform screens on highly defined tumor populations. The tumor microenvironment contains a diverse number of cell types, including epithelial



cells, stem cells, fibroblasts, stroma, endothelia, and immune populations. There is convincing evidence that cancer stem cells are involved in initiating and/or maintaining solid tumor growth<sup>49–51</sup> and are similarly responsible for tumor relapse after cancer patients receive treatments that cannot effectively target and ablate this population.<sup>52</sup> Thus, several chemical screens have focused on identifying drugs that can enhance viral replication and oncolysis specifically in cancer stem cell populations.

Pont et al<sup>53</sup> screened >400 drugs to identify potential targets that could enhance the replicative and oncolytic ability of the oncolytic adenovirus (Ad), Delta24-RGD, in both resistant and partially resistant patient-derived glioblastoma cancer stem-like cell (gCSC) cultures (Table 1). Four viral sensitizer compounds – including anagrelide, ebselen, fluphenazine, and indirubin – were identified that synergistically enhanced oncolytic activity across gCSC samples. Each compound impacted one or more core biological mechanisms, including enhancing virus attachment to host cells by increasing CAR expression levels, boosting replication efficiency, or increasing cell death. Fluphenazine emerged as a lead candidate since it could improve Delta24-RGD oncolysis in an extended panel of 12 gCSC cultures across a spectrum of virus sensitivity. Interestingly, fluphenazine significantly decreased Ad titers but nevertheless improved the degree of both apoptotic and necrotic tumor cell death and the oncolytic index of the virus in gCSCs, suggesting an uncoupling between the number of infectious virus particles and the oncolytic effect. These four sensitizer compounds were further validated with Delta24-RGD across a broader panel of tumor cell lines derived from other tissues. Strikingly, the four lead compounds could also synergize with other OV, including Newcastle Disease Virus and HSV-1-G47Δ,<sup>53</sup> suggesting that these drugs impacted conserved pathways that were important to the life cycle of multiple viruses.

Similarly, McKenzie et al<sup>54</sup> screened 73 chemotherapeutic compounds in preclinical or clinical development for their ability to sensitize the temozolomide-resistant patient-derived BT025 brain tumor-initiating cell (BTIC) culture to oncolytic MYXV (Table 1). A primary screen was performed at a drug dose of 1 μM to identify eight lead compounds, with a secondary screen at 100 nM to identify an additional three compounds, and a final screen at 10 nM, which identified no new candidate compounds. After further analysis, six of these eleven compounds achieved synergistic activity in combination with MYXV via Chou–Talalay analysis.<sup>54</sup> Several compounds acted on the same similar signaling hub – namely, the PI3K/Akt/mTOR pathway – consistent

with independent observations that rapamycin, which blocks mTOR, can increase rates of viral oncolysis.<sup>55,56</sup> Pemetrexed, a folate antimetabolite drug, increased both viral gene expression and viral titers but was the only drug that positively impacted both these viral parameters. Axitinib, a tyrosine kinase inhibitor, was chosen for further validation across three additional BTIC cultures and demonstrated increased killing in combination with MYXV across all tested cells. Interestingly, this enhanced death index was not accompanied by any change in apoptotic markers. Although markers of immunogenic cell death were not explored, certain chemotherapeutic drugs can enhance the immunogenicity of tumor cell death<sup>57</sup> to achieve synergistic anti-tumor efficacy during OVIT.<sup>58,59</sup>

## Manipulating the tumor microenvironment and enhancing therapeutic immune responses

Despite the clear importance of OV-mediated activation of antitumor immune responses through either inherent or engineered mechanisms, very few screens have been published that focus on defining new approaches to solidifying immune engagement or manipulating immunosuppressive cell populations within the tumor microenvironment, such as M2 macrophages, MDSCs, and Tregs. Several aspects of OV biology have the potential to impact immune engagement, including the type and immunogenicity of tumor cell death induced by the OV (eg, apoptosis, necroptosis, necrosis, autophagy) and the level of proinflammatory cytokine activation in infected cells. These, in turn, impact the establishment of the chemotactic gradient that drives the infiltration and activation of innate immune cell populations and the subsequent presentation of immunogenic TAAs by DCs to activate tumor- (or virus-) specific T-cells.

One of the few immune-related screens performed using an OV assessed which genes encoded by *Parapoxvirus ovis* (Orf virus [ORFV]) were involved in the establishment of small DC-rich immune cell depots at sites of infection. This screen is unique among the currently published literature for its focus on viral rather than host genes. This elegant screen was carried out entirely in vivo, delivering full ORFV ORFs as linear expression elements at spatially separated dermal sites in mice using gene gun technology.<sup>60</sup> Twenty-six pools of eight to ten ORFs were administered, and individual hits from pools that activated DC accumulation were rescreened. Ultimately, it was determined that the *B2L* gene from ORFV, a homolog of the VV *F13L* gene, was a direct driver behind the formation of these depots.



Given the importance of the immune response to the oncolytic effect,<sup>7,8,10</sup> and the clear ability of TAA-expressing OV<sub>s</sub> to engage therapeutically relevant antitumor memory immune responses,<sup>61</sup> in vivo screens have also been performed to identify optimal TAA targets during OVIT. Pulido et al created a VSV-expressed xenoantigen complementary DNA (cDNA) library mined from two human melanoma cell lines. Repeat intravenous treatment with this VSV library cured 60% of mice bearing syngeneic B16.F10 melanoma tumors. Individual VSV vectors within the cDNA library were screened for their ability to stimulate interleukin-17 production in splenocytes from treated mice restimulated with B16.F10 tumor lysates to identify the most potent encoded TAA targets. Three VSV vectors – encoding *NRAS*, *TYRPI*, and *CYCL* – were identified that, when used in combination, achieved the same therapeutic outcome as the complete cDNA library.<sup>62</sup> Importantly, using one or two of these vectors in combination was insufficient to achieve therapeutic efficacy, highlighting the importance of incorporating strategies designed to target multiple antigens. Indeed, this tallies with previous observations from the wider immunotherapeutic field.<sup>63,64</sup> Intriguingly, treating intracranial B16.F10 tumors with the three VSV-encoded TAAs that demonstrated efficacy against subcutaneously implanted B16.F10 tumors failed to achieve efficacy,<sup>65</sup> suggesting that the tissue in which the tumor resides has a significant role in selecting the nature of the TAAs that represent relevant therapeutic targets.

## Future directions

It is widely accepted that therapeutic approaches that focus on more than one malignant target achieve better control of complex, genetically diverse tumor masses. High-throughput screening has begun to play an important role in characterizing interventions that can impact multiple independent steps of the host–OV response. In the future, high-throughput screening will undoubtedly have a key role in identifying stackable interventions capable of impacting several cancer-associated pathways at temporally distinct periods during OVIT that could run to thousands of potential configurations. For example, prior to OV inoculation, innate immune responses could be targeted to minimize IFN responses in residual IFN-competent tumor cells to prevent the suppression of viral replication. During OV therapy, a chemotherapeutic agent could be administered to deplete suppressive Treg populations, together with a viral synergizer to solidify IFN suppression. Post-OV therapy, compounds capable of promoting immune memory formation may be administered to solidify tumor control and protect against relapse.

In vitro screens have provided tantalizing insights into the utility of high-throughput screening to identify novel therapeutic targets during OVIT, especially when these targets have been validated across multiple cell lines to determine broader relevance and across patient samples to explore potential clinical utility. The establishment of miniaturized cultures that mimic the in vivo environment will consolidate the utility of screening technologies for OVIT. Ultimately, in vivo screens have perhaps the greatest potential to reveal the most clinically relevant insights for OVIT, and further efforts in this area are needed.

The published host–virus interactome screens performed with OV vectors have predominantly focused on targeting the host genome. Yet, targeting the virus genome could also beneficially modulate responses, especially for OV<sub>s</sub> encoded by large genomes that have not been fully characterized. For example, numerous VV genes have unknown functions<sup>66</sup> and could be evaluated for their impact on oncolytic activity to generate a more potent OV.

Several other relatively new large-scale technological approaches have clear potential to provide insights for OVIT. Ribosome profiling is an emerging technology that provides a global snapshot of the mRNAs that are being translated in a cell at a given time. This is achieved by deep sequencing of all ribosome-protected mRNAs extracted from live cells, yielding mechanistic insights into viral and host gene expression and regulation during viral infection. In addition, genome-editing screens can employ CRISPR–Cas9 or transcription activator-like effector nuclease technology to completely remove or disrupt gene targets rather than knock them down, as in RNAi-based approaches. However, to our knowledge, none of these techniques have yet been applied in a primary OV screen.

## Conclusion

High-throughput screening has achieved clear benefits in the field of infectious disease research; yet up to this point, there have been relatively few publications that have used this technology to improve OVIT. OV screens have identified genes or pathways that have rational impacts on virological outcomes at the level of antiviral immune responses, mRNA or DNA synthesis, and metabolism. Yet, many of these genes have affected these pathways in unexpected or indirect ways. In addition, several novel nonrational pathways and mechanisms have been uncovered that may have otherwise eluded hypothesis-driven approaches, such as the impact of ER stress on viral oncolysis and the significance of nuclear pore proteins during VV replication. Taking a similarly

unbiased screening approach to focus on finding genes or pathways that consolidate immune system engagement is likely to provide important therapeutic insights to improve the long-term efficacy of OVIT and to guide the development and design of optimized clinical strategies.

## Acknowledgments

This work was supported by a Vanier Canada Graduate Scholarship from the Government of Canada (to KJA) and a grant from the Ontario Institute for Cancer Research, the Canada Foundation for Innovation, Angels of Hope, the Ottawa Regional Cancer Foundation, and the Terry Fox Research Institute (to DFS). The author's would also like to thank Scientific Illustration for the Research Scientist for providing the excellent open-source graphical resources that have been used in this manuscript.

## Disclosure

The authors report no conflicts of interest in this work.

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## CURRICULUM VITAE

**Kristina Allan**

**Languages:** English, French

## EDUCATION

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- |                   |                                                                                                                                                                                                                                                                                                         |
|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Fall 2010-present | Master's/PhD candidate in Biochemistry, University of Ottawa, Ottawa, Canada<br>Thesis Supervisor: Dr. David Stojdl<br>Thesis Title: <i>Enhancing oncolytic vaccinia virotherapy using functional genomic screening</i><br>Transferred from the Master's program to the PhD program in the Fall of 2012 |
| 2005-2010         | Bachelor of Science, Honours in Biopharmaceutical Sciences (Genomics/Option)/Co-op Education<br>University of Ottawa, Ottawa, Canada<br><i>Summa Cum Laude</i> – cGPA 9.8                                                                                                                               |

## Awards

- |           |                                                                                                                                                                        |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2013-2016 | Vanier Canada Graduate Scholarship                                                                                                                                     |
| 2010-2017 | Excellence Scholarship Research Award (University of Ottawa)                                                                                                           |
| 2013-2016 | CIHR Doctoral Research Award (declined)                                                                                                                                |
| 2012-2013 | Ontario Graduate Scholarship                                                                                                                                           |
| 2011-2012 | Frederick Banting and Charles Best Canada Graduate Scholarship-Master's Award                                                                                          |
| 2010-2011 | Ontario Graduate Scholarship                                                                                                                                           |
| 2010      | University Silver Medal/Science for the 2 <sup>nd</sup> highest standing in joint honours bachelor with major and multidisciplinary programs of the Faculty of Science |
| 2010      | Faculty Plaque in Biopharmaceutical Science (BPS) for the 1 <sup>st</sup> highest standing in BPS                                                                      |
| 2005-2010 | Canada's University Scholarship—French Immersion                                                                                                                       |
| 2005-2006 | Ontario Principals' Council Scholarship                                                                                                                                |

## RESEARCH EXPERIENCE

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### **Graduate student, University of Ottawa/Children's Hospital of Eastern Ontario (CHEO) Research Institute, Ottawa (September 2010-Present)**

- Developed significant expertise in human and vaccinia genome-wide RNAi screening (using siRNA and shRNA libraries) including optimization, logistics, image acquisition, image analysis, and operating robotics.
- Gained proficiency in working with recombinant DNA technology including engineering 35 novel vaccinia viruses, cloning multiple lentiviral vectors and CRISPR cell lines.
- Acquired experience in working with multiple tumour mouse models (xenograft and syngeneic) including implanting tumours, administering virus, measuring tumours, collecting specimens and *in vivo* imaging.
- Mastered skills in cell culture and virology techniques including generation of stable cell lines expressing shRNA; conducting wound healing, cytotoxic and virus spread assays, performing siRNA knockdown experiments, virus purification, virus manufacturing, and conducting plaque assays.
- Performed biochemistry techniques including PCR, qPCR, and ELISA.
- Gained experience in scientific writing

### **Co-op student, Monograph Development, Natural Health Products Directorate, Health Canada, Ottawa (May 2009-August 2009)**

- Assisted in the development and writing of monographs on Natural Health Products (NHPs)
- Reviewed and summarized clinical trials (in both French and English)
- Conducted literature research on the safety, efficacy and quality of NHPs

### **Co-op student, Children's Hospital of Eastern Ontario (CHEO) Research Institute, Ottawa (January 2008 - December 2008)**

- Developed the capacity to conduct high-throughput RNAi screens at CHEO
- Conducted the first high-throughput genome-wide RNAi screens in Ottawa

## PUBLICATIONS

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**Allan KJ**, Baird SB, Rowe KJ, Lefebvre CA, Labelle MC, Kmiecik J, Swift SL, McCart JA and Stojdl DF (2018). Genome-wide RNAi screens uncover MRAS as a novel target to enhance oncolytic vaccinia virotherapy. (In preparation)

**Allan KJ**, Mahoney DJ, Baird SD, Lefebvre CA, Stojdl DF (2018). Genome-Wide RNAi Screening to Identify Host Factors that Modulate Oncolytic Virus Therapy. *JoVE* 134: e56913

**Allan KJ**, Stojdl DF, Swift SL (2016). High-throughput screening to enhance oncolytic virus immunotherapy. *Oncolytic Virotherapy* 5:15-25

Mahoney DJ, Lefebvre C, **Allan K**, Brun J, Sinaei CA, Baird S, Pearce P, Gronberg S, Wilson B, Prakesh M, Aman A, Isaac M, Mamai A, Uehling D, Al-Awar R, Falls T, Alain T, Stojdl DF (2011). Virus-tumour interactome screen reveals ER stress response can reprogram resistant cancers for oncolytic virus triggered caspase 2-mediated cell death. *Cancer Cell* 20:443-56

## CONFERENCE PRESENTATIONS

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The 8<sup>th</sup> International Conference on Oncolytic Virus Therapeutics (2014). Oxford, England. *Enhancing the efficacy of oncolytic vaccinia virus therapy using functional genomics*

2013 Canadian Cancer Research Conference (2013). Toronto, Canada. *Enhancing the efficacy of oncolytic vaccinia virus using functional genomics*

Children's Hospital of Eastern Ontario (CHEO) Research Day (2013). Ottawa, Canada. *Enhancing the efficacy of oncolytic vaccinia virus therapy using functional genomics*

The 7<sup>th</sup> International Meeting on Replicating Oncolytic Virus Therapeutics (2013). Quebec City, Canada. *Enhancing the efficacy of oncolytic vaccinia virus therapy using functional genomics*

Ontario Institute for Cancer Research (OICR) Scientific Meeting (2013). Toronto, Canada. *Enhancing the therapeutic efficacy of oncolytic vaccinia virus using functional genomics*

The 6<sup>th</sup> International Conference on Oncolytic Viruses as Cancer Therapeutics (2011). Las Vegas, NV, USA. *Using Genome-wide RNAi Screening to enhance oncolytic virus therapy*