CHARACTERIZATION OF ΔM51-VSV EXPRESSING BECLIN1

CHARACTERIZATION OF ΔM51-VSV EXPRESSING BECLIN1

By
ELSPETH SMITH, B.Sc (Hons)

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree Master's of Science

McMaster University

MASTER OF SCIENCE (2011) McMaster University

Infection and Immunity Hamilton, Ontario

TITLE: Characterization of ΔM51-VSV Expressing Beclin1

AUTHOR: Elspeth Smith

SUPERVISOR: Dr. Brian Lichty

NUMBER OF PAGES: xi, 71

ABSTRACT

Autophagy is a cellular process in which cytoplasmic material is lysosomally degraded into its basic components. The primary functions of this process are cellular recycling and stress mitigation however it has roles in both viral pathogenesis and tumourigenesis. Beclin1 is a key mediator of autophagy and is involved in its initiation. In an attempt to examine the effects of an enhanced level of autophagy within infected cells on both the viral pathogenesis and oncolysis of VSV, a VSV mutant (ΔM51) expressing Beclin1 was constructed and characterized. However it was determined through western blot analysis of autophagy marker LC3, that while VSV infection enhanced autophagy in infected cells, Beclin1 expression results in what appears to be a transient increase in autophagy followed by markedly reduced levels of autophagy at mid to late time points. Still, Beclin1expression, either directly or possibly through altering the kinetics of VSV induced autophagy enhanced the pathogenesis of VSV *in vitro* in some cell lines, as determined by a cellular viability assay. Examination of the *in vivo* pathogenesis of VSV-Belcin1 elicited no differences from that of the parental virus. This discrepancy between the *in vitro* and *in vivo* results was likely due in part to the cell specific nature of the enhanced pathogenesis displayed *in vitro.* The oncolytic potential of VSV-Beclin1 was also explored. Despite enhanced pathogenesis in CT26 cells *in vitro*, VSV-Beclin1 displayed no improvement in the oncolysis of CT26 tumours *in vivo*, compared to VSV-GFP. Further considerations of these results can explain these findings. Most oncolytic viruses including VSV have poor penetration into solid tumours. Therefore it is unlikely that attempts to augment oncolysis via use of a transgene which

would only be expected to have an impact on the relatively few cells that are directly infected, would be successful. It is hoped that the conclusions drawn from this study will help direct future research aimed at exploring the relationship between autophagy and VSV pathogenesis as well as future attempts to arm VSV with the intent of augmenting its oncolytic potential.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Brian Lichty for giving me an opportunity to work in his lab despite my limited previous lab experience. He has been a huge support throughout my graduate work and I have very much appreciated his expertise and direction as well as his sense of humour over the past two years. I would also like to thank my committee members Dr. Dawn Bowdish and Dr. Jonathon Bramson for their suggestions, guidance and support.

I would like thank all members of Lichty lab for their training and support. In particular Kyle Stephenson has been extremely helpful with the animal work in this study and training me on many other techniques. I also thank Kyle for providing the VSV-IHC images in the appendix. Christina Engel and I worked very closely on this project during her brief stint in the Lichty lab. I thank her for her patient explanations and the translating she did for me. I would also like to thank the co-op students that did terms in our lab; Carly Tuinman, Jessica Breznic, Russell Sterrett, Eva Chan and Adam Hesch for helping make these past two years fun.

I am also particularly grateful to our lab technician Natasha Kazhdan, not only for preparing the viruses used in this study but also for her friendship and support. In addition, the Lichty-Wan research associate Fuan Wang has been a tremendous support to me throughout this process. He has been an extremely patient and knowledgeable teacher, and truly a great mentor. I cannot thank these two individuals enough.

I would also like to thank the members of the CGT including Mathieu Morissette for his assistance with the H&E and tunel staining photography and analysis, and Carly Horvath for her assistance with murine lung inflations. I thank members of the Ashkar, Rosenthal, Mossman and Wan labs for lending reagents, equipment and advice. I am especially grateful to Derek Cummings of Dr. Mossman's lab for generously providing the MEFs used in this study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABREIVIATIONS

29T3	-	Human Embryonic Kidney Cells
Ambra1	-	Activating Molecule in Beclin1 Regulated Autophagy
AMP	-	Adenosine Mono-phosphate
AMPK	-	AMP Dependent Kinase
Atg	-	Autophagy Related Gene
ATP	-	Adenosine Tri-phosphate
B16 F10	-	Murine Melanoma
Bcl-2	-	B-Cell Lymphoma-2
BH-3	-	Bcl-2 Homology Domain 3
BSA	-	Bovine Serum Albumin
CQ	-	Chloroquine
CT26	-	Murine Colon Carcinoma Cells
DMEM	-	Dulbecco’s Modified Eagle’s Media
DMSO	-	Dimethylsulfoxide
dsRNA	-	Double Stranded RNA
ER	-	Endoplasmic-reticulum
EtOH	-	Ethanol
FAST	-	Fusion Associated Small Transmembrane protein
FBS	-	Fetal Bovine Serum
GFP	-	Green Fluorescent Protein
H&E	-	Hematoxylin and Eosin Stains
Hrp	-	Horse Radish Peroxidase
HSV	-	Herpes Simplex Virus
IFN	-	Interferon
IL	-	Interluekin
IN	-	Intranasal
IT	-	Intratumoural

IV	-	Intravenous
L929	-	Murine Fibrosarcoma Cells
LC3	-	Microtubule Associated Protein Light Chain 3
mAtg13	-	Mammalian Ortholog of Atg 13
mAtg9	-	Mammalian Ortholog of Atg9
MAVS	-	Mitochondrial Anti-Viral Signaling Protein
MEF	-	Murine Embryonic Fibroblast
MOI	-	Multiplicity of Infection
mTOR	-	Mammalian Target of Rapamycin
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDV	-	Newcastle Disease Virus
O/N	-	Overnight
OT	-	Oncolytic Therapy
OV	-	Oncolytic Virus
PAS	-	Phagophore Assembly Site
PBS	-	Phosphate Buffered Saline
PCD	-	Programmed Cell Death
PCD-I	-	Apoptosis
PCD-II	-	Autophagy
Pfu	-	Plaque Forming Units
PI3K	-	phosphoinositide 3-kinase
PtdIns(3)P	-	Phosphatidylinositol-3 Phosphate
Rapamycin	-	Rapa
RIG-I	-	Retinoic Acid-Inducible Gene 1
RLR	-	RIG-I like Receptors
ROS	-	Reactive Oxygen Species
RT	-	Room Temperature
TBST	-	0.01% Tween - Tris Buffered Saline
TRAMP	-	Mouse Prostate Transgenic Adenocarcinoma cells

ULK1	-	Unc-51 Like Kinase 1
UVRAG	-	Ultraviolet Irradiation Resistance Associated Gene
Vero	-	Primate Kidney Epithelial Cells
VMP1	-	Vascular Membrane Protein 1
Vps	-	Vascular Protein Sorting
VSV	-	Vesicular Stomatitis Virus
VSV-G	-	VSV glycoprotein
VSV-Ind	-	VSV; Indiana Serotype
VSV-M	-	VSV Matrix Protein
VSV-NJ	-	VSV; New Jersey Serotype
Wt	-	Wild Type
αMEM	-	Alpha Minimum Essential Medium
ΔM51-VSV	-	Mutant VSV; Deleted Methionine 51 of VSV-M

Chapter 1: Introduction

1.1 Oncolytic Viruses

The concept of oncolytic viruses (OV) or viruses that selectively infect and lyse tumour cells was born over a century ago. Just after the turn of the century hematology professor, George Dock, noted a number of cases in which patients experienced complete spontaneous cancer remissions after natural viral infections (Dock, 1904). However, it was not until the emergence of DNA recombinant technology towards the end of the 20th century that research in this field made significant progress and oncolytic therapy (OT) became relevant (Hammill, 2010; Kelly, 2007; Moore, 1952). This technology along with a better understanding of virus-host relationships, has lead to the discovery and development of both naturally occurring and genetically manipulated attenuated viruses which are both safe and effective (Hammill, 2010; Kelly, 2007).

Today, the field of OT is a rapidly expanding area of research which shows great promise and has many advantages over more traditional cancer treatments. One of the key benefits of OT is the potential for broad based therapy (Wong, 2010; Kelly, 2007). Often OVs target tumour cells by taking advantage of defective anti-viral signaling pathways which are commonly disrupted in transformed cells but intact in the healthy surrounding tissue. For example the attenuated herpes simplex virus (HSV), HSV1716 selectively targets cancer cells with a dysfunctional PKR response, a phenomenon shared by an estimated 90% of cancers (He, 1997; Mundschau, 1992)

OVs also tend to employ several anti-tumour mechanisms. This increases their range of efficacy compared to traditional chemotherapies, which are often highly specific and

rapidly succumb to tumour resistance (Breitbach, 2010). In addition to direct tumour cytotoxicity it is now well understood that it is often the indirect bi-stander effects of OT that lead to the bulk of tumour clearance. For example, inflammation within the tumour vascular system caused by viral infection leads to vascular disruption, and ultimately tumour necrosis (Breitbach, 2007). Moreover, while most tumours tend to be immunosuppressive oncolytic infection creates a highly immune-stimulatory microenvironment that leads to the induction of anti-tumour immune responses (Gajeski, 2006; Prestwhich, 2008). In the case of some OVs, it is this anti-tumour immune response that is ultimately responsible for the vast majority of tumour clearance and has been proven necessary for OV efficacy (Diaz, 2007) This effect can also aid in the clearance of distant secondary tumors and limit relapse as well, (Hammill, 2010; Breitbach, 2010; Diaz, 2007; Toda, 1999), something that cannot be said for many traditional cancer therapies. Another benefit to OT is the ease in which viral genomes are manipulated. This has led to the development of not only safer attenuated viruses, but also enhanced tumour targeting, and a new generation of viruses “armed” with genes to increase their efficacy (Hammill, 2010; Wong, 2010; Diaz, 2007).

In the past 10 years, research in this field has achieved great strides. Preclinical research has led to the development of a several oncolytic viruses which show high efficacy in the treatment of a wide range of animal and human tumour models. In addition, phase I and II clinical trials in this fields have demonstrated that OT is extremely safe for human use (Eager, 2011; Breitbach, 2010; Kelly,2007). Still, OT has its disadvantages as well. Despite so much success in animal models, to date there has

been no completed phase III clinical trials in this field (Breitbach, 2010). While phase I and II clinical trials have demonstrated moderate success this has been somewhat anti-climactic compared to those celebrated in pre-clinical research. There are probably many explanations for this relative lack of efficacy in human trials, but the host immune system likely plays an important role (Wong, 2010; Prestwhich, 2008). OV often face rapid clearance in humans which can be augmented by the presence of serum antibodies (Prestwhich,2008; Aghi, 2005). In addition, viral particles which are relatively large compared to interstitial space within tumours generally have poor penetration into solid tumours (Smith, 2011; Wong, 2010; Prestwhich, 2008). Still, initial concerns in this field regarding safety have largely been absolved and now the focus has to shifted towards improving efficacy.

1.2 Oncolytic VSV

While Vesicular Stomatitis Virus (VSV) has been widely used in research for decades it has only been within the last several years that VSVs intrinsic oncolytic potential has been discovered and fully explored (Lichty,2004; Barber,2004; Stoidl, 2000). Indeed, wild type (wt) VSV has inherent tumour selectivity due to the lack of a robust innate anti-viral response common to many tumours. Furthermore both naturally occurring and genetically altered mutants exist with enhanced tumour targeting and oncolytic capacity.

VSV, most notably a virus infecting rodents and agricultural animals, is known to cause relatively mild disease in cattle, horses and swine. The two most common serotypes existing in North and Central America, are the New Jersey (VSV-NJ) and Indiana (VSV-

Ind) serotypes. VSV-NJ is more common in North America and is most often the cause of VSV outbreaks in this region (Parez, 2010, Lichty 2004), while VSV-Ind is the serotype used in research.

A member of the rhabdoviridae family, VSV is an enveloped negative sense, single stranded RNA (ssRNA) virus. The 11 kilobase-pair VSV genome codes for five proteins; the nucleocapsid protein (N), two polymerases (L) and (P), the surface glycoprotein (VSV-G) and the matrix protein (VSV-M) (Barber, 2005; Lichty, 2004). VSV-M has several important functions in various stages of infection including, regulating viral transcription and packaging viral proteins. VSV-M also plays an essential role in immune evasion by blocking cellular transcription and mRNA transport from the nucleus, (Barber, 2005; Lichty, 2004; von Kobbe, 2000). This prevents cellular production of innate anti-viral proteins such as type 1 Interferon (IFN). Without this function of VSV-M, VSV is extremely susceptible to the effects of type 1 IFNs and is highly attenuated in cells with intact IFN signaling (Stojdl, 2000). VSV-G is responsible for cellular entry via receptor mediated endocytosis (Barber, 2005; Roberts, P., 1999). While the cellular surface receptor of VSV-G is unknown it is considered to be ubiquitous. The broadly tropic nature of VSV-G protein enables VSV to infect a wide range of cells, however VSV's susceptibility to innate host responses restricts replication in humans (Lichty, 2004).

VSV demonstrates great potential as an oncolytic virus. To begin with, it is naturally oncolytic in a broad range of tumour types. Indeed, wtVSV is known to target cells with Ras, Myc and p53 defects which are estimated to encompass ~90% of all cancers (Balachandran, 2001). Furthermore, due to a broadly tropic G protein, mentioned above,

VSV is capable of infecting a wide range of human cell types and as such has the potential for effective use in many different tissues. Indeed, VSV is highly oncolytic, in many different human and animal tumour models, demonstrated in a vast number of i*n vitro* and *in vivo* studies (Stojdl, 2003). Despite high cytotoxicity in tumour cells, VSV is considered safe for human use. While wtVSV can cause human infection it does so with low virulence and infections are usually asymptomatic, (Barber, 2005; Lichty, 2004). In addition, many recombinant forms of this virus exist, which impart increased tumour targeting *in vivo*, and improved attenuation in healthy cells (Lichty; 2004; Stojdl, 2003). VSV is also non-transforming, another factor contributing to the safety of this virus (Balachandran, 2001; Burns, 1993). While naturally occurring human infections happen occasionally, these are rare outside of VSV enzootic regions of Central America and Ossabaw Island, Georgia (Perez, 2010; Rainwater-Lovett, 2007), and are largely confined to high risk populations such as those exposed to infected livestock and researchers directly working with VSV (Lichty,2004; Roberts, 1999). In addition these infections are most often attributed to VSV-NJ, as opposed to the serotype used in OT and laboratory research VSV-Ind. As such seroconversion rates among the general population are extremely low (Lichty, 2004; Roberts, 1999) which may be an important factor contributing to the success of an OV (Wong, 2010; Prestwhich, 2008; Aghi, 2005).

Both naturally occurring and genetically altered mutants exist with enhanced tumour targeting and oncolytic capacity (Lichty, 2004). One recombinant VSV mutant contains a single amino acid deletion of methionine-51 in the M protein (ΔM51-VSV) resulting in complete loss of the immune evasion function of this protein (Stojdl, 2003).

In healthy cells with robust IFN response systems VSV is highly attenuated. However, for reasons not entirely understood many cancers have dysfunctional anti-viral signaling pathways and are incapable of either responding to or producing type 1 IFN. For example, murine colon carcinoma CT26 cells are incapable of responding to type 1 IFN and as such are extremely sensitive to ΔM51-VSV infection. Conversely, primary murine embryonic fibroblasts (MEF) which have fully intact type 1 IFN signaling pathways are relatively resistant.

A growing trend in the field of OT is to "arm" viruses with genes that, when expressed, impart additional properties which augment either oncolysis or the anti-tumour immune response initiated by viral infection. The composition of the VSV genome enables this to be accomplished with relative ease (Lichty, 2004). There are many examples in which transgenes were successfully added to VSV in order to increase virulence, tumour targeting or oncolytic efficacy. For example is was found that the addition of the gene encoding the Reptilian Reovirus p14 fusion associated small transmembrane (FAST) protein to the VSV genome enhances the *in vivo* neurovirulence demonstrated by increased viral titers taken from brain tissue of infected mice compared to a control virus (Brown, 2009). Similarly, the addition of the gene encoding simian para-influenza virus protein F (SV5-F) to a replication restricted VSV (VSV-ΔG) enhanced the apoptosis and cytotoxic effect in mouse prostate transgenic adenocarcinoma (TRAMP) cells *in vitro* and significantly reduced tumour volumes *in vivo* compared to control virus (Chang, 2010). Ebert and colleagues found that VSV expressing a mutated Newcastle Disease Virus F protein (NDV/F-(L829A)) formed syncytia with rat and

human hepatocellular carcinoma cells which led to increased virulence *in vitro* and enhanced oncolytic efficacy *in vivo* (Ebert, 2004). Possibly more important than increasing direct cytotoxicity of tumour cells is altering the immune response to viral infection. Recent research has indicated that VSV OT is dependent on an intact immune system, and suggests that much of the tumour lysis is mediated through indirect mechanisms involving immune stimulation initiated by the viral infection (Diaz, 2007). Willmon et al. found that arming VSV with murine IFN-β resulted in enhanced oncolytic efficacy in a murine mesothemia model; an effect which was mediated by anti-tumour T cell responses (Willmon, 2009). In addition this group found that the VSV expressing mIFN- β was significantly safer than the control virus. Conversely, while host anti-tumour immune responses augment OT, anti-viral immune responses can inhibit viral replication and efficacy (Wong, 2010; Prestwhich, 2008). Wu et al. found that the addition of the murine gammaherpes virus-68 M3 protein to recombinant VSV (rVSV) MΔ51 inhibited the recruitment of anti-viral immune cells, namely neutrophil and natural killer cells, which led to significantly enhanced survival in a rat hepatocellular carcinoma model. Collectively, these studies and many more like them, indicate that the addition of trangenes is an effective method of altering the course of VSV infection to enhance OT.

1.3 Autophagy

Autophagy, a catabolic process which involves degradation of cellular components with lysosomal machinery, has received a lot of attention for its recently established roles in both viral pathogenesis and tumourgenesis. First described in the

1960's, most of the progress in the field of autophagy research has been made only in the last several years (Klionsky, 2005). In this time there has been an abundance of research exploring the intricate regulation, the many physiologic functions and the complex relationships between autophagy and other cellular processes. In addition research in this field has begun to shed light on the complex role of autophagy in human health and disease. However, its role in OT, a field that spans both virology and cancer therapy, has been widely overlooked.

1.3.1 Physiologic Function and Regulation

Autophagy is a process in which cytoplasmic proteins as well as aged and damaged organelles are lysosomally degraded into their basic components. These components, namely, free fatty acids and amino acids, are then recycled and used for protein synthesis and ATP production (Chen, 2011; Martinet, 2009; Klionsky, 2007a;) Autophagy is a highly conserved process, and occurs in all eukaryotic cells at a basal level (Klionsky, 2005). Its primary functions are in the maintenance of cellular homeostasis via the turnover of long-lived proteins and non-functional organelles and stress mitigation. Certain cellular stresses such nutrient depravation, hypoxia, and oxidative stress will result in the rapid induction of autophagy, which promotes survival in conditions that would otherwise lead to apoptosis (Martinet, 2009; Klionsky, 2007a). In addition to a cell survival mechanism, autophagy has well established roles in many other cellular functions including innate and adaptive immunity, development, and programmed cell death (Levine, 2004). Cellular deficiencies in this process have also

been implicated in many diseases including cancer, neurodegenerative diseases such as Alzhiemers disease, heart disease and some autoimmune disorders (Martinet, 2009; Huang, 2007)

Several forms of autophagy exist, including macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (Klionsky, 2007b, Klionsky, 2005). Macroautophagy, hence forth simply referred to as autophagy, is by far the best studied of these processes and refers to the bulk non-specific sequestration and degradation of cytoplasmic material via lysosomal fusion. This is in fact the only know cellular process capable of degrading organelles (Klionsky, 2007a).

Stress stimuli lead to the rapid induction of autophagy. This process begins with the formation of a double membrane known as the phagophore. The phagophore elongates engulfing cytoplasmic material in the process and finally closes forming a complete vesicle known as the autophagosome. The autophagasome then fuses with a lysosome, forming a structure known as the autophagolysosome. This leads to the degradation of the cytoplasmic contents as well as the inner membrane itself (Klionsky, 2005; Martinet, 2009).

Regulation of autophagy is largely post-translational. The molecular steps involved in this process are mediated by a tightly regulated family of genes known as Atg, or autophagy-related genes. First discovered and characterized in yeast, there are over 20 known Atg gene products, many of which have a mammalian orthologue, (Martinet, 2009; Levine, 2007; Klionsky, 2007; Chen, 2011). In addition to several kinases, phosphatages and GTPases, these proteins form the complexes which control

phagophore and autophagosome assembly, lysosome fusion and autophagolysosome degradation, (Martinet, 2009; Klionsky, 2007a). While many of the molecular steps involved were originally defined in yeast more recent research has indicated that the mammalian system is similar. The following summary of autophagy regulation consists of what is known about mammalian autophagy and the mammalian terminology is used whenever possible. This summary is illustrated in Figure 1.

The molecular regulation of autophagy can be broken down into 4 “core” complexes/systems required for induction and the normal progression of this process. These complexes/systems include first, the unc-51 like kinase (ULK1, ortholog of Atg1) complex; second, a class III phosphoinositide 3-kinase (PI3K), hVps34/Beclin1 (ortholog of Atg6) complex; third, two ubiquitin like conjugation systems, Atg 12 and microtubule associated protein light-chain 3 (LC3, ortholog of Atg 8); and forth, two trans-membrane shuttling proteins Atg9 and vascular membrane protein 1(VMP1) (Yang, 2010, Chen, 2011).

While the exact functions of this complex are unknown, the ULK1 complex is considered to be both an initiator of autophagy and thought to determine the extent of autophagy (Chen, 2011). The active form of this complex includes ULK bound to mammalian ortholog of Atg13 (mAtg13) and the scaffolding protein FIP200 (Jung, 2009; Hosokawa, 2009). Active mammalian target of rapamycin (mTOR) inhibits the activity of this complex via direct binding to ULK which is accompanied by several phosphorylation events (Jung, 2009; Hosokawa, 2009). AMP-dependent kinase (AMPK) is a key regulator of mTOR. As cellular AMP to ATP ratios increase in a low-energy state, AMPK is

activated and inhibits mTOR (Lum, 2005). Once inactive, mTOR disassociates from the ULK complex, resulting in the dephosphorylation of both mAtg13 and ULK. This as well as other phosphorylation events lead to the activation of the ULK/mAtg13 complex, and the induction of autophagy (Jung, 2009; Hosokawa, 2009). Another key regulator of mTOR is the class I PI3K/Atk system. Atk (also referred to as PKB) is activated in response to growth factor stimulation and in turn activates a class 1 PI3K and subsequently leads to mTOR activation (Lum, 2005). Active mTOR inhibits both autophagy and apoptosis. The PI3K/Atk pathway also regulates cellular nutrient up-take, which is increased during cell growth (Lum, 2005). These interconnect pathways regulate the induction of autophagy during times of starvation and its inhibition when nutrients are abundant.

Recent research suggests that the ULK1 complex likely regulates autophagy via interaction with the second core system the hVps34/Beclin1 complex (Fimia, 2011). In the absence of autophagy, hVps34 and Beclin1 are bound in a complex with activating molecule in Beclin1 regulated autophagy (Ambra1) and p150 (ortholog of Vps15). This complex is bound to cellular microtubules via Ambra1-dynein interactions. However upon the activation of the ULK1 complex as described above, interactions between ULK1 and Ambra1 lead to Ambra1 disassociation from dynein. At this point the Ambra1/hVps34/Beclin1 complex is relocated to the ER or other phagophore assembly sites (PAS) (Fimia, 2011). hVps34 held in its active state by Beclin1 generates phoshatidylinositol-3 phosphate (PtdIns(3)P) enriched membranes which are involved in

the recruitment of Atg proteins to the PAS and autophagy induction (Fimia, 2011, Yang, 2010).

The hVps34/Beclin1 system actually consists of several different complexes involving these two integral proteins. While these complexes share several key components, namely hVps34, Beclin1 and p150; they differ in additional subunits and function. Functions of the hVps34/Beclin1 system range from early stage phagaphore formation, to late stage autophagosome maturation and even inhibition of autophagy. For example, in addition to the phagophore assembly complex described above, another complex including hVps34, Beclin1, p150 and ultraviolet irradiation resistance associated gene (UVRAG), also required for autophagy, has been shown to associate with LC3 (Liang, 2008). This complex has a proposed role in the recruitment of LC3, among other Atg proteins, to the growing autophagosome and may be responsible for supplying membrane to the growing autophagosome by deforming existing membrane (Yang, 2010; Liang, 2008). Conversely, an hVps34/Beclin1 complex including the Beclin1 binding partner Rubicon, has been shown to negatively regulate autophagy (Yang, 2010; Matsunaga, 2009)

The third core system includes two ubiquitin-like proteins Atg12 and LC3. These systems are believed to be involved with elongation and closure of the autophagosome (Yang, 2010). In a reaction catalyzed by Atg7 and Atg10, Atg12 is conjugated to Atg5. This Atg12-Atg5 complex then interacts with Atg16 and forms large Atg12-Atg5-Atg16 oligomers referred to as Atg16L, in a process mediated by Atg16 self interactions (Geng and Klionsky, 2008). In a similar system LC3 is cleaved by Atg4 forming LC3-I which is

then conjugated to phosphatidylethanolamine (PE) by Atg7 and Atg3 (Geng and Klionsky, 2008). LC3-PE, known as LC3-II, is located on both the inner and outer membrane layers of the auophagosome and eventually becomes degraded along with the autophagosomal contents (Yang, 2010).

While their exact functions in autophagy are not fully understood it is clear that these two systems work in conjunction and are thought to be involved in phagophore elongation and autophagosome maturation. Atg16L is located on the phagophore and determines the location of LC3-II conjugation. Conversely, LC3-II and its associated proteins are required for formation of the Atg16L complex (Yang, 2010). Indeed, Atg3 -/- cells which are incapable of producing LC3-II have reduced Atg12-Atg5 conjugation and have smaller and non-closed autophagasomes (Sou, 2008); providing further evidence for the theory that these two systems are involved in phagophore elongation and autophagosome closure (Yang, 2010).

Finally, the forth system described in the literature involves the trans-membrane proteins the mammalian homolog of Atg9 (mAtg9) and VMP1, both of which are required for autophagy. Atg9 is normally found on the trans-golgi network but is reallocated to autophagosomes upon induction of autophagy (Yang, 2010; He, 2007). It is thought that this protein is involved in shuttling membrane to the expanding autophagosome. VMP1 is located on the plasma membrane but co-localizes with both Beclin1 and LC3 upon the induction of autophagy (He, 2007). Over-expression of VMP1 can induce autophagy, however this is dependent on interactions with Beclin1. It is

believed that this protein may be responsible for recruiting the hVps34/Beclin1 complex components to the autophagosome (He, 2007).

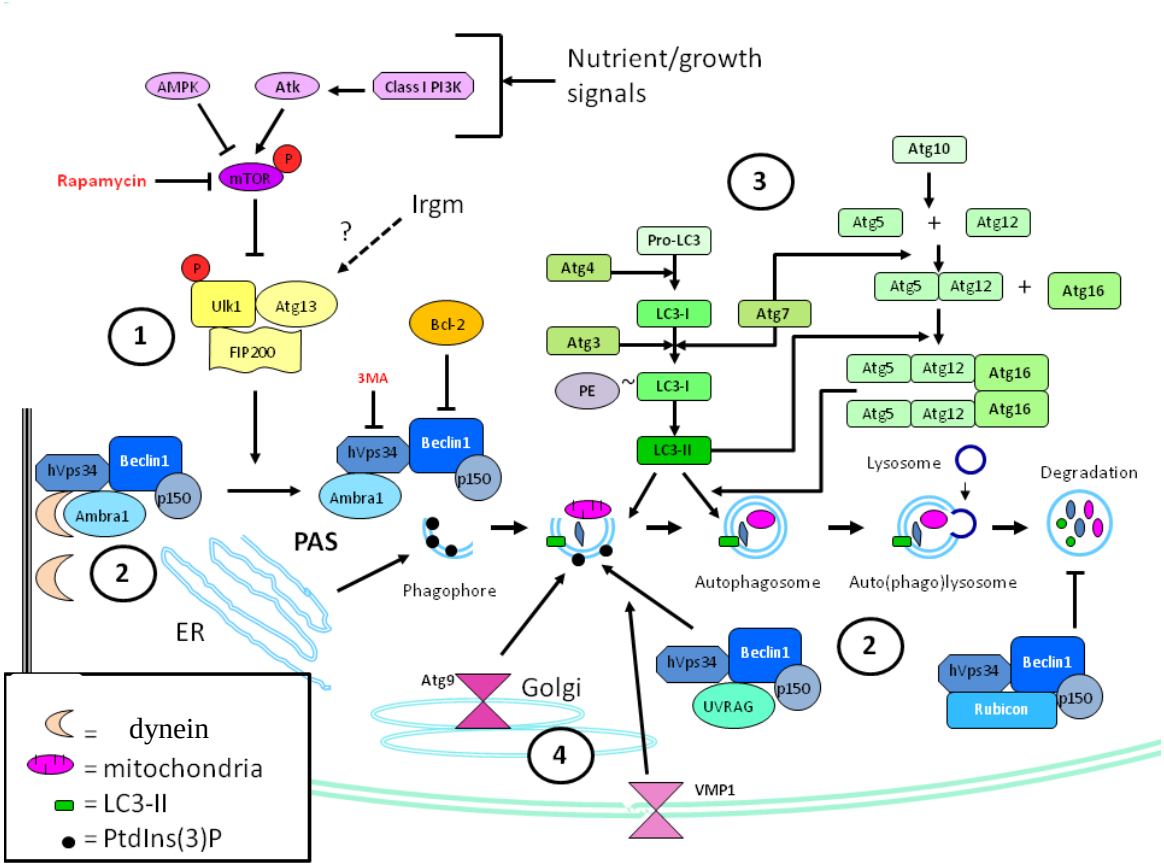


Figure 1: Cellular Regulation of Autophagy. 1) When activated the ULK1/Atg13 complex interacts with Ambra1 in a complex with Beclin1 and hVps34. The ULK1 complex releases the Ambra1 complex from microtubules by inhibiting dynein/Ambra1 binding (Fimia,2011). 2) Once free the Ambra1 complex relocates to the PAS (phagophore assembly site), where hVps34 creates PtdIns(3)P enriched membranes recruiting Atg proteins to this site and inducing autophagy (Fimia,2011). The Beclin1/hVps34 system consists of several complexes involving these key proteins, each with a different function. When bound to UVRAG the Beclin1/hVps34 complex has a role in autophagosome maturation. Conversely when bound to Rubicon this complex negatively regulates autophagy. 3) Two ubiquitin like conjugation systems, Atg5-Atg12 and LC3 work in conjunction with each other and are thought to play a role in

autophagosome maturation and closure (Yang,2010). 4) Two trans-membrane proteins Atg9 located on the golgi complex and VMP1 located on the plasma membrane, are thought to be involved in supplying membrane to the growing autophagosome and recruitment of Atg proteins (He, 2007).

1.3.2 The Role of Autophagy in Viral Pathogenesis

In addition to its primary functions of stress mitigation and cellular survival, autophagy also has a well established and important role in innate immunity and viral recognition (Martinet, 2009; Levine, 2007; Klionsky, 2006). Considered to be one the most evolutionarily ancient forms of innate immunity, autophagy acts to physically remove microbes from cells in the same way it degrades other cytoplasmic material, a function that is conserved in all eukaryotic cells (Delgado, 2007). In mammals and other vertebrates, with more complex immune systems autophagy plays a more involved role in cytokine signaling and antigen processing in adaptive immunity. However, just as the host immune system has evolved to utilize autophagy, so have pathogens evolved to evade or even exploit these defenses (Jackson, 2005, Prentice; 2004). It appears as though RNA viruses, such as VSV, have evolved to take advantage of autophagy in several ways. Most cells recognize ssRNA viruses through a family of cytoplasmic proteins, the RIG-I-like receptors (RLR), which includes the product of retinoic acid-inducible gene I (RIG-I) (Yoneyama, 2004). Upon binding double stranded RNA (dsRNA), a bi-product of RNA virus replication, RLRs are activated, turning on a signaling cascade that result in the development of an anti-viral cellular state mediated by type-I IFN signaling (Kawai, 2005; Yoneyama, 2004). Autophagy has been shown to play a role in this signaling at several levels. Jounai et al. demonstrated that interactions

between VSV dsRNA and the autophagic Atg5-Atg12 complex suppresses the RLR-IFN pathway. Shortly following VSV infection dsRNA binds RIG-I causing a conformational change that promotes RIG-I/Atg5-Atg-12 interactions. This prevents RIG-I activation of the RLR adapter mitochondrial anti viral signaling protein (MAVS) (Jounai, 2007). This effectively interrupts RLR signaling resulting in reduced type 1 IFN production, a phenomenon considered to be a vial immune evasion tactic (Jounai, 2007) Another group revealed that the absence of autophagy increases type-I IFN production following VSV infection compared to autophagy competent controls (Tal, 2009). They determined that the increase in type-I IFN production observed in autophagy deficient Atg5-/- MEFs was likely due to an increase in cellular levels of MAVS, which was attributed to the accumulation of aged mitochondria, due to lack of autophagic clearance (Tal, 2009). Accordingly, it follows that the opposite may also be true. It is conceivable that enhanced autophagy could result in the elimination of mitochondria and MAVS and consequently suppressed type 1 IFN signaling.

1.3.3 The Role of Autophagy in Cell death

Another more recently established function of autophagy is its role in cell death. Also known as programmed cell death (PCD) II, it is believed that under certain conditions autophagy can result in the over-digestion of cellular components and lead to an apoptosis and caspase independent cell death (Scarlatti, 2009). However, the relationship between apoptosis and autophagy is incredibly complex and not well understood (Eisenburg, 2009). These two systems share many of the same regulators including B-cell lymphoma-

2 (Bcl-2), mTor and p53, and are mediated by overlapping signaling pathways. For example, recent evidence indicates that caspases, the main group of proteases responsible for apoptosis signaling and execution, regulate autophagy as well. Wirwan et al. demonstrate that autophagy related proteins Beclin-1 and hVps34 are cleaved during apoptosis by caspase 3 and 8, leading to autophagy inhibition. Moreover, this group determined that a Beclin1 fragment produced in this cleavage reaction localizes to the mitochondria inducing the release of apoptosis inducer cytochrome-c (Wirawan, 2010). In this study the trigger for apoptosis, IL-3 deprivation, first induced autophagy. When autophagy was no longer able to mitigate this cell stress, apoptosis took over, followed by cell death. This is classic example of a scenario in which autophagy and apoptosis oppose each other, with autophagy functioning in its primary role of cell survival. However, these two systems can also act can act in parallel, independently of each or upstream/downstream of each other and in some cases have been shown to work in cooperation towards cell death (Eisenburg, 2009). Under some circumstances autophagy is necessary for the successful completion of apoptosis; and may fulfill the high energy demands of the apoptotic cell (Eisenburg, 2009). It is also evident that autophagic PCD II can compensate for apoptosis deficient cells (Scarlatti, 2009). While it is clear that these two systems are very closely linked, the details of their relationship are only now being uncovered. The complexity of this relationship makes it very difficult to predict how a cell or tissue might respond either by inhibiting or enhancing autophagy. Still, it is likely that the final fate of the cell, be it cell survival or death is dependent on the intricate

balance of many factors combined. This suggests that it may be possible to tip the balance from one dominant pathway to another.

In the context of OT, it is becoming increasingly clear that the dominant cell death pathway can have a significant impact on the outcome of treatment. Apoptosis or PCD-I is an inherently immuno suppressive process (Birge, 2008). Conversely, autophagy appears to be an immuno-stimulatory cell death pathway in comparison. Autophagy has a well established role in adaptive immunity and cross presentation, (Li, B., 2009; Uhl, 2009; Li, Y., 2008). Autophagic vesicles aid in the processing and transport of peptide to MHC loading sites (Li, Y.,2008; Delgado, 2007). One study located antigen in purified autophagosomes which were shown to be capable of cross-priming T cells. The authors of this study demonstrate that inhibitors of autophagy reduced cross-presentation from two antigen donor tumour cell lines, and inducers of autophagy increased cross-presentation, (Li, Y., 2008). Another study demonstrated that influenza infected MEFs dying via the autophagic pathway showed enhanced T cell priming compared to those dying via apoptosis, (Uhl, 2009). Collectively, these studies provide strong evidence that autophagy increases the cross-presentation from donor cells and is a more immune stimulatory form of cell death compared to apoptosis.

1.3.4 The Role of Autophagy in Tumourigenesis

Autophagy also plays an important but confounding role in tumourigenesis, and tumour maintenance. The gene Beclin1; whose protein product is an important in the initiation of autophagy has also been described as a tumour suppressor (Yue, 2003, Liang,

1999). Indeed, heterozygous Beclin1 knockout mice exhibit an increased rate of spontaneous tumour formation (Yue, 2003). In addition, many cancers including some types of breast, ovarian and prostate cancers have significantly low levels of autophagy compared to surrounding healthy tissue (Won, 2010; Aita, 1999). Furthermore, Beclin1 is a strong predictor of survival in patients with esophageal squamous cell carcinoma, (Chen, 2009).There are several theories that can explain the role of autophagy in tumour suppression. Its role in cell death may contribute, however it is likely that the maintenance of cellular homeostasis is more important, (Levine, 2007). One *in vitro* study illustrated that the absence of autophagy leads to an accrual of reactive oxygen species (ROS) due to the accumulation of damaged mitochondria, (Tal, 2009). The accumulation of ROS, known mutagens, is a likely contributor to tumourigenesis in autophagy deficient cells, explaining the tumour suppressive role of Beclin1. In addition its role in antigen cross presentation from tumour cells suggests that the down-regulation of autophagy in certain cancers may aid in tumour immune evasion, (Li, Y., 2008; Levine, 2007)

On the other hand there is an expanding body of evidence indicating that many different cancers types have relatively high levels of autophagy compared to surrounding tissue (Ahn, 2007). It is believed that these cancers may be exploiting this process to evade apoptosis and possibly to convey resistance to some cancer treatments (Zhang, 2010; Geng,2010; Carew, 2007; Ahn,2007; Paglin, 2001). Also autophagy has been shown to aid in tumour cell evasion of drug induced apoptosis, (Deganhardt, 2006). One theory that may explain this paradox is that autophagy protects against early tumour formation by eliminating old and damaged mitochondria that would otherwise lead to the

accumulation of ROS promoting DNA damage and tumouregenesis. However, once a tumour is established, autophagy may promote tumour maintanence and growth by providing a mechanism for coping with poor blood supply, low nutrient and oxygen availability, high energy requirements and chemotherapies (Martinez-Outschoorn, 2010).

1.3.5 Beclin1

Beclin1 is a 60 kD coiled coil protein encoded by a gene located on chromosome 17q21 (Aita,1999). Most well known for its regulation of autophagy, Beclin1 was first discovered through its interaction with the apoptosis inhibiting protein Bcl-2 (Liang, 1998). Still, it was not until several years later that Beclin1 was discovered to contain the Bcl-2 homology domain-3 (BH-3); placing it in the Bcl-2 family of proteins which interact via these BH domains (Oberstein, 2007). Unlike Bcl-2 which contains several BH domains, Beclin1 is a BH-3 only protein. As such it falls into a BH-3 only subfamily of proteins which have pro-apoptotic functions, mediated via inhibition of other anti-apoptotic BH proteins (Kang, 2011). Still, Beclin1's role in apoptosis regulation is under debate. Bcl-2 is a well established inhibitor of Beclin1, and does so via BH3 binding which results in the disassociation of Beclin1 from hVps34 and autophagy inhibition (Yang, 2010). However, whether or not these BH3 interactions can also inhibit the anti-apoptotic functions of Bcl-2, thereby promoting apoptosis is a matter of debate and there is evidence on both sides of the argument (Vazquez, 2010).

Beclin1 is often described as an autophagy initiator. As illustrated above it associates with the proteins hVps34 and p150 in several different complexes which are

involved in the initiation and progression of autophagy. Indeed, Beclin1 is so strongly associated with this process that it is a commonly used marker of autophagy (Klionsky, 2007b). Several groups have successfully used the over-expression of Beclin1 to up-regulate autophagy in a range of different experimental scenarios. Spencer et al. found that genetransfer of Beclin1 with a Lenti virus vector initiated autophagy in a rat neuroblastoma cell line and corrected pathogenesis caused by protein accumulation in these cells (Spencer, 2009). Gene transfer of Beclin1, was also able to restore normal levels of autophagy in autophagy deficient human breast carcinoma cells (Liang,1999). These examples provide evidence that Beclin1 may be a useful tool in studying autophagy in the context of OT.

1.4 Experimental Rational

In the context of OT, there is evidence to suggest that promoting autophagy within tumour cells could benefit both the viral replication and the anti-tumour immune responses. Upon viral infection most cells will initiate an anti-viral signaling cascade which involves the activation of MAVS located on the mitochondria (Seth, 2005; Tal, 2009). Because of its importance in the innate anti-viral response it is plausible that inhibition of MAVS, through increased autophagy and the degradation of mitochondria, could result in enhanced susceptibility to viral infection (Tal, 2009). In addition, there is a large body of evidence that indicates that autophagic cell death is more immuno-stimulatory than apoptosis (Birge, 2008; Scarlatti, 2009; Ulh, 2009; Delgado,2007). If the delicate balance that exists between these two cell death pathways could be tipped to

favor autophagy this could have profound effects on the oncolysis of VSV which is so dependent on anti-tumour immune response (Wongthida, 2010, Diaz, 2007). Therefore, a virus armed with a gene that could enhance autophagy represents a method by which the viral infection of relatively few tumour cells could aid in the destruction of the tumour mass.

To that end, we have constructed ΔM51-VSV expressing Beclin1; VSV-Beclin1, in an attempt to explore the ramifications of autophagy induction on both viral pathogenesis and subsequently oncolysis of VSV.

We hypothesize that the over-expression of Beclin1 within VSV infected cells will induce an enhanced state of autophagy. We believe this will augment viral pathogenicity possibly by interfering with type 1 IFN signaling. In addition to enhanced pathogenicity we expect that a high level of autophagy present in dying tumour cells will improve the oncolytic efficacy of VSV.

Chapter 2: Material and Methods

2.1 Cell Lines and Viruses

All cell lines were cultured at 37° C with 5% CO_2 in media supplemented with 2mM L-glutamine, 100μg/mL penicillin and 100μg/mL streptomycin. Murine colon carcinoma (CT26) cells, murine fibrosarcoma cells (L929), and primate kidney epithelial cells (Vero), all supplied by Dr. John Bell (University of Ottawa; Ottawa Hospital Research) were maintained in Alpha Minimum Essential Medium (αMEM) supplemented with 8% heat inactivated Fetal Bovine Serum (FBS). Murine melanoma B16 F10 cells generously supplied by Dr. Yonghong Wan (McMaster University) were maintained in MEM-F11 media supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% vitamins and 0.1% 10x β-mercapthenol. Murine embryonic fibroblasts (MEF) were generously donated by Dr. Karen Mossman (McMaster University) and were cultured in α-MEM supplemented with 12% FBS. Human embryonic kidney cells, (29T3) also supplied by Dr. John Bell, were cultured in Dulbecco's Modified Eagle's Media (DMEM) with 8% FCS.

All viruses used in this research; ΔM51 VSV-Beclin1 (VSV-Beclin1), ΔM51 VSV-GFP (VSV-GFP) and wild type VSV-GFP (wtVSV-GFP), were propagated and sucrose gradient purified as follows. 293T cells were seeded onto 15cm culture dishes. 24 hours later or when cells were 85-90% confluent the media was removed from each plate and cells were infected with the viral construct with a multiplicity of infection (MOI) of 0.1 in 500μL of media at 37°C for 45 minutes. Plates were rocked every 15 minutes to

prevent the cells from drying out. Immediately following infection 20mL of media was added back and cells were incubated at 37°C overnight (O/N) or until cells displayed signs of cytopathic effect. Supernatants were collected and centrifuged at 440 x g for 12 minutes at 4°C. Supernatants were filtered with .22μm filter and 0.5M EDTA pH8 is added to a final concentration of 5%. Samples were centrifuged at 18,592 x g for 1.5 hours at 4°C. Media was decanted and the virus pellet from each 250mL of media was re-suspended in 1mL of sterile PBS and vortexed for 30 seconds. Virus was kept on ice while a discontinuous sucrose gradient from 3% through 40% with a 75% cushion all made in PBS was prepared in ultra-centrifuge tubes. Virus was added to tubes which were then centrifuged at 80,496 x g for 30 minutes at room temperature (RT). Virus band was collected and dialyzed in dialysis cassettes (Thermo Scientific) against sterile PBS at 4°C. After 1 hour fresh PBS was added and virus was dialyzed O/N at 4°C. Virus was then dialyzed in 15% glucose in PBS for 4 hours. Virus was aliquoted and stored at -80°C. All viruses were titrated on Vero cells.

2.2 *In vitro* Viral Growth Assay

Vero cells were seeded onto 10cm culture dishes; 1 x 10^6 cells/plate and cultured for 24 hours. MEFs were seeded onto 10cm dishes; 5x 10^4 cells/plate and cultured for 24 hours. MEFs and Vero cells were infected with either VSV-GFP or VSV-Beclin1 at an MOI of 5. Supernatants were collected at 4, 8, 12, 24 hours and stored at -80°C. At a later time a viral plaque assay (described below) was performed using five 10-fold dilutions of each sample, in duplicate.

2.3 *In vitro* Infections and Cell Lysate Preparation

Cells cultured in 15cm cell culture dishes or T-150 flasks were infected with an MOI of 5 of either VSV-Beclin1 or VSV-GFP in 100μL α-MEM for 45 minutes at 37°C, mock treated or treated with Rapamycin (Rapa);100μM (CT26, L929) or 200μM (MEF). After infection 20mLs of the appropriate media for each cell line was added back and cells were incubated as per usual. At 4, 8 and 24 hours after infection or beginning of treatment one plate from each treatment group received chloroquine (CQ); 50 μM. After 4 hours of CQ treatment (t = 8, 12 and 28 hours) the media was collected from one CQ positive and one CQ negative plate from each treatment group. Cells were washed twice with PBS and trypsinized for 3-5 minutes. Cells were rinsed from plates and added to the corresponding media. Cell suspensions were centrifuged at 440 x g for 5 minutes at 4°C, decanted and cell pellets washed with PBS. Cells were centrifuged and washed twice more. After the third wash cells were centrifuged as above and PBS decanted. Cell pellets were lysed in RIPA lysis buffer (12 mM sodium deoxycholoate, 10% octylphenoxypolyethoxyethanol (Igepal), 1% SDS in PBS) 50-75μL/million cells. Lysates were sheared and centrifuged for 10 minutes at 6708 x g, at 4°C. Supernatants were aliquoted and stored at -80°C. Protein was quantified with a Protein Assay kit (Bio-Rad).

2.4 Western Blot Analysis

2x loading buffer (25% 0.5 M Tris-HCL pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenyl Blue in ultra-purified H_2O) was added to 40μg of protein from each sample, which was then boiled at 95°C for 5 minutes. For LC3 analysis samples were run onto 12% polyacrylamide gels and wet transferred onto .22 μm nitrocellulose membrane (Santa Cruz) with 350 mA for 1hr. For the analysis of all other proteins the samples were run onto 8% polyacrylamide gels and wet transferred onto .45 μm nitrocellulose membrane (Santa Cruz) with 350 mA for 1hr. All membranes were blocked with 5% skim milk in 0.01% tween-tris buffered saline (TBST) for 1 hr at RT. Membranes were incubated with primary antibody; rabbit anti-LC3 (Abcam), rabbit anti-Beclin1 (Cell Signaling), or Rabbit anti-Hsp70 (Pierce) diluted to 1:1000 antibody to 5% Bovine Serum Albumin (BSA) in TBST O/N, at 4°C. All blots were also incubated with murine anti-β-actin (Sigma-Aldrich) diluted to 1:40,000 in 5% skim milk in TBST for 1 hour at RT. Membranes probed for LC3 were incubated with anti-rabbit and anti-mouse antibodies conjugated to horse radish peroxidase (hrp) (Pierce) diluted to 1:5000 in 5% milk in TBST for .5 hour at RT and visualized using a chemiluminesence detection kit (Bio-Rad). Membranes probed for all other proteins were incubated with anti-rabbit and anti-mouse fluorescent conjugated antibodies (Pierce) and visualized using a Licor Odyssey scanner.

2.5 Caspase 3/7 Activity Assays

MEF and CT26 cells were plated onto 96 well dishes at a density of 2×10^4 cells/well and cultured for 24 hours. Five 10-fold serial dilutions of VSV-Beclin1 and

VSV-GFP were prepared in α-MEM supplemented with 10% FBS. Media was removed from 96 well dishes and wells were infected in duplicate with 20μL of each dilution, resulting in a range of MOIs from 0.001 to 10 for 45 minutes at 37°C. A mock infection was also included in duplicate for each cell type. Media was added back to wells for a total volume of 100μL and plates were incubated for 48 hours. Plates were centrifuged for 5 minutes at 440 x g and 50μL of media was removed from the top of each well. 50μL of Caspase-Glo® 3/7 caspase activity reagent (Promega) prepared according to manufactures instructions, was added to each well and plates were incubated on a shaker for 1 hour at RT. Plates were read on a luminometer and caspase activity relative to mock infection was calculated.

2.6 MTT Assays

Cells were plated onto 96 well dishes at a density of approximately 2 x 10^4 cells/well. The following day each virus was diluted in a 10-fold serial dilution. After media was removed from plates and wells were infected in triplicate with a range of MOIs from 0.0001 to 100, or mock infected in 20μL of media for 45 minutes at 37°C. Directly following infection media was added back to 100μL. After 48 hours of incubation at 37°C 10μL of 5mg/mL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Invitrogen) was added and incubated for 4 hours at 37°C. Media was removed from plates and 150μL of dimethyl sulfoxide (DMSO) was added; plates were incubated at RT for 10 minutes. Plates were read with a Saffire plate scanner with an absorbance wave length of 570ηm and a reference wavelength of 670ηm

in order to determine the cellular reduction of MTT. Percent of cells alive relative to mock infected cells was calculated.

2.7 Type 1 IFN Bio-Assay

Supernatants collected from previously infected cells (described above in 2.2) were centrifuged at 13,148 x g for 20 min at 4°C. Viral particles within supernatants were inactivated by dialyzing samples in 50mM glycine in H_2O; pH2, using dialyzer tubes (Thermo Scientific), for 6 hours at 4°C. Supernatants were then neutralized by dialysis in PBS for 1 hr at 4°C and again with fresh PBS O/N. L929 cells were seeded onto 96 well dishes at a density of approximately 2 x 10^4 cells/well and cultured for 24 hours. pH neutralized supernatants were diluted 1:10 followed by eleven, 3-fold serial dilutions in α-MEM supplemented with 8% FBS. Likewise, a type-1 IFN standard (SD) was serial diluted with a starting concentration of 750 U/mL. Media was removed from L929 cells and 100μL of each supernatant and SD dilution was added to wells in duplicate; plates were incubated at 37°C for 24 hours. Again, media was removed and cells were infected with 5 x 10^5 plaque forming units (pfu) of wtVSV-GFP in of α-MEM. Plates were incubated for an additional 24h hours at 37°C. Plates were read on a Typhoon Fluorescence Reader and fluorescence was quantified using ImageQuant software. Protection from wtVSV infection was used as a marker for type-1 IFN activity.

2.8 Viral Plaque Assays

Approximately 4 x 10^5 Vero cells were seeded onto 6cm dishes and cultured for 24 hours. Frozen murine lungs were thawed, weighed and homogenized with an Ultra-Turrax T-25 (IKA-Werke) homogenizer. Homogenates were vortexed for 15 seconds and centrifuged for 10 minute at 440 x g. Supernatants were removed, vortexed and centrifuged at 6708 x g for 10 min. The volumes of the resulting supernatants were measured and these homogenates were diluted five times in a 10-fold serial dilution in duplicate. Media was removed from Vero cells which were then infected with 100µL of each dilution and incubated for 45 minutes at 37°C. Plates were overlaid with one part 1% molten agarose to one part 2X-F11 media supplemented with 20% FBS, heated to 42°C. After 24 hours incubation at 37°C plaques are counted and viral pfu/gram of tissue was calculated. Each plaque represents a single infectious viral particle.

2.9 Tissue Staining

Murine lungs fixed in 10% para-formaldehyde were embedded in paraffin, cut into 4µm cross-sections and mounted on glass slides. For detection of apoptotic cells, tunel staining was performed using ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) according to the manufactures protocol for paraffin embedded tissues. For assessment of inflammation and signs of infection slides were stained with hemotoxylin and eosin (H&E).

2.10 *In vivo* Infections and Oncolytic Experiments

In order to assess the *in vivo* pathogenesis of VSV-Bec, female Balb-c mice received an intranasal (IN) infection of 5 x 10^8 pfu in 30 μL of saline with either VSV-Beclin1 or VSV-GFP. Mice were sacrificed and lungs removed at 48, 72 and 96 hours. The right lungs were removed; flash frozen and stored at -80°C. At a later time a viral plaque assay (described above) was performed in order to detect viral load. Left lungs were perfused with 500μL of 10% para-formaldehyde in PBS and fixed in this solution for 72 hours. Lungs were then washed in 50% ethanol (EtOH) and again in 70% EtOH. Finally lungs were cross sectioned and paraffin embedded to prepare for sectioning and tissue staining (described above). To assess weight loss following infections, Balb-c mice were infected IN with 5 x 10^8 pfu in 30 μL of saline with either VSV-Beclin1 or VSV-GFP and weights were monitored for 10 days or until mice recovered to their original weight.

To evaluate the oncolytic potential of VSV-Beclin1 compared to the parental virus, female Balb-c mice were engrafted with 5 x 10^5 CT26 cells subcutaneously in the left hind limb flank. Once tumours reached an average of 100mm^3 (day 0) mice were randomly placed into one of the following treatment groups: VSV-Beclin1, intravenous (IV) delivery; VSV-Beclin1, intratumoural (IT) delivery; VSV-GFP, IV; VSV-GFP (IT) or a mock treatment group. Mice receiving IV treatment received 5 x 10^8 pfu in 200μL of saline on day 0 and again on day 2 via tail vein injection. Mice receiving IT treatment received 1 x 10^7 pfu in 50μL of saline also on days 0 and 2. Mock treated mice received

two 50μL IT injections of saline. Tumours were measured and volume calculated for 45 days or until mice reached endpoint (1500 mm^3).

2.11 Statistical Analysis

GraphPad Prism (version 4; GraphPad Software, Inc.) was used for statistical analysis. Survival curves were generated by the Kaplan-Meier method. Statistical tests used are reported in the figure legends. All reported *P* values were considered statistically significant at $P < 0.05$.

Chapter 3: Results

3.1 Preliminary Characterization of VSV-Beclin1

3.1.1 Beclin1 expression does not impact the in vitro replication of VSV

In order to confirm Beclin1 expression following infection with VSV-Beclin1, a range of cell types MEF, CT26 and L929 cells were infected with an MOI 5 with either VSV-Beclin1 or the parental virus, VSV-GFP. Cells were lysed at 8, 12 and 24 hours and lysates were probed for Beclin1 via western blot analysis. Each cell line exhibited strong expression of Beclin1 compared to VSV-GFP infected cells. This expression began at least as early as 8 hours post infection expression and continued until at least 28 hours, the last time point tested (not all time points shown; Figure 2A). Because the addition of a transgene can in some cases interfere with viral replication we sought to confirm the normal *in vitro* replication of VSV-Beclin1. Type 1 IFN signaling in host cells is a major determinant of VSV replication. For this reason we chose Vero cells which are incapable of producing Type 1 IFN, and MEFs which have an intact IFN signaling system in order to test the replication of VSV-Beclin1 in type 1 IFN deficient and sufficient systems. Each cell line, Vero and MEF, was infected with either virus at an MOI of 5. Supernatants were collected at 4, 8, 12, 24, and 31 hours. Plaque assays were performed on supernatants and pfu/ml of each sample was calculated. VSV-Beclin1 exhibited a replication pattern typical to parental VSV infection in both cell lines; there were no differences in the replication between the two viruses (Figure 2B). A comparison of the two cell lines indicated that MEFs were less permissive to both VSV-Beclin1 and VSV-

GFP replication compared to type 1 IFN deficient Vero cells. At four hours post infection supernatants from Vero cells contained significantly more viral progeny then the MEF supernatants at the same time point, 9.5 x 10^7 and 3.12 x 10^2 pfu/ml respectively (P value = 0.0026, student's t test). Also MEF viral production peaked much lower at 1.0 x 10^8 pfu/ml than Vero viral production at 5.95 x 10^9 pfu/ml (P value = 0.0014, student's t test). This indicates that as expected, cells lacking a functional IFN signaling system were much more permissive to ΔM51-VSV infection than those with a fully intact system and Beclin1 expression appeared not to modify this

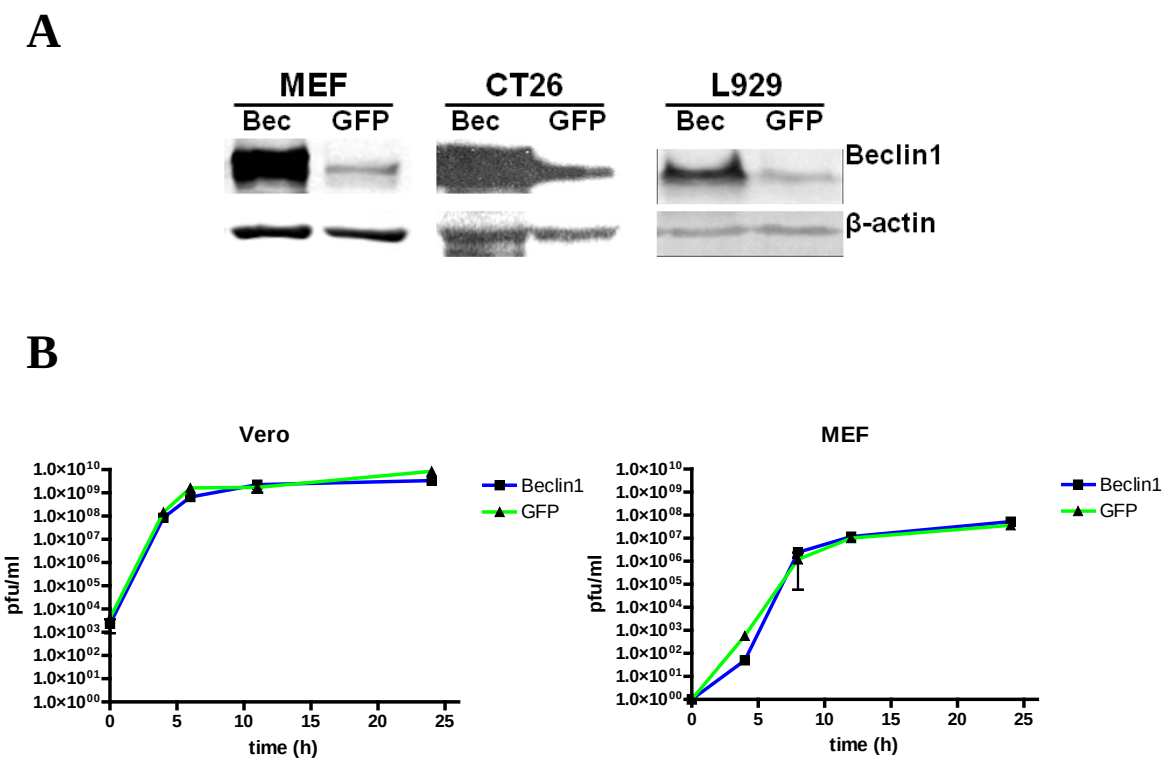


Figure 2: Beclin1 expression does not alter the in vitro replication of VSV. A) Beclin1 expression in VSV-Beclin1 infected cells. Cells were infected with MOI 5 with either VSV-Beclin1 or the parental virus for 12 hr. Western blot analysis was performed on cell lysates and membranes were probed for Beclin1. B) *In vitro* viral growth curve. MEF and Vero cells were infected with either VSV-Beclin1 or the parental virus, MOI 5.

Supernatants were collected at times indicated and viral load was determined via plaque assay.

3.1.2 Host innate type 1 IFN response following VSV infection is not altered by the expression of Beclin1.

Autophagy has a proven role in the host innate immune response to viral infection and type 1 IFN signaling (Delgado,2009). The absence of autophagy has been shown to enhance RLR signaling following viral infection leading to enhanced type 1 IFN production within infected cells (Tal, 2009). Thus it follows that an increase in autophagy could reduce RLR signaling and subsequent type 1 IFN production and potentially lead to enhanced permissiveness to viral infection in host cells. In addition, it has been demonstrated that autophagic machinery interferes with viral recognition pathways, a phenomenon which led to enhanced VSV replication host cells (Jounai, 2007). Given the importance of type 1 IFN signaling in VSV pathogenesis and host cell permissiveness as well as the complex relationship between autophagy and these innate immune pathways, we sought to explore host type 1 IFN responses following VSV-Beclin1 infection. In order to determine if Beclin1 expression altered these responses, L929 and MEFs were infected with an MOI of 5 with either VSV-Beclin1 or VSV-GFP. Supernatant was collected at 8, 12 and 28 hours after infection. Total acid-resistant bioactive type 1 IFN in supernatants was determined by use of a type 1 IFN bio-assay. L929 cells were pre-treated with cell supernatants or a dilution of type 1 IFN standard, followed by an infection with wtVSV-GFP. Fluorescence was measured 24 hours later. Lack of fluorescent signal was considered protection mediated by active type 1 IFN present in

supernatant pre-treatments. Protection was compared to a recombinant IFN standard and active type 1 IFN within samples was quantified using ImageQuant software. IFN production begins at least as early as 8 hours post infection and increases throughout the course of infection. There was a similar amount of IFN produced by the two cells lines at each time point. However, there were no significant differences in the type 1 IFN produced following VSV-Beclin1 infection compared to VSV-GFP infection in MEFs nor L929 cells (P value = 0.1409, 0.8231; two way ANOVA; Figure 3).

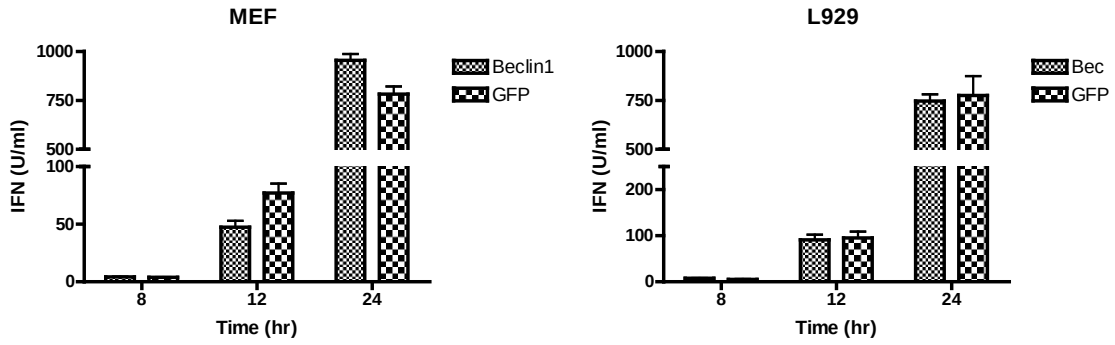


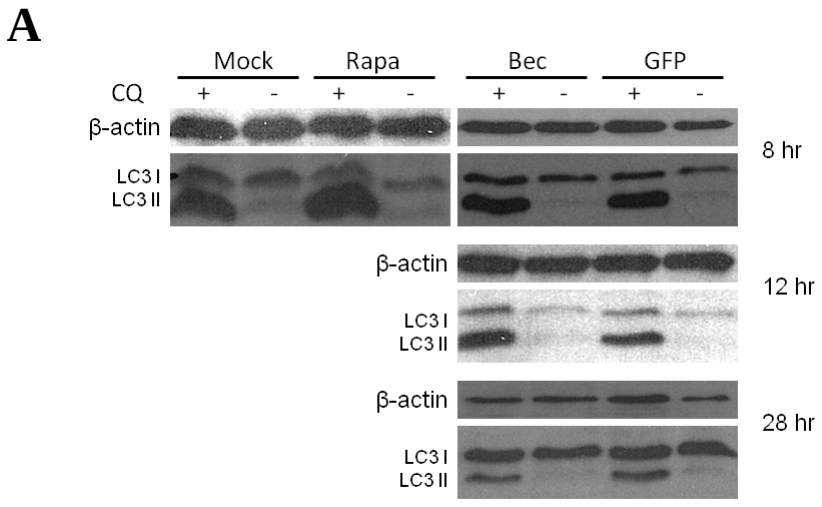
Figure 3: Beclin1 expression has no impact on IFN production in MEF and L929 cells. Cells were infected with either virus; MOI 5, and supernatants collected at 8,12,24 hours. Type-1 IFN activity of each sample was determined in duplicate via IFN bio-assay. Data is representative of 3 separate experiments. IFN production was not significantly different following infection with either virus in both MEF and L929 cells (two way ANOVA).

3.1.3 Expression of Beclin1 alters the kinetics of VSV induced autophagy in some cell lines.

Beclin1 is considered to be a key mediator in both the initiation and the progression of autophagy (Yang, 2010; Klionsky, 2011). Furthermore, several groups have demonstrated that over-expression of Beclin1 can enhance autophagy (Spencer,

2009; Liang, 1999). We hypothesized that the over-expression of Beclin1 in VSV infected cells would up-regulate this process. To test this hypothesis we examined LC3 levels in VSV-Beclin1 cells compared to those infected with VSV-GFP. LC3 is an integral part of the autophagic machinery and the most widely used marker of autophagosomes and autophagy (Klionsky, 2007b). In the early stages of autophagy LC3-I is converted to LC3-II which localizes to the autophagosomes and other autophagy related vacuoles (Chen, 2011). However, LC3-II itself becomes degraded along with the cytoplasmic contents of the autophagosome. The use of the lysosomal/vacuole protease inhibitor chloroquine (CQ) prevents the degradation of the autophagsomal contents resulting in the accumulation of LC3-II thereby enabling the visualization of this protein (Klionsky, 2007b). It is the conversion from LC3-I to LC3-II as well as LC3-II turnover that are indicative of autophagy and can be examined by assessing cellular levels of these proteins. Rapamycin, a potent inhibitor of mTOR and inducer of autophagy was used here as a positive control. Given the high expression of Beclin1 in all cell types tested it was hypothesized that this would translate into a dramatic induction of autophagy expressed as an increase in LC3-II. However this was not necessarily the case. In MEFs rapamycin treatment (200 ηM) stimulated an increase in LC3-II levels, visible in the presence of CQ, indicating an increase in autophagy. However, infection with neither VSV-Beclin1 nor VSV-GFP enhanced LC3-II levels at any time points tested, indicating that neither VSV infection nor Beclin1 expression induced autophagy in this cell line (Figure 4A). CT26 cells treated with rapamycin (100 ηM) demonstrated a dramatic induction of autophagy which was most obvious without the presence of CQ (Figure 4B) In this cell line at 12

hours post infection there were higher levels of LC3-II (-CQ) in cells infected with VSV-Beclin1 compared to mock and VSV-GFP infected cells, indicated a Beclin1 enhanced autophagy at this time point (Figure 4B). Interestingly, by 28 hours LC3-II expression was markedly reduced in VSV-Beclin1 infected cells compared to VSV-GFP infected cells which have very high expression at this time (Figure 4B). Clearly VSV infection itself enhances autophagy, however this data suggests that this induction occurs at an earlier time point in the VSV-Beclin1 infected cells. Similar to MEFs, L929 cells also required the use of CQ in order to visualize a rapamycin induced enhancement of LC3-II levels and autophagy induction. By 12 hours post infection there was a dramatic increase in LC3-II levels (-CQ) in VSV-GFP infected cells, indicating that similar to the CT26 cells VSV infection induced autophagy (Figure 4C). Likewise, there was dramatically less LC3-II present in VSV-Belcin1 infected cells at this time point compared to the VSV-GFP infected cells (Figure 4C). It is possible that similar to the CT26 cells, Beclin1 was altering the kinetics of VSV enhancement of autophagy in L929 cells as well.



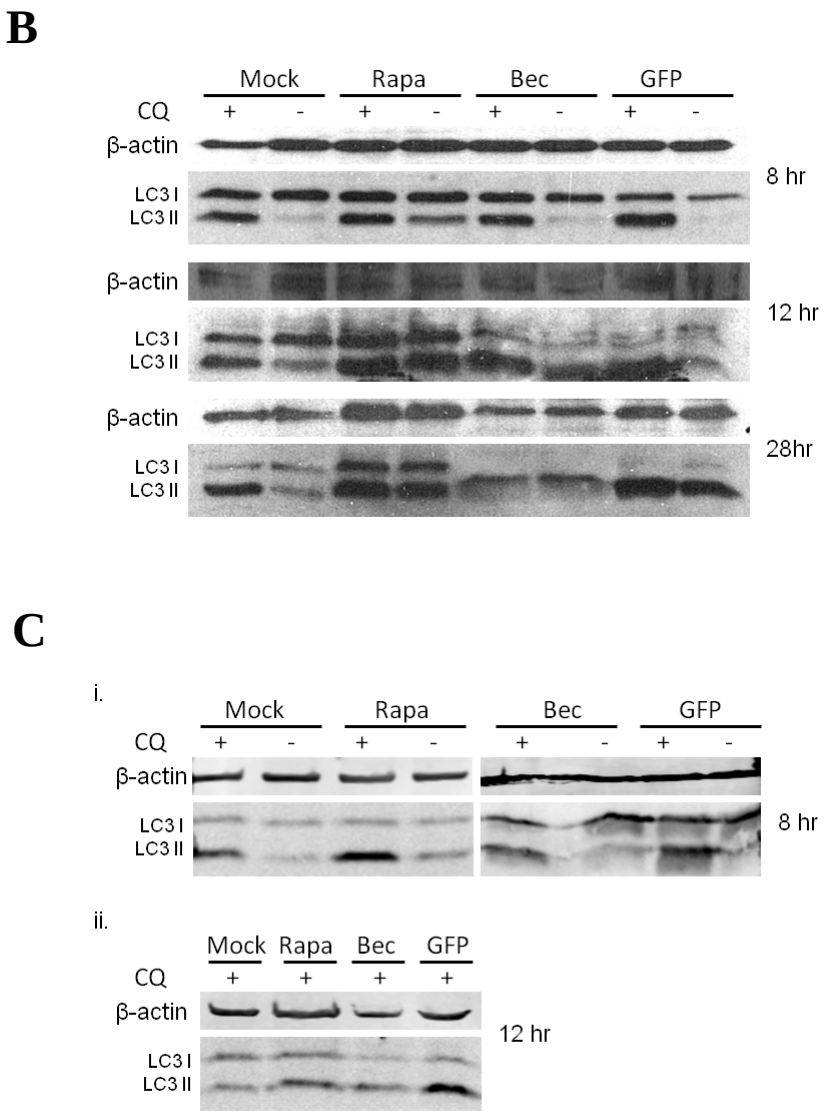


Figure 4: *Beclin1 expression alters the kinetics of VSV induced autophagy in some cell types.* Cells A) MEF, B) CT26 and C) L929 were treated with rapamycin (200ηM for MEF, 100ηM for L929 and CT26) for 28hrs; mock treated or infected with either VSV-Beclin1 or VSV-GFP;MOI 5, for lengths of time indicated. 4 hours prior to cell lysis cells were treated with 50μM chloroquine (CQ). Western blot analysis was performed and membranes were probed for LC3.

3.2 Pathogenesis of VSV-Beclin1

3.2.1 Expression of Beclin1 enhances a caspase independent cell death in some cells lines tested.

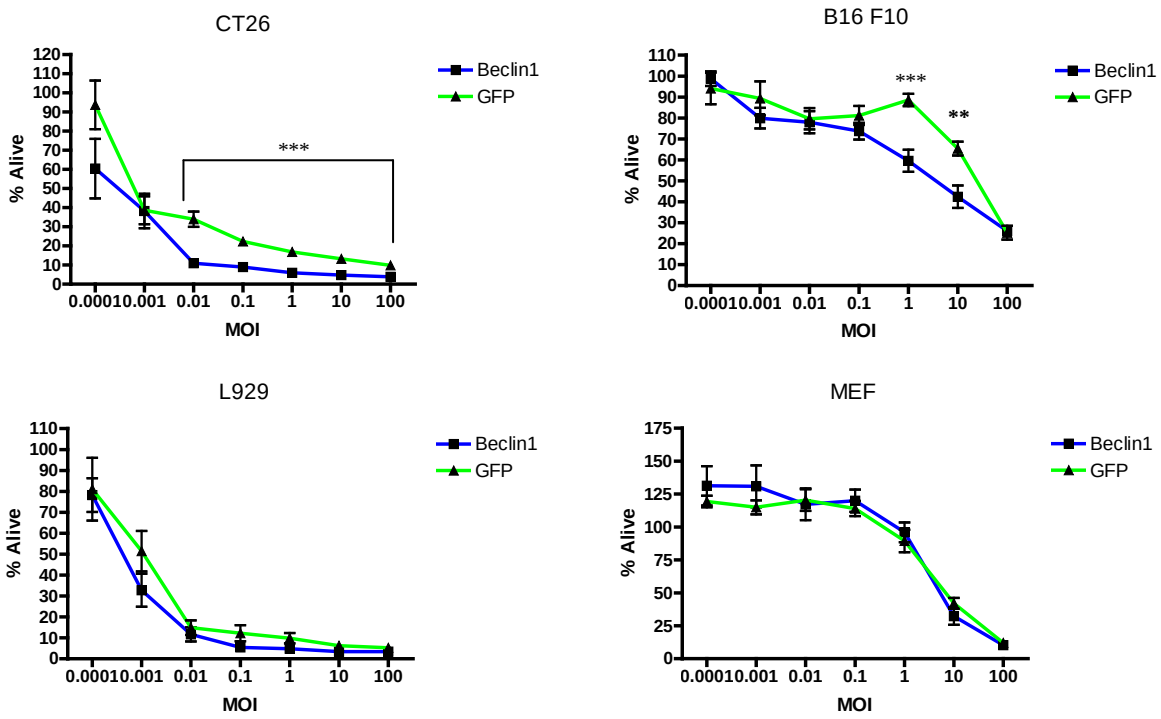
Under certain circumstances autophagy can cause cell death that is independent from apoptosis and caspase activation (Scarlatti, 2009; Eisenburg, 2009). In addition, Beclin1 is a known binding partner of the key apoptosis regulator Bcl-2 (Liang, 1998) and is involved in the regulation of apoptosis (Kang, 2011). Indeed, these two cell death pathways are closely linked, sharing many common regulators and overlapping signaling pathways. Because of the complex and not well understood relationship between Beclin1/autophagy with cell death and apoptosis we investigated the impact of Beclin1 expression on the cell death induced by VSV infection. To that end, CT26, MEF, L929 and B16F10 cells were infected with either VSV-Beclin1 or VSV-GFP with a range of MOIs from 0.0001 to 100. 48 hours after infection an MTT assay was used to determine and compare the extent of cell death induced by the two viruses. An MTT assay measures the reduction of MTT via mitochondrial enzyme succinate dehydrogenase and is a widely used method of monitoring both metabolic activity and cellular viability. Neither MEFs nor L929 cells show any differences in the extent of cell death induced by the two viruses (P value = 0.4268 and 0.0683, two way ANOVA; Figure 5A). However, the extent of cell death in both CT26 and B16F10 cells was significantly enhanced by Beclin1 expression (P value = 0.0001 and 0.0007, two way ANOVA; Figure 5A). Infection of CT26 cells with an MOI of 0.01 resulted in 1/3 as many viable cells after 48hours with VSV-Beclin1 compared to VSV-GFP; 11.0 and 33.9 % alive respectively (P value $\leq$ 0.0001, students t

test; Figure 5A). VSV-Beclin1 enhanced cell death in CT26 cells following infections with MOIs of 0.1, 1, 10 and 100 as well (P value ≤ 0.0001, student's t test). This trend was also seen in B16F10 cells which displayed significantly more cell death following infections with MOI of 1 and 10 compared to VSV-GFP (P value ≤ 0.001, 0.01, student's t test respectively, Figure 5A).

Beclin1 is a BH3-only protein, and thus belongs to a subfamily of BH protein known for their pro-apoptotic function (Kang, 2011; Oberstein, 2007) On the other hand autophagy is also a type of programmed cell death. Therefore, there is evidence to suggest that the enhanced cell death observed following VSV-Beciln1 infection could be due to a Beclin1 mediated induction of apoptosis or possibly due to an increase in autophagic cell death. In order to get some indication of what type of cell death is being enhanced we evaluated apoptosis induction following infections with both viruses. Caspase activation is central to both intrinsic and extrinsic apoptosis pathways and is a commonly used marker for either pathway (Kolher, 2002; Ola, 2011). We evaluated caspase 3 and 7 activation as a marker of apoptosis in MEF and CT26 cells. Each cell line was infected with either VSV-Beclin1 or VSV-GFP with a series of MOI, ranging from 0.001 to 10 for 48 hours, at which point caspase 3 and 7 activation was assessed using CaspaseGlo 3/7 kit (Promega). The caspase activation induced by each virus across this range of MOI was very similar in both viruses in MEFs (P value = 0.7159, two way ANOVA; Figure 5B). At 48 hours post infection caspase activity peaks with an MOI of 0.1 and decreases with higher MOIs of 1 and 10 where it is almost at uninfected levels (Figure 5B). In CT26 cells the pattern of caspase activation at 48 hours across this range of MO1 appeared to be

slightly lower in VSV-Belcin1 infected cells compared to VSV-GFP, a trend that was close to significance (P value = 0.0563, two way ANOVA; Figure 5B). The pattern observed in CT26 cells was highly dissimilar to that of MEFs. In these cells, caspase activation decreases with higher MOIs following infection with either virus. This is likely because the cells which received the higher MOIs were already beyond peak caspase activity at 48 hours post infection. At any rate, the enhanced cell death observed following VSV infection in CT26 cells was not paralleled with a corresponding increase in caspase activation.

A



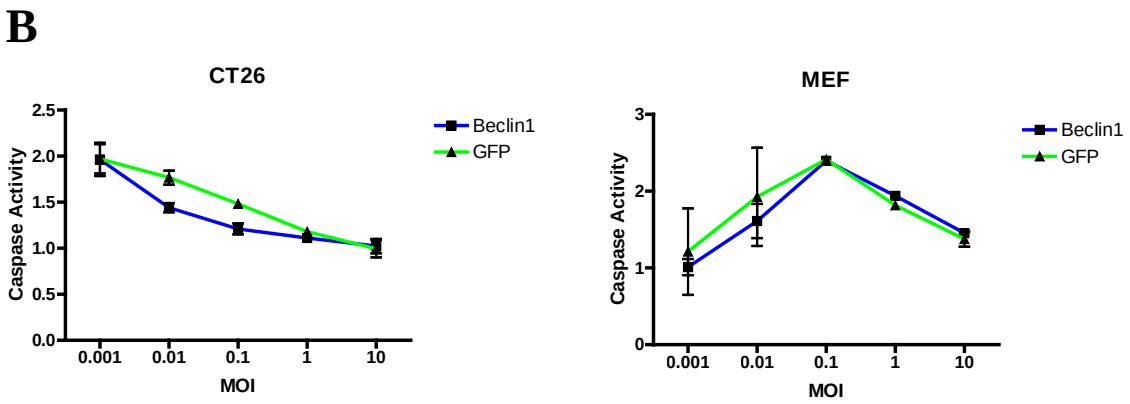


Figure 5: Beclin1 expression enhances a caspase independent cell death in some cell lines infected with VSV in vitro. A) CT26, MEF, L929, and B16 F10 cells were infected with either VSV-GFP or VSV-Beclin1 with a range of MOI in triplicate. Cell death was detected with MTT labeling, and percent alive relative to mock infection was calculated. Unpaired t-tests were performed for each MOI; ** = P value < 0.01, *** = P value < 0.001. This data is from two independent experiments. B) MEF and CT26 cells were infected with either VSV-Beclin1 or VSV-GFP at a range of MOI in duplicate. Caspase 3/7 activity was detected and fold change relative to mock infection calculated. Unpaired student t-tests were performed for each MOI, viruses were not significantly different from each other.

3.2.2 In vivo pathogenesis and replication of VSV was not altered by Beclin1 expression

As described above, Beclin1 expression enhanced the *in vitro* pathogenesis of VSV in some cell lines. In order to determine if this phenomenon transpired into the *in vivo* setting we evaluated the pathogenesis of VSV-Beclin1 in female Balb-c mice. A high volume IN infection results in lung pathology in mice infected with VSV. Mice typically loose 10 -15% of their body weight, a measure which is analogous to viral pathology (Brown, 2009). Mice were infected IN with 5 x 10^8 pfu of either virus and weights were monitored for 10 days. Most of the weight loss occurred within the first 24 hours in both groups of mice, with both groups reaching minimum weights at around 72 hours. Mice infected with VSV-Beclin1 or VSV-GFP lost an average of 11.2% and 14.2% of their

body weight respectively (Figure 6A). These results were not significantly different (P value = 0.3598, two way ANOVA).

Viral replication can also be indicative of viral pathogenesis (Brown, 2009). As a second measure of *in vivo* pathogenesis we evaluated the viral load in murine lungs infected with either virus IN. Mice were infected with 5×10^8 of either VSV-Beclin1 or VSV-GFP in 30 μl of saline. Mice were sacrificed and their left lungs removed at 48, 72 and 96 hours post infection. A viral plaque assay was used to compare the viral load of lungs infected with each virus at these time points. Analysis of these results indicates that the viral load in lungs infected with either virus were not statistically different from each other at all time points tested, 48, 72 and 96 hours (p value = 0.0608,0.6316, and 0.1489, student's test, Figure 6B). In addition the overall viral load pattern demonstrated across these time points was also similar between the two viruses (p value = 0.0618, two way ANOVA, Figure 6B). The viral load of VSV-Beclin1 or VSV-GFP infected mice was also examined in the brains and olfactory bulbs following IN infections, as well as in the lungs and spleens following IV infections; results were similar (Data not shown).

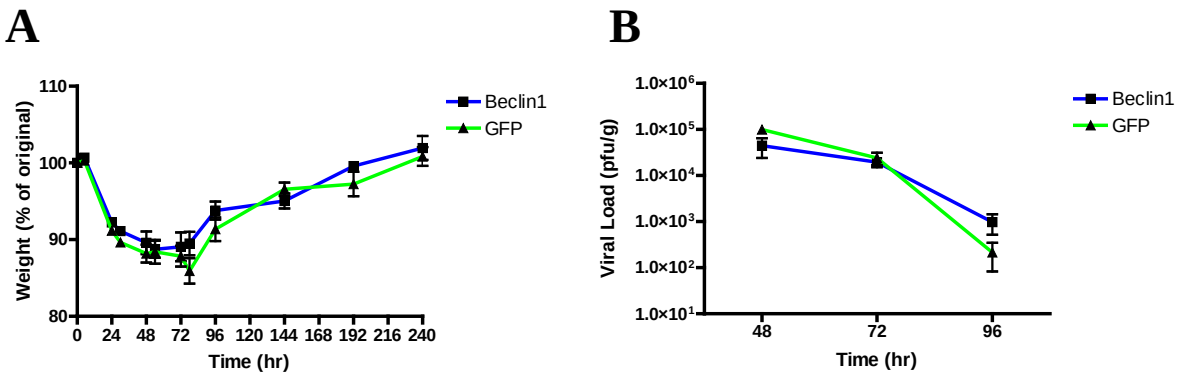


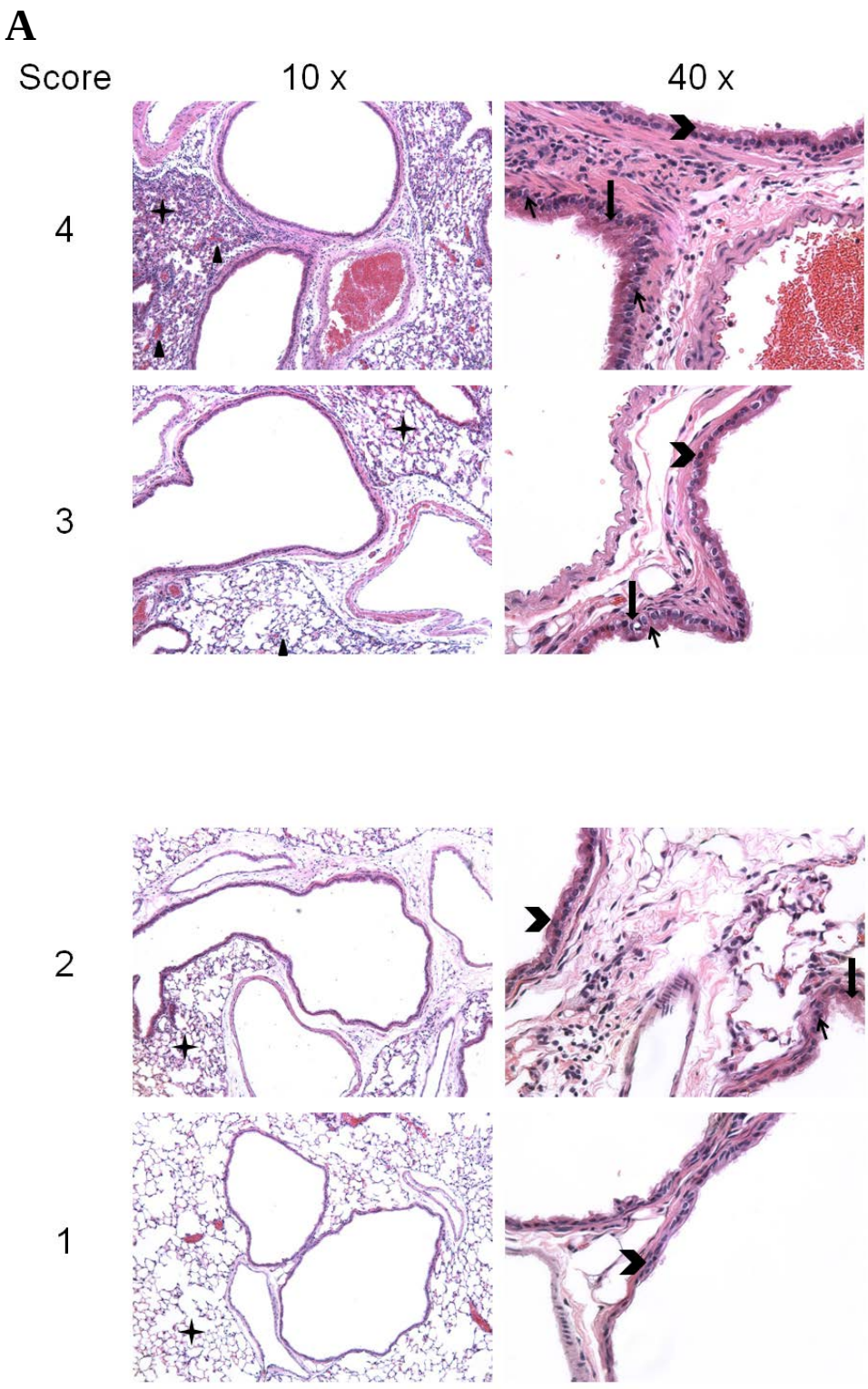
Figure 6: In vivo pathogenesis of VSV-Beclin1 is similar to parental virus. Balb-c mice were infected via intranasal administration with 5 x 10^8 pfu/ml in 30 µL of saline of either VSV-Beclin1 or VSV-GFP. A: Weights were monitored, n=5. Results were not significantly different (*P* value = 0.3598, two way ANOVA). (B) The right lungs were removed at 48 (n=3), 72(n=5), and 96 hours (n=5). Lungs were homogenized and viral load was determined via plaque assay. At no time point was there a significant difference (*P* value = 0.0608,0.6316, and 0.1489, student's test).

3.2.3 Beclin1 expression did not alter the tissue damage nor the extent of apoptosis in VSV infected lungs of Balb-c mice.

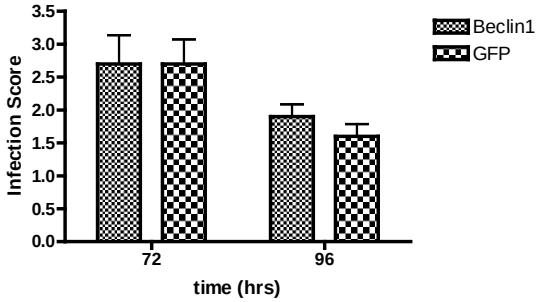
Given the Beclin1 induced augmentation of cell death *in vitro* we wanted to further examine the *in vivo* pathogenesis of VSV-Beclin1 and more specifically the cell death taking place in infected tissues. Lungs infected as above were inflated and fixed in para-formaldehyde. In order to examine the tissue damage and immune cell infiltration sections were stained with H&E stains. Each section was given a score from 1-4 corresponding to its relative signs of infection by a blinded observer. This score was based on pathological signs indicative of infection induced inflammation and tissue damage. These signs include, airway thickening and immune cell infiltration, extent of hemorrhaging, loss of epithelial cilia and large airway epithelial damage and repair. Images representing each score are presented in Figure 7A. For details of signs of

infection refer to the figure legend. The scores from each group were tabulated and statistical analysis performed. At 72 hours post infection lungs infected with VSV-Beclin1 and VSV-GFP had average infection scores of 2.9 and 2.7 respectively. By 96 hours the average infection scores had reduced to 1.9 and 1.6 respectively, indicating mice are recovering at this time point (Figure 7B). This correlates with the weight loss data which demonstrated weight gain in between 72 and 96 hours indicative of recovery at this point in the infection (Figure 6A). Still, infections with both viruses resulted in similar tissue damage and immune cell infiltration at each time point tested, 72 and 96 hours (P value = 0.7860 and 0.2897; students t-test, Figure 7B).

We also examined the extent of apoptosis in VSV-Beclin1 infected murine lungs. Balb-c mice were infected intranasally with 5×10^8 pfu of either VSV-Belcin1 or the control virus. At 72 and 96 hours post infection lungs were removed, inflated and fixed with para-formaldehyde. Fixed lungs were paraffin embedded, sectioned and tunel stained for DNA fragments digested at caspase cleavage sites; a marker of apoptosis. Images presented in Figure 7C are representative of each group. Evaluation of positive staining demonstrates that at 72 hours post infection there is wide spread apoptosis that is concentrated around the airways (Figure 7C). By 96 hours there are fewer apoptotic cells in both groups, indicating that the infection is being cleared. However, there were no apparent differences in the extent of apoptotic cells between lungs infected with either VSV-Belcin1 or VSV-GFP at either time point (Figure 7C).

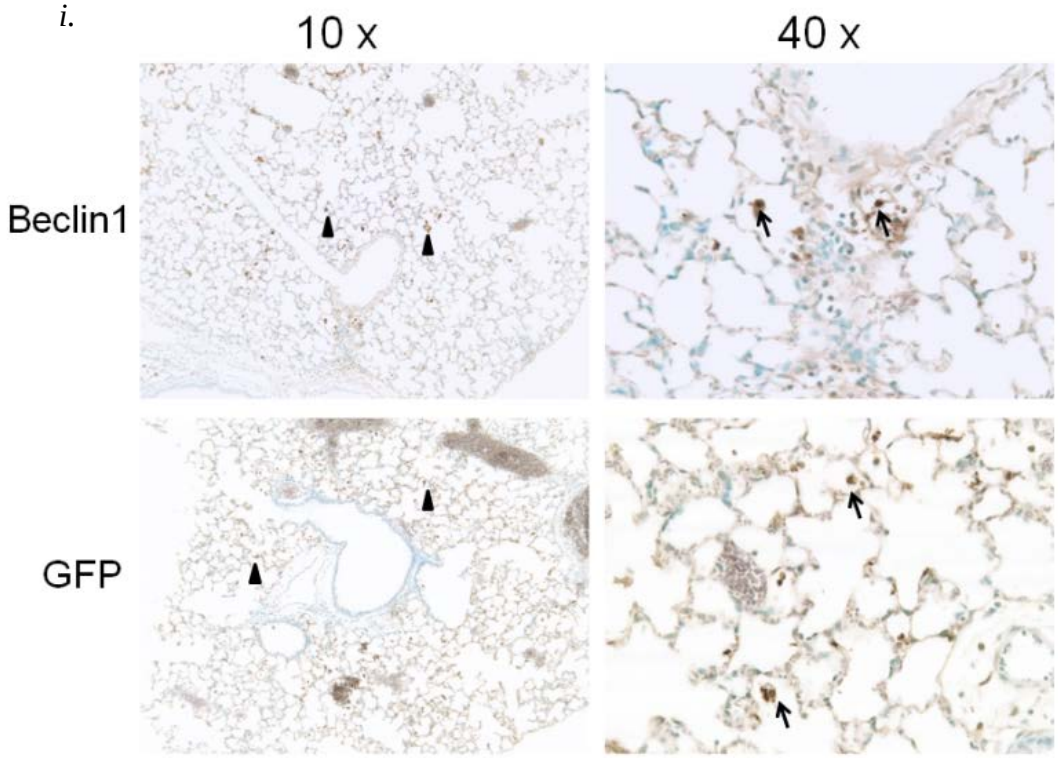

A
Score
10 x
40 x
4
3
2
1

B


Beclin1
GFP
Infection Score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
72
96
time (hrs)

C

i.


10 x
40 x
Beclin1
GFP

ii.

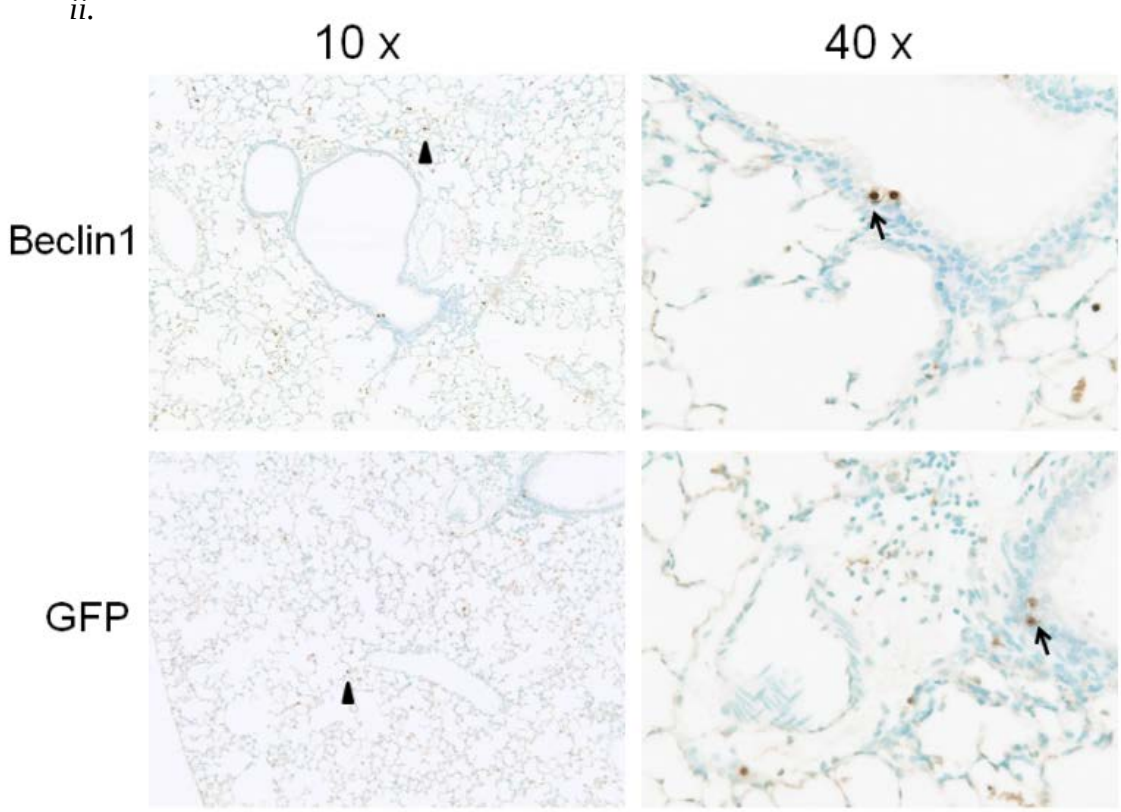


Figure 7: *Beclin1 expression does not alter the tissue damage nor the extent of apoptosis in the VSV infected lungs of Balb-c mice.* Balb-c mice were infected with 5 x 10^8 of either VSV-Beclin1 or VSV-GFP. At 72 and 96 hours post infection mice were sacrificed and left lungs were inflated and formalin fixed. A-B) Paraffin embedded sections were stained with H & E stains. Each slide received an infection score by a blinded observer. A) Representative images of each score are shown; details of each score are as follows:

4- indicates dense parenchyma with very little open space indicative of severe immune cell infiltration (4-point star); wide spread hemorrhaging (triangles); extreme thickening of airway epithelial layer (chevron); large areas of epithelial sloughing (large arrow) and many multi nuclear epithelial cells (small arrows) indicative of epithelial damage and repair.

3- indicates less dense parenchyma with more open space (4-point star); some hemorrhaging (triangles); thick airway epithelial layer (chevron); moderate epithelial sloughing (large arrow) and some multi nuclear cells (small arrow).

2- Parenchyma is mostly clear of immune cell infiltration (4-point star); some airway epithelial layer thickening (chevron); small areas of epithelial sloughing (large arrow); few multi nuclear cells (small arrow) and very limited hemorrhaging.

1- Little/no immune cell infiltration in parenchyma, alveolar structure clearly visible (4-point star); thin airway epithelial layer (chevron); no hemorrhaging, very few/no multi nuclear cells, very limited epithelial sloughing.
The average infection scores from both viruses were similar at 72 and 96 hours (*P* value = 0.7860 and 0.2897; students t-test).
B) Cumulative scores from each group (n=5), there was no statistically significant differences between virus groups. C) Paraffin embedded sections were tunel stained to detect apoptotic cells. Images of 10x and 40x magnification are representative of each group; n=5. Triangles indicate large areas of tunel positive cells. Higher magnification reveals the dark nuclear staining indicative of tunel positive cells (small arrows).

3.3 Evaluation of the oncolytic potential of VSV-Beclin1 compared to VSV-GFP

3.3.1 Oncolytic efficacy of VSV-Beclin1 was not different from the parental virus

While there were no observed differences in the *in vivo* pathogenesis of VSV-Beclin1 compared to the GFP expressing counterpart it is clear that the enhanced cell death observed in the *in vitro* pathogenesis experiments was cell type dependent. Indeed the most significant differences in the cell death caused by VSV infection with the two viruses was observed in CT26 cells. Therefore we hypothesized that the Beclin1 expression dependent enhanced cell death observed *in vitro* would enhance the oncolytic efficacy of VSV *in vivo* as well. To test this hypothesis, Balb-c mice were engrafted with 5×10^5 CT26 cells. Once tumours reached an average of 100 mm^3 mice received either IV (5×10^8pfu) or IT (1×10^7pfu) injections of VSV-Beclin1, or VSV-GFP, or IT injections of saline on days 0 and 2. Tumour volumes and survival were monitored. All treatments; IT VSV-Beclin1, IT VSV-GFP, IV VSV-Beclin1, and IV VSV-GFP, slowed tumour growth which led to significantly prolonged survival compared to saline treated mice (p value: 0.0377, 0.0150, 0.0067, 0.0067; log rank test; Figure 8A). However, there were no significant differences in the control of tumour growth between VSV-Beclin1 and VSV-

GFP with either route of injection, IT or IV (p value = 0.8118, 0.8979 ANOVA, Figure 8B). This was reflected in the survival outcomes which also demonstrated no significant differences between the two viruses with either injection route, IT or IV (p value: 0.9659, 0.6124; log rank test; Figure 8A). Interestingly the route of administration had a significant effect on mouse survival. The use of IV injections was significantly more effective at controlling tumour growth and prolonging survival compared to the IT injections (p value: 0.0029; log rank test).

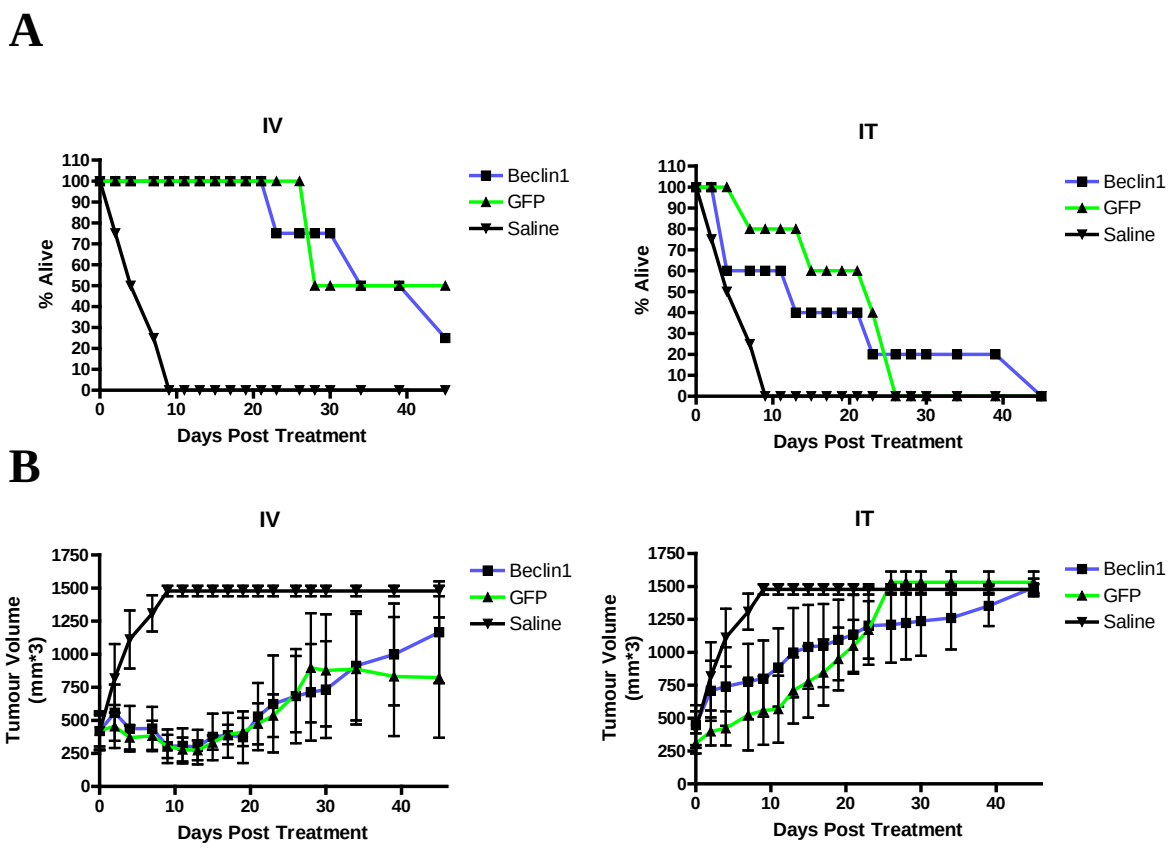


Figure 8: Oncolysis of VSV-Beclin1 in CT26 tumour cells compared to VSV-GFP. Balb-c mice were engrafted with 5 x 10^5 CT26 cells. Once tumours reached an average volume of 100 mm^3 treatment began (day 0). Mice received 2 doses of either IV administration of 5 x 10^8 pfu/200 µl of either VSV-Beclin1 or VSV-GFP, IT administration of 5 x 10^7

pfu/50 µl of either virus or 200µl of saline on day 0 and day 2. Tumour volumes A) IV and B) IT and mice survival C) IV and D) IT was recorded. Neither the tumour volumes (p value = 0.8118 (IT), 0.8979 (IV) ANOVA) nor mice survival (p value: 0.9659 (IT), 0.6124 (IV); log rank test) with either administration of VSV-Beclin1 were significantly different from those of the VSV-GFP treated mice.

Chapter 4: Discussion

4.1 Characterization of VSV-Beclin1

Prior to this study there had been no characterization of VSV expressing Beclin1. As a first step in the preliminary characterization of VSV-Beclin1, this study confirmed strong Beclin1 expression in infected cells (Figure 2A). In addition the normal replication of this virus was confirmed in both incomplete and intact type I IFN systems (Figure 2B).

A primary aim of this study was to determine the impact of Beclin1 expression on the induction of autophagy within the context of VSV infection. It was hypothesized that Beclin1 over-expression would stimulate an enhanced state of autophagy. Surprisingly, this was not necessarily the case. In MEFs, despite strong Beclin1 expression there was no induction of autophagy compared to controls. In these cells infection with neither of the two viruses had any impact on LC3-II levels within infected cells compared to mock infected controls, up until 28 hours post infection, the last time point examined (Figure 4A). In CT26 cells the induction of autophagy following rapamycin treatment is most obvious in CQ negative cells which exhibit a clear increase in LC3-II. In these cells, similar to MEFs, there are no changes in LC3-II levels at 8 hours post infection with either virus. However, by 12 hours VSV-Beclin1 infected cells displayed enhanced LC3-II levels (CQ –ve) compared to both mock and VSV-GFP infections indicating enhanced autophagy at this time point (Figure 4B). Interestingly, by 28 hours there was dramatically less LC3-II in VSV-Beclin1 infected cells (Figure 4B). One plausible explanation for these observations is that due to a period of relatively intense autophagy

detected at 12 hours, by 28 hours post infection cellular stores of LC3 were spent in the VSV-Beclin1 infected cells. Because at this point in the infection host machinery would have been overwhelmed by viral protein production the ability of the host cell to replace LC3 was been limited. This is verified by low levels of LC3-I in all infected cells compared to both mock and rapamycin treated cells at 28 hours. In addition to its role in autophagy initiation, when bound in a complex with Rubicon Beclin1 is also involved in the negative regulation of autophagy (Yang, 2010). Another possible explanation for the sharp decrease in LC3-II levels at 28 hours is that over-expression of Beclin1could have led to autophagy inhibition. It may be that initially autophagy was enhanced (12 hours) but once cellular Beclin1 reached a critical level its function shifted to inhibition. Beclin1 plays a critical role in autophagy at many stages of the process, including phagophore elongation and autophagosome maturation (Figure 1). It is possible that strong Beclin1 expression merely sped up the process rather than actually initiating an increase in total autophagy. Still, regardless of the exact mechanism, it is clear that by 28 hours there was significantly less LC3-II in VSV-Beclin1 infected CT26 cells compared to those infected with VSV-GFP, even in the presence of CQ which prevented the autophagic degradation of LC3-II. This indicates there was a significant reduction of autophagy at this time. This was also observed in L929 cells at 12 hours (Figure 4C). This suggests that similar to the CT26 cells there may have been an enhancement of autophagy in the VSV-Beclin1 infected cells prior to 12 hours in L929 cells. However the transient nature of this induction requires the use of more time points to fully examine this phenomenon. Why this pattern was not observed in MEFs is not clear though it is likely due in part to their

relative resistance of to VSV infection. It is possible that at a time point later than 28 hours this trend would transpire.

Interestingly, VSV-GFP did induce an increase in LC3-II levels compared to mock infected cells in both L929 (12 hours; Figure 4C) and CT26 cells (28 hours; Figure 4B), indicating a VSV induced initiation of autophagy in these cell lines. Autophagy is an innate defense mechanism which protects against intracellular pathogens and is induced in response to pathogen associated molecular pattern receptors; toll like receptor (TLR) stimulation (Delgado, 2007). In addition, VSV has been demonstrated to induce autophagy despite cellular recognition through the RLR system, suggesting that this signaling pathway is also capable of inducing autophagy. Though the exact mechanism for this is unknown (Jounai, 2009). On the other hand, autophagy may also act to block IFN signaling by degrading mitochondria and the associated protein MAVS; a key adaptor in RLR/IFN signaling. Furthermore, Jounai et al. demonstrated that the autophagic Atg5-Atg-12 conjugate can block the IFN pathway following VSV infection an event which was considered to be a viral mediated immune evasion tactic. Still, given the evolutionarily conserved role of autophagy as an innate host defense, likely the induction of autophagy described in this study was host cell mediated. Though whether or not VSV is capable of blocking or utilizing this process to its advantage remains to be determined.

While this study demonstrated a Beclin1 dependent shift in the kinetics of VSV induced autophagy the exact nature of this shift are not entirely understood. The fact that there is some ambiguity regarding the exact effect of Beclin1 over-expression on the

induction and rate of autophagy speaks to the limitations of the assay used. Western blot analysis of LC3 levels is useful in examining the conversion of LC3-I to LC3-II, which occurs early in the process. In addition, comparing the accumulated levels of LC3-II in the presence of CQ to LC3-II cellular levels in the absence of CQ aids in the visualization of LC3-II turnover, indicative of autophagy completion. However, this assay says little about the steps in between LC3-I conversion and lysosomal degradation. Moreover, the nature of this assay as well as the fact that LC3-II continues to be produced in this process makes it difficult to determine a quantitative level of autophagy. As mitochondria are degraded through the process of autophagy, detecting cellular levels of this organelle is one method of monitoring autophagy (Klionsky, 2007b). However, the process of mitophagy, or autophagic degradation of mitochondria is thought to be a selective process with regulation systems distinct from that of macroautophagy (Kim, 2007). While some triggers of autophagy, such as starvation, also result in the rapid induction of mitophagy this is not true for all autophagy inducers. In this study an attempt was made to detect cellular mitochondria levels via western blot analysis of mitochondria associated protein Hsp70, however no detectable differences in mitochondrial levels were elicited between any of the treatments including rapamycin treated controls (data not shown). This suggests that the regulators of rapamycin and VSV induced autophagy are separate from those of mitophagy. Another useful method in the study of autophagy is a long-lived protein degradation assay. Autophagy is largely a non-specifc cytoplasmic degradation pathway, as such all proteins undergo autophagic degradation at the same rate. Still, proteins with long half lives tend not to be degraded through other degradation pathways

such as proteasomal degradation (Klionski, 2007b). Therefore the turnover of these long lived proteins can be a reasonable indicator of the amount of autophagy taking place within a cell over a given period of time. A pulse/chase technique using a radio labeled amino acid is one way of monitoring the turnover of long lived proteins (Klionsky, 2007b). However, this is fairly complicated and requires a large amount of optimization for each step including length of incubation (pulse) and chase periods.

Since the beginning of this study more information has come to light regarding the regulation of autophagy. While it is clear that Beclin1 is a key regulator of autophagy it does not appear to be the initiator it was once thought to be. The autophagy regulating function of Beclin1 requires the activity of hVps34 (Yang, 2010; Fimia, 2011). Furthermore, activation of the Beclin1/hVps34 complex requires activated ULK/Atg13 complex. This new information that has come to light in the past year regarding the initiation of autophagy suggests there may be other protiens that are more likely to significantly and sustainably enhance autophagy when over expressed. As most cellular triggers of autophagy signal through the mTOR system, one of these regulators of mTOR would be a likely candidate. In addition Irgm1 in murine cells and IRG1 in human cells have been demonstrated to be strong inducers of autophagy though the mechanisms for this are unknown (Delgado, 2007; Singh, 2006). Still, because VSV itself induces autophagy it is unclear whether additional measures would be capable of enhancing this process significantly.

The status of cellular IFN signaling is an important determinant of permissiveness to VSV infection (Barber, 2005). MAVS, located on the mitochondria is a key adapter in

RLR mediated recognition of ssRNA viruses and the ensuing downstream signaling leading to the anti-viral state (Seth, 2004). Therefore it was hypothesized that induction of autophagy would result in mitochondrial/MAVS digestion, thereby blocking this pathway and augmenting viral pathogenesis. Consequently, this study sought to determine if host IFN production is altered by the expression of Beclin1 in the context of VSV infection. A type 1 IFN bio-assay was used to assess the IFN response to VSV-Beclin1 infection in MEF and L929 cells compared to the control virus. It was determined that Beclin1 expression had no impact on IFN production of VSV infected MEF or L929 cells (Figure 3). This was likely because as described above mitophagy, while once thought to be synonymous to autophagy is actually a separate, albeit very closely related system (Kim, 2007). Neither VSV induced nor Beclin1 enhanced autophagy appear to include mitophagy, as determined by western blot analysis of hsp70 (data not shown). Therefore it is unlikely that autophagy would have an impact on cellular levels of MAVS and IFN signaling in this setting.

4.2 Pathogenesis of VSV-Beclin1

This study also sought to characterize the pathogenesis of VSV-Beclin1. To that end, cell death induced by infection with either VSV-Beclin1 or VSV-GFP was assessed via an MTT assay. It was hypothesized that Beclin1 expression would enhance VSV pathogenesis by blocking IFN signaling and increasing cellular permissiveness to VSV.

However this study demonstrated that there were no differences in the IFN produced by cells infected VSV-Beclin1 compared to the control virus (Figure 3). Still, MTT assays established a significant difference in the cell death induced by these two viruses in CT26 and B16 F10 cells, though no differences were exhibited in MEF and L929 cells (Figure 5A). Autophagy, also known as PCD-II, is capable of causing an apoptosis independent cell death. Furthermore, Beclin1 is a BH-3 domain only protein which belongs to a subfamily of proteins with pro-apoptosis functions (Kang, 2011). Indeed, Beclin1is a well established binding partner of Bcl-2 a key inhibitor of apoptosis (Liang, 1998). While it is evident that Bcl-2 also inhibits autophagy via interactions with Beclin1, it is not clear whether this relationship is reciprocal (Vazques, 2010). In order to obtain further insight into the type of cell death enhanced by Beclin1 expression, caspase activation was assessed in cells infected with either VSV-Beclin1 or VSV-GFP. However, it was determined that there were no significant differences in the level of caspase activation in either CT26 or MEFs when infected with either virus. There are two primary apoptosis pathways, extrinsic and intrinsic. While the triggers for each of these pathways differ, both result in the activation of executioner caspases 3 and/or 7 (Ola, 2011) The assay used in this study measures activation of caspases 3 and 7. These results suggest that neither the intrinsic nor extrinsic apoptosis pathways accounted for enhanced cell death in VSV-Beclin1 infected cells. Although less common, caspase independent apoptosis pathways do exist, therefore this assay does not rule out apoptosis altogether. Another possibility is that the enhanced autophagy demonstrated in CT26 cells, albeit transient could have resulted in PCD type II in some cells, enhancing the total cell death in the population.

It is also possible that a different time point would have yielded slightly different results. In MEFs caspase activation was low at lower MOIs but increased and peaked at an MOI of 0.1, finally decreased with higher MOIs after this point (Figure 5B). Similarly, CT26 caspase activation decreased with higher MOIs (Figure 5B). A comparison of the caspase activation and MTT results explains this apparent contradiction. Infections with MOIs on the downward slope of caspase activation; 1 and 10 for MEFs, 0.001 to 10 for CT26 cells; resulted in significant cell death by 48 hours. Likely, caspase activation was low at these MOIs because cells were already dead at this point. Therefore, there may have been higher caspase activation in CT26 cells at either an earlier time point or a lower MOI than examined in this study. It is possible that there would have been differences in peak caspase activation between VSV-Beclin1 and VSV-GFP that were not elicited on the declining slope of caspase activation.

Regardless of the type of cell death induced, it is evident that Beclin1 dependent enhanced cell death was cell type specific as it was observed in CT26 and B16 F10 cells but not in MEF or L929s (Figure 5A). Still, the physiological reason for this specificity remains unclear. Both CT26 and B16 F10 cells are transformed cell lines, but L929 cells are as well. CT26 and L929 cells are similarly permissive to VSV infection, while B16 F10 and MEFs are similarly resistant to infection (Figure 5A). Interestingly the cell line which exhibited the most dramatic differences in pathogenesis between the two viruses, CT26 cells, also displayed the most evident enhancement of LC3-II levels induced by Beclin1 expression. Based on this correlation it is tempting to believe that it is in fact an

autophagic cell death that was responsible for the increase in the cell death induced by this virus. Still, further study of this trend will be required to elicit the true cause.

Given the differences in viral pathogenesis demonstrated in some cell lines *in vitro* this study also examined the pathogenesis of VSV-Belcin1 in an *in vivo* model. Weight loss was monitored in IN (lung) infected mice. Mice which receive an intranasal infection demonstrate lung pathology in the form of immune cell infiltration and tissue damage which correlates with viral replication within the lungs. As a result these mice loose a significant proportion of their body weight. If VSV-Beclin1did in fact produce a significantly greater extent of cell death i*n vivo*, this would likely have been reflected in greater weight loss. However this was not the case, in fact VSV-Beclin1 mice lost less weight in average than VSV-GFP infected mice (11.2 vs .14.2 % respectively), though these results were not statistically significant (Figure 6A). In addition, the viral load within infected lungs at 48, 72, and 96 hours was examined as a second measure of *in vivo* pathogenecity, however no differences were observed between the two viruses (Figure 6B)

Because of the differences observed in the cell death induced by infections with VSV-Beclin1 and VSV-GFP *in vitro*, tissue staining was utilized to examine the cell death induced by these viruses in the lungs of IN infected mice. H&E staining of infected tissues can give a good indication of tissue damage, visible via airway epithelial shedding and repair, loss of cilia and associated inflammation which manifests as immune cell infiltration. Infection scores based on these factors demonstrated that lung infection with either virus produced similar levels of tissue damage and immune cell infiltration in the

lungs (Figure 7A). Tunel staining, to assess apoptosis induced in infected lungs also demonstrated similar levels of this cell death pathway between the two viruses (Figure 7B).

Collectively these experiments demonstrate convincingly that VSV-Beclin1 displayed similar *in vivo* pathogenesis to the control virus in the tissues tested. This apparent discrepancy between the *in vitro* and *in vivo* results is explained by the fact that the enhanced in vitro pathogenesis exhibited in VSV-Beclin1 infected cells was evidently cell type specific (Figure 5A). Indeed MEFs demonstrate no differences in the cell death induced by these two viruses. The reason for this specificity are not known but likely the factors required for Beclin1 dependent enhancement of cell death are not present in the cells of the lung.

4.3 Oncolysis of VSV-Beclin1

VSV-Beclin1 demonstrated enhanced cell death in some cell lines *in vitro*, particularly in CT26 cells. The reasons for this discrimination are not clear as there were no obvious similarities between those that do and those that do not exhibit this phenomenon. While none of the assays used to test the *in vivo* pathogenesis of this virus demonstrated any differences between the two viruses this is likely due to at least in part to the cell specific nature of the enhanced pathogenesis of VSV-Beclin1. As the greatest differences between the two viruses in the cell death induced was observed in CT26 cells it is conceivable that this would transpire into the *in vivo* setting in these cells. This study sought to determine if VSV-Beclin1 would induce significantly more cell death in a CT26

tumour model *in vivo*. However, VSV-Beclin1 did not prove to be any more effective in controlling tumour volumes, nor prolonging survival than the control virus VSV-GFP (Figure 8). Given that this study demonstrated up to 60% more cell death induced by VSV-Beclin1 in CT26 cells *in vitro* it is curious why this virus did not exhibit a better ability to control tumour volume *in vivo*. However, closer examination of this system may explain this contradiction. As mentioned above, the oncolytic efficacy of many OV, VSV in particular, is largely due to the bystander effects of viral infection such as vascular disruption and anti tumour immune responses (Diaz, 2007; Wongthida, 2009). The structure and density of most solid tumours is such that in general the penetration of OVs into solid tumours is poor (Smith, 2011). This is certainly true for VSV infection of CT26 tumours. Immunohistochemistry (IHC) for VSV protein in VSV infected CT26 tumour sections demonstrates that IV administration of VSV-GFP resulted in the infection of a limited region of the tumour that was restricted to the perimeter of the tumour (Figure 9, unpublished data; Kyle Stephenson). Therefore, an increase in the pathogenesis of VSV is unlikely to have a significant impact on the oncolyis of an entire tumour given that such a small region of the tumour is exposed to virus.

Chapter 5: Conclusions

The aim of this study was to determine the impact of Beclin1 expression on the state of autophagy in VSV infected cells and explore the consequences of this expression on the pathogenesis and oncolytic potential of VSV-Beclin1. This study first sought to establish whether or not Beclin1 over-expression enhances autophagy in VSV infected cells. It was determined that while Beclin1 does appear to enhance autophagy in some cell lines this is a transient effect that is followed by a dramatic reduction of autophagy in VSV-Beclin1 infected cells compared to those infected with VSV-GFP. It is unclear whether this short term enhancement is capable of having a significant effect on the course of VSV infection.

Recent research in the field of autophagy regulation has shed more light on the factors which control the initiation of this process. It is now clear that there are key signaling events upstream of Beclin1 activation. It may be that some of the proteins involved in these events would be more promising candidates to induce a long term enhancement of autophagy in the context of OT. It is hoped that attempts to modulate levels of autophagy with the use of a transgene will benefit from these results.

This study did however establish convincingly that VSV infection itself results in a strong induction of autophagy within 12 hours of infection in some cell lines. This induction is comparable to that induced by rapamycin treatment (Figure 4B,C). Given the evolutionarily conserved role of autophagy in the innate clearance of intracellular pathogens and the evidence from research with other viruses, it is most likely that this is a host defense mechanism. Because of this strong induction of autophagy mediated by VSV

infection it is unclear whether any additional transgene would be capable of augmenting this process further. Therefore, any future attempts to study the relationship between autophagy and VSV infection should bare these results in mind.

This study also sought to determine the effects of Beclin1 expression on VSV pathogenesis. *In vitro* analysis of cell death following infection revealed that VSV-Beclin1 was more pathogenic in some cell lines than VSV-GFP. Analysis of caspase 3/7 activation exhibited similar activation induced by both viruses, indicating that the enhanced cell death displayed by VSV-Beclin1 infection may be independent of extrinsic and intrinsic apoptosis pathways. It is possible that, albeit short term, the enhanced autophagy in CT26 cells mediated by Beclin1 expression could have led to PDC-II in some of these cells augmenting the total cell death in the population. However further analysis would be required to fully explore this possibility. Given the relationship between Beclin1 and apoptosis inhibitor Bcl-2 it is most likely that the over-expression of Beclin1 is enhancing apoptosis in a mechanism that was not elicited in the assay used.

The analysis of the *in vivo* pathogenesis of VSV-Beclin1 elicited no differences between the two viruses. It is most likely that the discrepancy between the *in vitro* and *in vivo* results is explained by the fact that the Beclin1 mediated enhancement of death is cell type specific.

The final aim of this study was to explore the oncolytic potential of VSV-Beclin1. Despite Beclin1 mediating enhanced cell death in CT26 cells *in vitro*, VSV-Beclin1 exhibited no significant differences in neither the control of tumour volumes nor the survival of mice in this tumour model compared to the control virus (Figure 8). IHC

analysis of VSV penetration into CT26 tumours demonstrates that this virus infects only a very limited region of the tumour with little to no penetration of the core (Figure 9). Therefore it is unlikely that an event that only affects those cells infected would have a significant impact on the destruction of the entire tumour. In order to truly change the course of VSV oncolysis one would most likely have to alter the secondary immune responses elicited by OV infection, which are known to account for the bulk of tumour clearance. It is hoped that future endeavors to arm VSV or any OV with a transgene in an attempt to augment oncolysis will benefit from these conclusions.

Chapter 6: References

Aghi M, Martuza RL. Oncolytic viral therapies - the clinical experience. *Oncogene*. 2005;24(52):7802-7816.

Ahn CH, Jeong EG, Lee JW, et al. Expression of beclin-1, an autophagy-related protein, in gastric and colorectal cancers. *APMIS*. 2007;115(12):1344-1349.

Aita VM, Liang XH, Murty VV, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics*. 1999;59(1):59-65.

Balachandran S, Porosnicu M, Barber GN. Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis. *J Virol*. 2001;75(7):3474-3479.

Barber GN. VSV-tumor selective replication and protein translation. *Oncogene*. 2005;24(52):7710-7719.

Barber GN. Vesicular stomatitis virus as an oncolytic vector. *Viral Immunol*. 2004;17(4):516-527.

Birge RB, Ucker DS. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ*. 2008;15(7):1096-1102.

Breitbach CJ, Paterson JM, Lemay CG, et al. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol Ther*. 2007;15(9):1686-1693.

Breitbach CJ, Reid T, Burke J, Bell JC, Kirn DH. Navigating the clinical development landscape for oncolytic viruses and other cancer therapeutics: no shortcuts on the road to approval. *Cytokine Growth Factor Rev*. 2010;21(2-3):85-89.

Brown CW, Stephenson KB, Hanson S, et al. The p14 FAST protein of reptilian reovirus increases vesicular stomatitis virus neuropathogenesis. *J Virol*. 2009;83(2):552-561.

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A*. 1993;90(17):8033-8037.

Carew JS, Nawrocki ST, Kahue CN, et al. Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. *Blood*. 2007;110(1):313-322.

Chang G, Xu S, Watanabe M, Jayakar HR, Whitt MA, Gingrich JR. Enhanced oncolytic activity of vesicular stomatitis virus encoding SV5-F protein against prostate cancer. *J Urol*. 2010;183(4):1611-1618.

Chen Y, Klionsky DJ. The regulation of autophagy - unanswered questions. *J Cell Sci*. 2011;124(Pt 2):161-170.

Chen Y, Lu Y, Lu C, Zhang L. Beclin-1 expression is a predictor of clinical outcome in patients with esophageal squamous cell carcinoma and correlated to hypoxia-inducible factor (HIF)-1alpha expression. *Pathol Oncol Res*. 2009;15(3):487-493.

Delgado M, Singh S, De Haro S, et al. Autophagy and pattern recognition receptors in innate immunity. *Immunol Rev*. 2009;227(1):189-202.

Diaz RM, Galivo F, Kottke T, et al. Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. *Cancer Res*. 2007;67(6):2840-2848.

Dock G. The influence of complicating disease upon leukemia. *AM JMed Sci.* 1904; 127: 563-592

Eager RM, Nemunaitis J. Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther*. 2011;18(5):305-317.

Ebert O, Shinozaki K, Kournioti C, Park MS, Garcia-Sastre A, Woo SL. Syncytia induction enhances the oncolytic potential of vesicular stomatitis virus in virotherapy for cancer. *Cancer Res*. 2004;64(9):3265-3270.

Fernandez M, Porosnicu M, Markovic D, Barber GN. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *J Virol*. 2002;76(2):895-904.

Fimia GM, Di Bartolomeo S, Piacentini M, Cecconi F. Unleashing the Ambra1-Beclin 1 complex from dynein chains: Ulk1 sets Ambra1 free to induce autophagy. *Autophagy*. 2011;7(1):115-117.

Gajewski TF, Meng Y, Harlin H. Immune suppression in the tumor microenvironment. *J Immunother*. 2006;29(3):233-240.

Geng Y, Kohli L, Klocke BJ, Roth KA. Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. *Neuro Oncol*. 2010;12(5):473-481.

Hammill AM, Conner J, Cripe TP. Oncolytic virotherapy reaches adolescence. *Pediatr Blood Cancer*. 2010;55(7):1253-1263.

He C, Klionsky DJ. Atg9 trafficking in autophagy-related pathways. *Autophagy*. 2007;3(3):271-274.

Hosokawa N, Hara T, Kaizuka T, et al. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell*. 2009;20(7):1981-1991.

Huang J, Klionsky DJ. Autophagy and human disease. *Cell Cycle*. 2007;6(15):1837-1849.

Jackson WT, Giddings TH,Jr, Taylor MP, et al. Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol*. 2005;3(5):e156.

Jounai N, Takeshita F, Kobiyama K, et al. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci U S A*. 2007;104(35):14050-14055.

Jung CH, Jun CB, Ro SH, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell*. 2009;20(7):1992-2003.

Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ*. 2011;18(4):571-580.

Kawai T, Takahashi K, Sato S, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol*. 2005;6(10):981-988.

Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. *Mol Ther*. 2007;15(4):651-659.

Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys*. 2007;462(2):245-253.

Kim KW, Hwang M, Moretti L, Jaboin JJ, Cha YI, Lu B. Autophagy upregulation by inhibitors of caspase-3 and mTOR enhances radiotherapy in a mouse model of lung cancer. *Autophagy*. 2008;4(5):659-668.

Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol*. 2007;8(11):931-937.

Klionsky DJ. The molecular machinery of autophagy: unanswered questions. *J Cell Sci*. 2005;118(Pt 1):7-18.

Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human. *Autophagy*. 2007;3(3):181-206.

Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol*. 2007;7(10):767-777.

Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6(4):463-477.

Li B, Lei Z, Lichty BD, et al. Autophagy facilitates major histocompatibility complex class I expression induced by IFN-gamma in B16 melanoma cells. *Cancer Immunol Immunother*. 2009.

Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM. Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res*. 2008;68(17):6889-6895.

Liang C, Lee JS, Inn KS, et al. Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat Cell Biol*. 2008;10(7):776-787.

Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 1999;402(6762):672-676.

Lichty BD, Power AT, Stojdl DF, Bell JC. Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med*. 2004;10(5):210-216.

Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: cell survival in the land of plenty. *Nat Rev Mol Cell Biol*. 2005;6(6):439-448.

Martinet W, Agostinis P, Vanhoecke B, Dewaele M, De Meyer GR. Autophagy in disease: a double-edged sword with therapeutic potential. *Clin Sci (Lond)*. 2009;116(9):697-712.

Martinez-Outschoorn UE, Whitaker-Menezes D, Pavlides S, et al. The autophagic tumor stroma model of cancer or "battery-operated tumor growth": A simple solution to the autophagy paradox. *Cell Cycle*. 2010;9(21):4297-4306.

Matsunaga K, Saitoh T, Tabata K, et al. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol*. 2009;11(4):385-396.

Mundschau LJ, Faller DV. Oncogenic ras induces an inhibitor of double-stranded RNA-dependent eukaryotic initiation factor 2 alpha-kinase activation. *J Biol Chem*. 1992;267(32):23092-23098.

Oberstein A, Jeffrey PD, Shi Y. Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J Biol Chem*. 2007;282(17):13123-13132.

Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol Cell Biochem*. 2011;351(1-2):41-58.

Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res*. 2001;61(2):439-444.

Perez AM, Pauszek SJ, Jimenez D, Kelley WN, Whedbee Z, Rodriguez LL. Spatial and phylogenetic analysis of vesicular stomatitis virus over-wintering in the United States. *Prev Vet Med*. 2010;93(4):258-264.

Prentice E, Jerome WG, Yoshimori T, Mizushima N, Denison MR. Coronavirus replication complex formation utilizes components of cellular autophagy. *J Biol Chem*. 2004;279(11):10136-10141.

Prestwich RJ, Errington F, Harrington KJ, Pandha HS, Selby P, Melcher A. Oncolytic viruses: do they have a role in anti-cancer therapy? *Clin Med Oncol*. 2008;2:83-96.

Rainwater-Lovett K, Pauszek SJ, Kelley WN, Rodriguez LL. Molecular epidemiology of vesicular stomatitis New Jersey virus from the 2004-2005 US outbreak indicates a common origin with Mexican strains. *J Gen Virol*. 2007;88(Pt 7):2042-2051.

Roberts A, Buonocore L, Price R, Forman J, Rose JK. Attenuated vesicular stomatitis viruses as vaccine vectors. *J Virol*. 1999;73(5):3723-3732.

Roberts PC, Kipperman T, Compans RW. Vesicular stomatitis virus G protein acquires pH-independent fusion activity during transport in a polarized endometrial cell line. *J Virol*. 1999;73(12):10447-10457.

Scarlatti F, Granata R, Meijer AJ, Codogno P. Does autophagy have a license to kill mammalian cells? *Cell Death Differ*. 2009;16(1):12-20.

Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*. 2005;122(5):669-682.

Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science*. 2006;313(5792):1438-1441.

Smith E, Breznik J, Lichty BD. Strategies to Enhance Viral Penetration of Solid Tumors. *Hum Gene Ther*. 2011.

Sou YS, Waguri S, Iwata J, et al. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol Biol Cell*. 2008;19(11):4762-4775.

Spencer B, Potkar R, Trejo M, et al. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J Neurosci*. 2009;29(43):13578-13588.

Stojdl DF, Lichty B, Knowles S, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med*. 2000;6(7):821-825.

Stojdl DF, Lichty BD, tenOever BR, et al. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell*. 2003;4(4):263-275.

Tal MC, Sasai M, Lee HK, Yordy B, Shadel GS, Iwasaki A. Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. *Proc Natl Acad Sci U S A*. 2009;106(8):2770-2775.

Toda M, Rabkin SD, Kojima H, Martuza RL. Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. *Hum Gene Ther*. 1999;10(3):385-393.

Uhl M, Kepp O, Jusforgues-Saklani H, Vicencio JM, Kroemer G, Albert ML. Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. *Cell Death Differ*. 2009;16(7):991-1005.

Vazquez CL, Colombo MI. Beclin 1 modulates the anti-apoptotic activity of Bcl-2: insights from a pathogen infection system. *Autophagy*. 2010;6(1):177-178.

von Kobbe C, van Deursen JM, Rodrigues JP, et al. Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Mol Cell*. 2000;6(5):1243-1252.

Wennier ST, Liu J, McFadden G. Bugs and Drugs: Oncolytic Virotherapy in Combination with Chemotherapy. *Curr Pharm Biotechnol*. 2011.

Willmon CL, Saloura V, Fridlender ZG, et al. Expression of IFN-beta enhances both efficacy and safety of oncolytic vesicular stomatitis virus for therapy of mesothelioma. *Cancer Res*. 2009;69(19):7713-7720.

Wirawan E, Vande Walle L, Kersse K, et al. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. *Cell Death Dis*. 2010;1:e18.

Won KY, Kim GY, Kim YW, Song JY, Lim SJ. Clinicopathologic correlation of beclin-1 and bcl-2 expression in human breast cancer. *Hum Pathol*. 2010;41(1):107-112.

Wong HH, Lemoine NR, Wang Y. Oncolytic Viruses for Cancer Therapy: Overcoming the Obstacles. *Viruses*. 2010;2(1):78-106.

Wu L, Huang TG, Meseck M, et al. rVSV(M Delta 51)-M3 is an effective and safe oncolytic virus for cancer therapy. *Hum Gene Ther*. 2008;19(6):635-647.

Yang PM, Liu YL, Lin YC, Shun CT, Wu MS, Chen CC. Inhibition of autophagy enhances anticancer effects of atorvastatin in digestive malignancies. *Cancer Res*. 2010;70(19):7699-7709.

Yoneyama M, Kikuchi M, Natsukawa T, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*. 2004;5(7):730-737.

Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A*. 2003;100(25):15077-15082.

Zhang XQ, Dunner K,Jr, Benedict WF. Autophagy is induced by adenoviral-mediated interferon alpha treatment in interferon resistant bladder cancer and normal urothelial cells as a cell death protective mechanism but not by the bystander factors produced. *Cancer Gene Ther*. 2010.

Chapter 7: Appendix

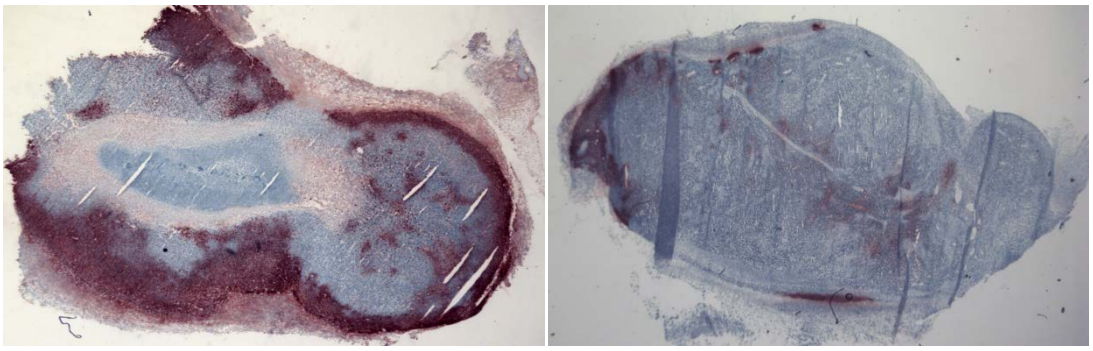


Figure 9: VSV has poor penetration into solid tumours following IV infection. IHC of VSV proteins in CT26 tumours engrafted in Balb-c mice, infected with 5 x 10^8 pfu IV (Kyle Stephenson, unpublished data).