THE DEVELOPMENT OF COXSACKIEVIRUS TYPE B3 AS A NOVEL ONCOLYTIC VIRUS AGAINST *KRAS*-MUTANT NON-SMALL-CELL LUNG CANCER

by

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. Despite a better understanding of the molecular mechanisms of lung cancer and the subsequent emergence of targeted therapies, treatment responses are typically short-lived. Oncolytic virotherapy provides a possible alternative direction for controlling this incurable disease. Coxsackievirus type B3 (CVB3) is a common human pathogen associated with viral myocarditis in young adults. Due to its highly lytic nature and ability to selectively replicate within cancerous cells, I hypothesize that CVB3 can be developed as an oncolytic virus. Here we demonstrated that *in vitro,* CVB3 specifically targets *KRAS*-mutant (*KRAS*^{mut}) non-small-cell lung cancer (NSCLC), a subtype of NSCLC with limited treatment options. Furthermore, we showed *in vivo* that intratumoral injection of CVB3 significantly reduces tumor volumes in patient-derived *KRAS*^{mut} NSCLC xenograft models. Mechanistically, we found that aberrant activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling and elevated expression of the coxsackievirus and adenovirus receptor (CAR), the primary receptor for CVB3 internalization, are associated with preferential replication of CVB3 within *KRAS*^{mut} NSCLC. However, despite a satisfactory tumor regression rate, CVB3 treatment leads to the onset of viral myocarditis in immunocompromised mouse models, indicating that potential safety issues need to be addressed prior to its potential application in lung cancer therapy. It is known that CVB3 subverts host machinery to gain survival advantages, and this process is highly associated with a spectrum of human disorders. We reported that Grb2-associated binding protein 1 (GAB1), a scaffolding adaptor protein responsible for intracellular signaling assembly and transduction, plays a crucial role in regulating compensatory cardiac response to aging and hemodynamic stress. Furthermore, we

demonstrated that both GAB1 and Grb2-associated binding protein 2 (GAB2, a functional homologue of GAB1), are proteolytically cleaved after CVB3 infection by virus-encoded protease $2A^{pro}$, independent of caspase activation. We showed that virus-induced cleavage of GAB1 is beneficial for viral growth as the resulting cleavage fragment (GAB1-N_{1-174}) further enhances ERK1/2 activation and promotes viral replication. Taken together, our findings suggest that CVB3 is a potent oncolytic agent against *KRAS*^{mut} NSCLC, and that elimination of CVB3-induced cardiotoxicity would significantly enhance the safety of this virotherapy.

Lay Summary

Lung cancer is the leading cause of cancer-related deaths worldwide, with few effective treatment options. Recently, virus-based cancer therapies provide a promising new treatment option. In this dissertation, I showed that coxsackievirus (CVB3) treatment destroys a subset of lung cancer, but caused heart inflammation in mice with a defective immune system. My study then further pursued the mechanism by which CVB3 induces heart damage. GAB1 is a protein responsible for maintaining heart function. I found that CVB3 disrupts the structure of GAB1 and generates a short form of this protein that further facilitates CVB3 multiplication by activation of molecules that the virus uses to infect cells. Taken together, my study suggests that CVB3 is a potent tool for lung cancer treatment, but it remains necessary to develop a combined strategy to prevent CVB3 infection in the heart before CVB3-based cancer therapy can be considered safe for humans.

Preface

All the work presented in this dissertation was primarily completed by me under the supervision of Dr. Honglin Luo. Together with Dr. Luo, I developed the projects and designed the experiments. All of the *in vitro* experiments were performed by me except for the plaque assays. I performed the majority of the *in vivo* experiments with the help of Tatjana Bozin and Jingchun Zhang. I contributed to all of the data collection and analysis. The content of this dissertation contains three original research papers. Chapter 2 is based on a manuscript entitled "CVB3 Is a Novel Oncolytic Virus against *KRAS*-Mutant Non-Small-Cell Lung Cancer" [**Deng, H.,** Tanya, S., Liu, H., Xue, Y., Mohamud, Y., Zhang J., Jia, W., Lockwood, W., and Luo H]. The manuscript is under preparation at the time of the online submission of my doctoral dissertation. Chapter 3 is based on a research paper published in FASEB J [**Deng, H.,** Fung, G., Shi, J., Xu, S., Wang, C., Yin, M., Hou, J., Zhang, J., Jin, ZG., and Luo, H. (2015) Enhanced Enteroviral Infectivity via Viral Protease-Mediated Cleavage of Grb2-Associated Binder 1. *FASEB J*, 29(11), 4523-4531]. Another research paper [**Deng, H.,** Fung, G., Qiu, Y., Wang, C., Zhang, J., Jin, ZG., Luo, H. Cleavage of Grb2-Associated Binding Protein 2 by Viral Proteinase 2A during Coxsackievirus Infection. *Front Cell Infect Microbiol*. 2017;7:85] is incorporated into Chapter 5. During my PhD, I was also involved in several additional projects in collaboration with other laboratory members and contributed to five peer-reviewed articles as co-author.

The reuse and reprint of all published work is with permission from the journals as indicated. The *in vivo* work was approved by the University of British Columbia Animal Care Committee (A15-0015). All CVB3 studies were approved by the University of British Columbia Research Ethics Board (Certificate # B14-0190).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADP: adenosine diphosphate

AUF1: (AU)-rich element RNA binding factor 1

CAR: coxsackievirus and adenovirus receptor

CD: cytosine deaminase

CEA: carcinoembryonic antigen

CRC: colorectal cancer

CVA21: coxsackievirus serotype A21

CVB3: coxsackievirus serotype B3

DAF: decay accelerating factor

DAPI: 4’ 6-diamidino-2-phenylindole

DAP5: death-associated protein 5

DCM: dilated cardiomyopathy

DMEM: Dulbecco’s modified eagle medium

DOS: daughter of sevenless

EGFR: epidermal growth factor receptor

eIF4G: eukaryotic initiation factor 4 gamma

eIF5B: eukaryotic initiation factor 5B

EMT: epithelial-mesenchymal transition

ERK1/2: extracellular signal-regulated kinase 1/2

FBS: fetal bovine serum

GAB: growth factor receptor bound protein 2 associated binding protein

GAB1: growth factor receptor bound protein 2-associated binding protein 1

GAB2: growth factor receptor bound protein 2-associated binding protein 2

GEM: genetically engineered mouse

GM-CSF: granulocyte macrophage colony stimulating factor

GTP: guanosine triphosphate

GRB2: growth factor receptor bound protein 2

G3BP1: ras-GTPase activating protein SH3 binding protein 1

HA: human influenza hemagglutinin

HCC: hepatocellular carcinoma

H&E: hematoxylin and eosin

hTERT: human telomerase reverse transcriptase

HSV-1: herpes simplex virus-1

IA: intraarticular injection

IHC: immunohistochemistry

IκBα: inhibitor of κBα

IP: intraperitoneal injection

IRS1: inulin receptor substrate 1

IT: intratumoral injection

IV: intravenous injection

JNK: c-Jun NH_2-terminal kinase

KRAS: kirsten rat sarcoma 2 viral oncogene homologue

LC: lung cancer

LRP6: low-density lipoprotein receptor-related protein 6

MAPK: mitogen activated protein kinase

MAVS: mitochondrial antiviral signaling protein

MBD: c-Met binding domain

MDA5: melanoma differentiation-associated protein 5

MEK1/2: mitogen-activated kinase kinase

MOI: multiplicity of infection

mTOR: mammalian target of rapamycin

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MV: measles virus

NBR1: neighbor of BRCA1 gene 1

NDV: Newcastle disease virus

NFAT-5: nuclear factor of activated T-cell 5

NF-κB: nuclear factor kappa beta

NK: natural killer

NSCLC: non-small-cell lung cancer

ORF: open reading frame

OV: oncolytic virus

PABP: poly-A binding protein

PBS: phosphate-buffered saline

PD-1: programmed cell death protein 1

PD-L1/PD-L2: programmed death ligand 1/2

PFU: plaque forming unit

PH: pleckstrin homology

PIP_2: phosphotidylinositol 4,5-bisphosphate

PIP_3: phosphotidylinositol 3,4,5-trisphosphate

PI3K: phosphatidylinositiol-3-kinase

PLCγ: phospholipase C γ

PKB: protein kinase B

PMS: phenazine methosulfate

PSA: prostate-specific antigen

RAF: rapidly accelerated fibrosarcoma

RIP3: receptor interacting protein kinase-3

RTK: receptor tyrosine kinase

ROS1: c-ros oncogene 1

RPMI: Roswell park memorial institute

SCID: severe combined immune deficient

SH2: src-homology-2

SH3: src-homology-3

SHP2: src-homology-2 (SH2)-containing protein tyrosine phosphatase 2

SOC1: suppressor of clear 1

SQSTM1/p62: sequestosome 1

SRF: serum response factor

SSCHN: squamous cell carcinoma of the head and neck

SSTR: somatostatin receptor

STAT1: signal transducer and activator of transcription 1

STAT5: signal transducer and activator of transcription 5

TDP43: transactive response DNA-binding protein-43

TK: thymidine kinase

TKI: tyrosine kinase inhibitor

TRIF: Toll/Il-1 receptor domain-containing adaptor inducing interferon-beta

WT: wild type

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Last but not least, I would like to appreciate my family and thank them for their love, and also for their understanding and tolerance.

Chapter 1: Introduction

1.1 Lung Cancer

1.1.1 The Nature of Lung Cancer

Lung cancer (LC) is the leading cause of cancer-related mortality, with an estimated 1,690,000 deaths per year world-wide[1]. According to Cancer Statistics (Canada) in 2017, an estimated ~28,600 new cases of LC were diagnosed. Many of these patients (~21,100) will die of this disease even with multiple therapies available today. To date, cigarette smoking has proven to be the most important risk factor for LC, while other factors, including air pollution, mutations in susceptibility genes, and occupational exposures are also highly associated with the onset of the disease[2]. Clinically, presenting symptoms of LC largely depend on the location of the tumor within the lung as well as the stage of the disease. Although approximately 10% of patients are asymptomatic when diagnosed, most patients may present with nonspecific systemic symptoms of fatigue, anorexia, and weight loss, or with local symptoms caused by the primary tumor and its metastasis[1]. The typical manifestations of a primary LC include chest discomfort, cough, dyspnea, and hemoptysis. Histologically, LC has been subdivided into either small-cell lung cancer or non-small-cell lung cancer (NSCLC) with the latter being further classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma[3, 4]. NSCLC is the most predominant type of LC, accounting for over 85% of all LCs[5-7]. Further LC subcategorization has been applied after the realization that NSCLC can be driven by specific gene mutations responsible for both initiation and maintenance of the cancer[8-10]. These genes include two of the best-studied proto-oncogenes, kirsten rat sarcoma 2 viral oncogene homologue (*KRAS*) and epidermal growth factor receptor (*EGFR*)[10, 11]. Of note, the frequencies of mutant alleles of *KRAS* and *EGFR* occur separately in NSCLCs, indicating a mutually exclusive relationship between *EGFR* and *KRAS* mutations[12]. This

finding has led to more efficient stratification of the patients for therapeutic purposes. Although *EGFR* mutations in NSCLCs were identified 20 years later than *KRAS* mutations, they have been widely established in the clinic as valuable markers for enhanced sensitivity to targeted therapies (gefitinib and erlotinib). On the contrary, no direct *KRAS* inhibitor is available in the clinical setting. The *KRAS* gene encodes membrane-bound 21kDa guanosine triphosphate (GTP) binding protein that regulates cell proliferation, differentiation, motility, and apoptosis[13]. Mutant *KRAS* in NSCLCs is oncogenic due to the consistent activation of its downstream signaling effectors that mainly include rapidly accelerated fibrosarcoma (RAF*)* / mitogen-activated kinase kinase (MEK1/2) / extracellular signal-regulated kinase 1/2 (ERK1/2*)* and phosphatidylinositiol-3-kinase (PI3K) / protein kinase B (Akt) / mammalian target of rapamycin (mTOR) cascades, resulting in a variety of biological abnormalities responsible for cancer initiation, maintenance and progression[14]. More importantly, it was found that *KRAS* mutations are associated with poor responses to chemotherapies and targeted therapies, such as erlotinib, and correlate with poor clinical prognosis[14]. Therefore, there is an urgent need to develop alternative approaches to target NSCLCs with *KRAS* mutations.

1.1.2 Treatment Options for NSCLCs

Treatment options for NSCLCs vary according to several factors, including LC histologic type, stage at presentation, and molecular profile[15]. Traditionally, surgical resection remains the first choice of treatment for patients at early stage, while management options for patients with locally advanced or metastatic disease include systematic chemotherapy, radiotherapy and chemoradiotherapy[16, 17]. Due to the nature of NSCLCs characterized by aggressive local invasion, early lymphatic and hematogenous dissemination, as well as chemotherapeutic resistance, little

effect has been made on the mortality rate of this disease by routine therapeutic approaches[18-20]. The 5-year survival rate for NSCLCs is ~ 15% and overall median survival only ranges from 10 to 12 months[21-24]. There is therefore an urgent need to develop novel therapeutic strategies. Recently, with a better understanding of the molecular biology of NSCLC, targeted therapies and immunotherapies have emerged promising options in this regard[25, 26].

1.1.2.1 *ERK1/2* Inhibitors

Several oncogenes can constitutively activate ERK1/2 signaling, promoting malignant transformation of NSCLC[27, 28]. Hence, it is not surprising that the inhibition of ERK1/2 signaling has the potential to prevent tumor growth, progression and metastasis. Pre-clinical pharmacological studies have shown that the blockage of ERK1/2 signaling pathway using specific MEK1/2 inhibitors can significantly inhibit tumor cell proliferation, which provides a strong rationale for the development of small-molecule inhibitors of MEK1/2 for NSCLC treatment[29, 30]. To date, eleven MEK1/2 inhibitors have been tested in clinical trials[31-36]. Unfortunately, their insufficient antitumor activity, severe toxicity, and the lack of improvement seen in disease progression preclude further clinical development of these inhibitors[29, 37].

1.1.2.2 *KRAS* Inhibitors

KRAS mutations are commonly observed in NSCLCs, and therefore also become attractive candidates for targeted therapy[8, 38]. Thus far, however, little progress has been made to develop therapeutic inhibitors of *KRAS*[36]. Given the obstacles in the development of specific *KRAS* inhibitors, recent studies have designed a new strategy to inhibit downstream molecule effectors of mutant *KRAS* using a combination of multiple specific molecule inhibitors[39]. Preclinical studies

have shown that a combinatorial approach using a MEK1/2 inhibitor and a fibroblast growth factor receptor 1 inhibitor can significantly enhance tumor cell death *in vitro* and *in vivo*, but testing in humans is still in an early phase[39].

1.1.2.3 *EGFR* Inhibitors

EGFR targeted therapy is distinct from conventional cancer therapies in its potential to provide increased tumor specificity[40]. Two predominant classes of *EGFR* inhibitors widely used in clinic are monoclonal antibodies that specifically target the extracellular domain of *EGFR*, while small molecule tyrosine kinase inhibitors (TKIs) target the receptor catalytic domain of *EGFR*[41, 42]. Although the mechanisms by which these two inhibitors elicit antitumor activity are slightly different, their final action to inhibit *EGFR* signaling is the same. Clinical studies reported that TKI treatment in patients with advanced *EGFR*-mutant NSCLCs is associated with a 75% response rate and a 10-14 month median progression-free survival, indicating that patients with TKI treatment have a better outcome over those who received traditional chemotherapy[43-46]. However, recent evidence showed that the majority of patients who respond well to TKI treatment have disease progression within 2 years after the initial treatment due to acquired TKI resistance. Thus, subsequent treatment options are limited for these patients[47, 48].

1.1.2.4 Programmed Cell Death Protein 1 (PD-1) Inhibitors

The PD-1 receptor is an immune checkpoint inhibitor expressed on adaptive immune cells and is normally used for the regulation of excessive immune responses[49]. Expression of its ligand (programmed death ligand 1/2, PD-L1/PD-L2) on tumor cells is an evolved strategy for the tumors to evade host antitumor immunity by binding to PD-1[50-53]. A recent clinical trial has demonstrated

that pembrolizumab, a highly selective, humanized, IgG4 monoclonal antibody against PD-1, can prolong overall survival in patients with PD-L1-positive , advanced NSCLCs[54, 55]. However, the long-term efficacy is still controversial and largely depends on the expression profile of PD-L1 within the tumor microenvironment[56].

1.1.3 Oncolytic Therapeutics

Since the first observation that cancer patients who contracted a viral infection went into brief periods of clinical remission, significant progress has been made in the field of oncolytic therapeutics during the past few decades[57]. An oncolytic virus (OV) is clinically defined as a virus that is capable of inducing lysis of malignant cells through its self-replication process without causing damage to normal tissues[58-60]. To date, it has become increasingly clear that in addition to direct lysis of tumor cells by viral infection, activated innate and adaptive immune responses triggered by the virus are also a critical component of OV-mediated clinical benefit[61]. Additionally, a better understanding of tumor biology and the molecular mechanisms of viral cytotoxicity has provided us with a scientific rationale to develop more efficient OVs as potent, self-amplifying antitumor agents[62, 63]. As a result, several viruses, including adenovirus, herpes simplex virus-1 (HSV-1), measles virus, and reovirus, have demonstrated varying degrees of success in clinical trials against multiple solid tumors, while a modified HSV-1 has been approved by the US Food and Drug Administration after Phase III clinical trials for the treatment of melanoma in October 2015[64-69] (**Table 1**). Considering that little survival benefit has been achieved by current antitumor therapeutics against NSCLCs, novel oncolytic virotherapy offers hope in this regard.

Table 1: Ovs in Clinical Trials[59]

Virus	Name	Modification	Phase	Route	Tumor
Adenovirus	Oncorine (H101)	E1B-55k	2	IT	SCCHN
		E3	3	IT	SCCHN
	Onyx-015	E1B-55k	1	IV	Lung metastases
		E3B	1,2	Multiple	Multiple tumors
	CG7060	PSA control	1	IT	Prostate cancer
	CG7870/CV787	Rat probasin-E1A	1/2	IV	Prostate cancer
	Telomelysin	hTERT	1	IT	Solid tumors
	Ad5-CD/TKrep	CD/TK	1	IT	Prostate cancer
	Ad5-D24-RGD	RGD, Delta-24	1	IP	Ovarian cancer
NDV	NDV-HUJ	n/a	1/2	IV	Glioma
	PV701	n/a	1	IV	Solid tumors
	MTH-68/H	n/a	2	Inhalation	Solid tumors
	NV1020	n/a	1	IV	Solid tumors

Virus	Name	Modification	Phase	Route	Tumor
Reovirus	Reolysin	n/a	1,2	IT/IV	Multiple tumors
Vaccinia	JX-594	TK(-)	1	IT/IV	Multiple tumors
MV	MV-CEA	CEA	1	IP	Ovarian Cancer
CVA21	CAVATAK	n/a	1	IT	Melanoma
HSV	OncoVEX	GM-CSF	1	IT	Solid tumors
		ICP34.5(-)	2	IT	Melanoma
		Us11	1/2	IT	SCCHN
	G207	ICP34.5(-), ICP6(-)	1/2	IT	Glioma
		LacZ (+)	1	IT	Glioma
	HSV 1716	ICP34.5(-)	1	IT	SCCHN/Glioma
	HF10	HSV-1 HF strain	1	IT/IA	Multiple
	NV1020	n/a	1	IA	CRC liver metastases

NDV: Newcastle disease virus; MV: measles virus; CVA: coxsackievirus serotype A21; SCCHN: squamous cell carcinoma of the head and neck; PSA: prostate-specific antigen; hTERT: human telomerase reverse transcriptase; CD: cytosine deaminase; TK: thymidine kinase; CRC: colorectal cancer; HCC: hepatocellular carcinoma; ADP: adenosine diphosphate; GM-CSF: granulocyte macrophage colony stimulating factor; SSTR: somatostatin receptor; CEA: carcinoembryonic antigen; IT: intratumoral injection; IV: intravenous injection; IP: intraperitoneal injection; IA: intraarticular injection.

1.1.4 Mechanism of OV Action

OV-mediated tumor destruction lies in the ability of a virus to selectively kill tumor cells and subsequently release viral progeny to infect adjacent cancerous cells[70] **(Figure 1)**. The inherent capacity of OVs to infect, and subsequently lyse cancerous cells is driven by several factors. First, specific membrane receptors that mediate virus internalization are usually highly expressed on susceptible tumor cells[70]. Second, tumor cells usually have higher metabolic activities than quiescent cells, a characteristic which supports increased viral propagation. Third, many tumors have impaired antiviral signaling, such as type I interferon signaling, thus supporting selective infection of tumor cells[71]. On the other hand, host immunity can be activated by recognizing viral antigens within the immunosuppressive tumor microenvironment, contributing to the establishment of host antitumor immunity. Although it is generally believed that activated host innate and adaptive immunity may eliminate viral spread and consequently attenuate virus-mediated oncolytic effect, the presence of virus particles, cell debris, as well as subsequent release of tumor antigens and danger-associated molecular patterns enhances antitumor immunity[70]. Of note, host-virus interaction is a complex process, the success of OV therapeutics is determined by the balance between pre-existing antiviral immunity and antitumor immunity.

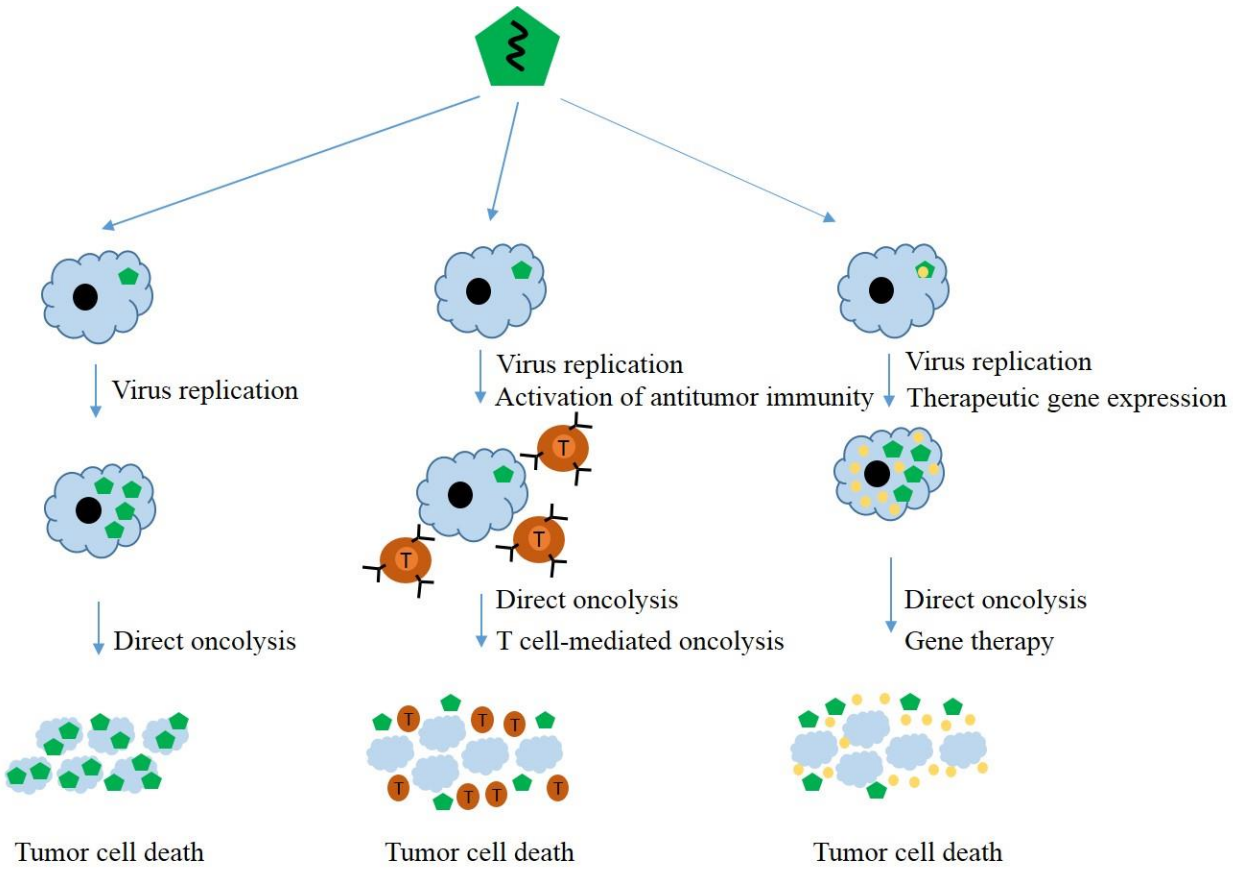


Figure 1. Mechanism of OV Action

Oncolytic viruses mediate tumor destruction by several potential mechanisms: 1) The virus itself can directly lyse tumor cells as a result of viral replication; 2) The virus can cause tumor cell death by the induction of innate and adaptive antitumor immunity; 3) The virus can mediate antitumor activity by the expression of therapeutic transgenes inserted into the viral genome.

1.1.5 Strategies to Improve the Safety of OVs

The selectivity to cancerous cells with minimal damage and destruction to normal tissues is always the first consideration when choosing human pathogenic viruses as therapeutic OVs. Evidence generated from preclinical trials has shown that existing oncolytic virotherapies are generally well-tolerated after both local and systemic administration[72-74]. Although serious

toxicities have been documented in several cases, the common adverse effects are usually mild and self-healing[75-80]. Furthermore, the transmission of an OV from a patient to close contacts was not observed in preclinical trials.

Over recent decades, with a better understating of the mechanisms by which viruses replicate within the host cells, targeted modification by molecular biology techniques has been widely exploited to improve the specificity of OVs[62, 81]. For example, adenoviruses have been rendered tumor-selective by the deletion of E1 genes, whose products are essential for viral replication in normal but not cancerous cells[82, 83]. A modified HSV-1 with the deletion of neurovirulence factor ICP34.5 is characterized by its tumor-specific cell lysis capability while leaving central nervous systems uninfected[84]. However, these approaches have also met some difficulties in terms of efficacy. First, extensive manipulation of viral genome often results in a strong attenuation of viral replication, which compromises the virus's effect against tumor cells. Second, viruses usually have a high spontaneous mutation rate during their replication process, which may destroy the stability of gene modification[85, 86]. Additionally, tumor cells vary widely depending on their histological type, molecular profile, and stage, as a result, each tumor type may respond differentially to different OVs.

1.1.6 OVs for NSCLCs

So far, four OVs have been used in clinical trials for the treatment of NSCLCs: 1) Oncorine (H101) is a modified adenovirus with deletion of ΔE1B-55kDa and ΔE3, and is characterized by its selective targeting of cells that have low expression levels of tumor suppressor protein p53[87]; 2) Seprehvir (HSV1716) is a $\gamma_1$34.5 null mutant with an intact U_L39 gene and replicates selectively in actively dividing cells[88]; 3) Maraba virus (MG1) contains both G protein (Q242R) and M protein

(L123W) gene mutations that attenuate its virulence in normal diploid cells, while maintaining hyper-virulence in cancerous cells[87]; and 4) reovirus type 3 Dearing strain (ReoT3D) is a ubiquitous, non-enveloped *dsDNA* virus with an enhanced oncolytic effect potentiated by chemotherapeutic agents[89]. Despite impressive progress, key challenges, such as poor tropism for the lung and pre-existing host immunity to eliminate effective viral spread, need to be overcome before these OVs can be applied to the patients[90].

1.1.7 Potential Candidates for Developing Novel OVs

The challenges of applying current OVs to patients with NSCLC provide an opportunity to identify better OV candidates with greater therapeutic potential. *Picornaviruses* have a natural ability to mediate cell death through combinatorial strategies, including shutdown of host cellular protein synthesis, proteolytic cleavage of transcription factors, and induction of apoptosis[91, 92]. All of these features of *Picornaviruses* make this family of viruses excellent candidates for oncolytic therapies. A recent phase I study reported that CVA21 belonging to the *picornavirus* family is a promising novel oncolytic immunotherapeutic agent for unresectable melanoma as it exhibits both local and distant durable tumor responses[93]. Further, a large-scale screening of 28 enterovirus strains identified coxsackievirus serotype B3 (CVB3) as one of the most potent OVs against NSCLCs[94]. CVB3 exhibits several advantages over other viruses as an oncolytic agent: 1) CVB3 infection in NSCLCs provides immunologic damage-associated molecular pattern signals, provoking host antitumor immunity[94]; 2) CVB3 preferentially infects and induces apoptosis in actively dividing cells rather than quiescent cells, and its replication rate significantly increases in tumor cell lines due to the activation of oncogenic signaling pathways[95]; 3) CVB3 infection is profoundly inhibited by type-I interferon, as a result, normal cell lines with intact interferon

signaling are more resistant to CVB3 infection than tumor cells that feature impaired interferon signaling[96-98]; and 4) CVB3 infection in adults is generally asymptomatic or causes mild flu-like symptoms[99].

1.2 Coxsackievirus

1.2.1 CVB3 Life Cycle

CVB3, a member of the *picornavirus* family, compasses a 7.4kb single-stranded positive-sense RNA genome (ssRNA(+)) that comprises a large open reading frame flanked at both 3' and 5' termini by untranslated regions[99, 100]. CVB3 has a short life cycle **(Figure 2)**. Following entry into the cells via attachment to the coxsackievirus and adenovirus receptor/decay accelerating factor (CAR/DAF) complex, the virus undergoes uncoating for the purpose of release of the viral genome[101, 102]. CVB3 expresses its viral gene products via internal ribosome entry site (IRES)-dependent translation of a single open reading frame (ORF) that encodes a viral polyprotein[103]. The newly synthesized polyprotein is rapidly processed both during and after the translation by virus-encoded proteases in an exquisitely specific manner[104, 105]. This process is initiated by several primary cleavage events carried out in *cis*, followed by *trans* cleavage events that generate mature polypeptides, as well as several precursor molecules[106, 107]. The resulting viral products render host cells a favorable intracellular environment for viral replication and progeny-virus production[100]. Ultimately, the viral genome is packaged into a tight icosahedral capsid (~30nm in diameter) composed of 4 structure proteins to initiate a new round of infection.

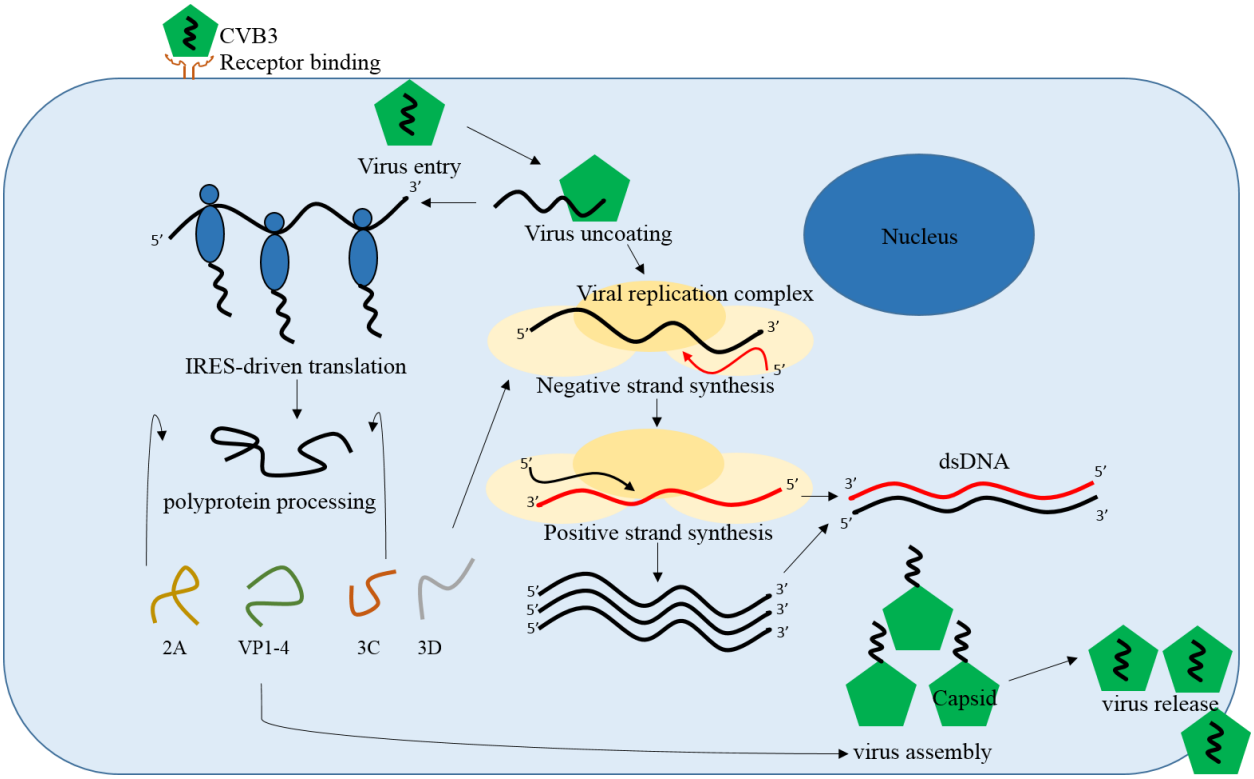


Figure 2: CVB3 Life Cycle

CVB3 life cycle begins with its internalization via the CAR/DAF complex, followed by the translation of a viral genome into a large viral polyprotein. The newly synthesized polyprotein is processed by viral proteases into structural and non-structural proteins. The intermediate single-stranded negative-sense RNA genome (ssRNA (-)) serves as a template for the synthesis of viral progeny genomes (ssRNA(+)). The resulting viral transcripts together with viral structural proteins are integrated to make complete virions. The life cycle of CVB3 is completed by virion release to initiate a new round of infection of adjacent cells.

1.2.2 The Functions of Each Viral Protein

The viral genome is composed of only 11 genes encoding a polyprotein that includes four structural and seven non-structural proteins. The polyprotein is processed by virus-encoded proteases, yielding mature viral proteins. Functionally, the viral polyprotein is divided into three regions, which includes P1, P2 and P3[108]. P1 encodes four capsid proteins (VP1, VP2, VP3 and VP4) that are responsible for virus assembly; P2 and P3 encode several functional proteins that are mainly used for protein processing, viral replication, and host-virus interaction[109]. The P1 region is the most variable part of the polyprotein, while the P2 and P3 regions are much more conserved[110]. Proteins derived from the P2 and P3 regions include two viral proteases ($2A^{pro}$ and $3C^{pro}$), an RNA-dependent-RNA polymerase (3D), an ATPase (2C), two membrane modifiers (2B and 3A), and a small peptide for the initiation of RNA synthesis (3B)[108]. Viral protease $2A^{pro}$ is a cysteine protease that cleaves in *cis* to separate the P1 region from the polyprotein and is involved in virus-induced pathogenesis via disrupting host cell protein synthesis[111, 112]. Viral protease $3C^{pro}$ is a cysteine protease as well. It cleaves P2 from the polyprotein and plays a crucial role in virus-induced blockage of host transcription[113]. The viral RNA-dependent RNA polymerase 3D is a major component of the viral replication complex with an elongation activity[114]. It can also uridylylate VPg and use the resulting VPg-pUpU as a primer for viral RNA replication[115, 116]. 2B and 3A are two membrane-associated proteins responsible for the regulation of plasma membrane permeability, cellular protein secretion and the presentation of membrane proteins during viral infection[117-121]. 3B is a small peptide containing 21 to 23 amino acids. 3B interacts with 3D, providing VPg-pUpU as a primer for both positive- and negative-stranded RNA synthesis[115, 122]. A schematic of the polyprotein products and their associated functions is provided in **Figure 3**.

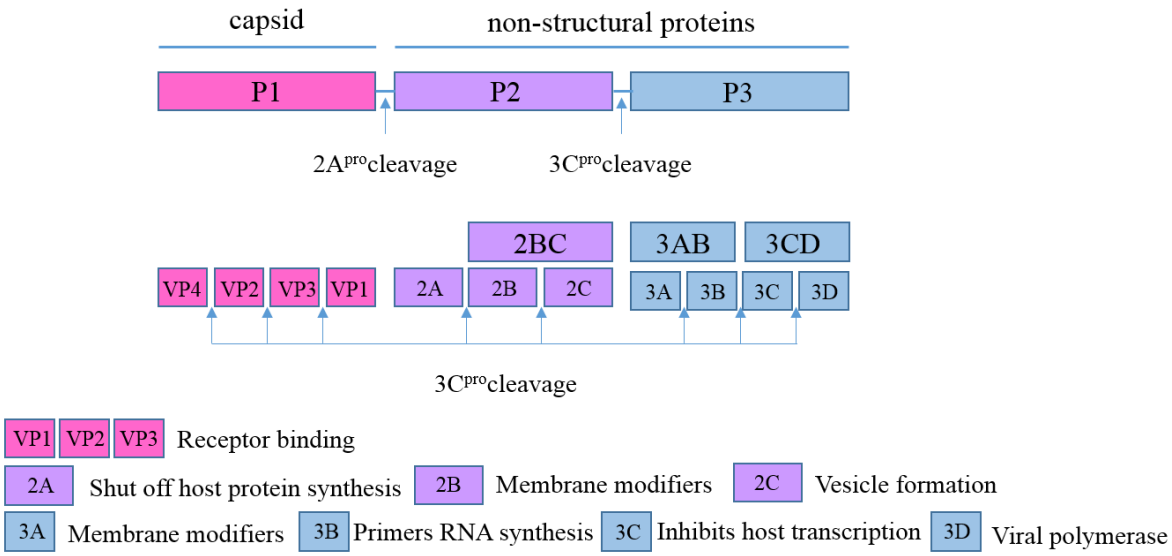


Figure 3: The Functions of Each Viral Protein

Viral proteins play crucial roles in regulating various steps of CVB3 life cycle, which includes viral RNA transcription and translation, virus assembly, and virus release. The functional roles of each viral protein are denoted.

1.2.3 Viral Strategies to Overcome Host Surveillance

Viruses are abundant, diverse and rapidly evolving pathogens that the host can be challenged by and they therefore represent a severe threat to human health. Paradoxically, they obligatorily depend on host cells for survival. Throughout evolution, mammalian cells have developed a variety of defense mechanisms to protect themselves from microbial pathogen infection[123, 124]. For example, inflammation usually comes as a host defense machinery to clear infected cells[125, 126]. To overcome the obstacle of host surveillance, CVB3 has also evolved a number of different strategies to maximize its survival advantage. For example, CVB3 cleaves innate immune adaptor molecules, mitochondrial antiviral signaling protein (MAVS), and Toll/Il-1 receptor domain-containing adaptor inducing interferon-beta (TRIF), as a mechanism to escape

host innate immune recognition[127]. Furthermore, viral protease $2A^{pro}$ directly cleaves eukaryotic initiation factor 4 gamma (eIF4G) to abolish cap-dependent mRNA translation that is essential for host protein synthesis[128]. Sophisticatedly, CVB3 does not merely disable its prey, but takes advantages of the remains[91]. The cleaved products stimulate the translation of uncapped viral mRNA using a novel internal ribosome entry mechanism to benefit viral replication. Likewise, host-cell transcription is dramatically reduced by the cleavage of several cellular transcription factors between Gln-Gly pairs mediated by CVB3-encoded protease $3C^{pro}$, creating a disproportionately higher number of viral RNA molecules in the host cells[91, 129]. By effectively terminating host cell translation and transcription, CVB3 not only commandeers abundant cellular resources, but protects itself against host immune responses by limiting antigen presentation to T cells as well[130].

Table 2: The Targets of CVB3-Encoded Viral Proteases

Viral Protease	**Gene Symbol**	**Full Gene Name**	**Function**
$2A^{pro}$	eIF4GI/eIF4GII	Eukaryotic Initiation Factor 4G I/II	Viral translation
$2A^{pro}$	PABP[112]	Poly-A binding protein	Viral translation
$2A^{pro}$	DAP5[131]	Death-associated protein 5	Viral translation
$3C^{pro}$	eIF5B[132]	Eukaryotic initiation factor 5B	Viral translation
$2A^{pro}$	SRF[133]	Serum response factor	Viral transcription
$3C^{pro}$	AUF1[134]	(AU)-rich element RNA binding factor 1	Viral transcription
$3C^{pro}$	TDP43[135]	Transactive response DNA-binding protein-43	Viral transcription

Viral Protease	Gene Symbol	Full Gene Name	Function
2A^{pro}	NFAT-5[136]	Nuclear factor of activated T-cell 5	Viral transcription
3C^{pro}	G3BP1[137]	Ras-GTPase Activating Protein SH3 binding protein 1	Viral transcription
2A^{pro}	Dysferlin[138]	Dysferlin	Cellular integrity
2A^{pro}	Dystrophin[139]	Dystrophin	Cellular integrity
2A^{pro}	SQSTM1/p62[140]	Sequestosome 1	Protein dynamics
2A^{pro}, 3C^{pro}	NBR1[141]	Neighbour of BRCA1 gene 1	Protein dynamics
3C^{pro}	RIP3[142]	Receptor interacting protein kinase-3	Protein dynamics
2A^{pro}	MAVS/MDA5[143]	Mitochondrial antiviral signaling/ Melanoma differentiation-associated protein 5	Cellular defense
3C^{pro}	RIG-1[143]	Retinoic acid-inducible gene 1	Cellular defense
3C^{pro}	TRIF[127]	TIR domain-containing adaptor inducing beta interferon	Cellular defense
3C^{pro}	IKBα[144]	Inhibitor of κBα	Cellular defense
2A^{pro}	GAB1[145]	Growth factor receptor bound protein 2-associated binding protein 1	Host Signaling
2A^{pro}	GAB2[146]	Growth factor receptor bound protein 2-associated binding protein 2	Host Signaling

1.2.4 The Immune Responses to CVB3

CVB3, like other microbes, induces exquisite host responses to infection. These responses are basically divided into innate and adaptive immunity. The innate immune responses are usually activated by general sensor pathways, in which toll-like receptors and retinoic acid-inducible gene 1 like receptors are involved[147, 148]. Triggering of these receptors alters the expression of a variety of genes that encode cytokines, chemokines and other proteins, contributing to the production of interferons and the activation of adaptive immunity[149]. NK cells are effector lymphocytes of host innate immunity that controls CVB3 infection by limiting its spread[150]. Previous studies found that depletion of NK cells in mice can significantly increase virus load in the heart and pancreas[151, 152]. In addition to their direct cytotoxicity to infected cells, NK cells can sensitize both dendritic cells and T cells, causing the transition from innate immunity to adaptive immunity[153]. The adaptive immunity is mainly performed by B cell-mediated humoral immunity and T cell-mediated cellular immunity. Both types of adaptive immunity contribute to the protection from virus attack. They play complementary roles with each easing the load on the other. Evidence showed that CVB3 infection of B cell-deficient mice results in persistent and severe damage to the organs, including the heart, lung and pancreas, and that transfer of B cells can transiently suppress viral replication and rescue organ functions[154-156]. These findings highlight a protective role for B-lymphocytes in CVB3 infection. However, the functional role of T-lymphocytes in limiting CVB3 infection is complex and the mechanism remains controversial[157]. Murine studies demonstrated that mice infected with recombinant CVB3 expressing a CD 8^+ cytotoxic T-lymphocyte epitope fail to generate a strong immune response, indicating that host adaptive immunity is dispensable for CVB3 clearance[158]. Nevertheless, another study showed that depletion of CD 8^+ T cells leads to a

marked increase in CVB3 titres, and pre-existing CVB3-specific CD 8^+ T-cells protect the host against virus challenges[158, 159].

1.2.5 Diseases Associated with CVB3 Infection

Based on the pathogenic characteristics of infected neonatal rat, Coxsackievirus can be divided into two serotypes, A and B[160]. Clinically, coxsackievirus serotype B (CVB) infection is associated with a more severe disease spectrum of myocarditis, pancreatitis, insulin-dependent diabetes mellitus, and aseptic meningitis than coxsackievirus serotype A (CVA)[161, 162]. Among all six serotypes of CVB, CVB3 is the most predominant serotype causing myocarditis and dilated cardiomyopathy (DCM), particularly in infants and children. Emerging evidence showed that CVB3 infection is responsible for approximately 20%-40% of sporadic cases of acute heart diseases and DCM in children[163-165]. Nevertheless, most CVB3 infection is asymptomatic, or may lead to mild symptoms such as rash, myalgia or upper respiratory symptoms[166].

1.2.6 Viral Myocarditis

Viral myocarditis is clinically defined as a cardiac disease caused by viral infection with inflammation of the myocardium and necrosis and/or degeneration of adjacent myocytes in the absence of an ischemic event[167-169]. DCM is a common sequela of viral myocarditis, which is characterized by left ventricular dilatation and impaired cardiac output[170]. Myocarditis is often caused by common viral infection, including the infection by enterovirus, particularly CVB3, adenovirus, parvovirus B19 and human herpesvirus 6[171, 172]. Endomyocardial biopsy remains the gold standard for diagnosis of myocarditis[173]. Therapeutically, there are few effective therapies available for viral myocarditis and DCM. Treatment options include either supportive therapies

for left ventricular dysfunction or heart transplantation for end-stage heart failure[174-176]. The progression of CVB3-induced viral myocarditis is an extremely complex process involving both direct viral infection and host immune-mediated mechanisms. Both factors contribute to the pathogenesis of acute injury and subsequent tissue remodeling of target organs[177, 178]. Therefore, development of a rational therapeutic strategy and a better understanding of the molecular pathogenesis of the disease must be achieved.

1.2.7 Pathogenesis of Viral Myocarditis

Overall, little is known about the pathophysiology of acute myocarditis in humans, and the only existing understanding of the pathogenesis of viral myocarditis is largely generated from animal studies[179, 180]. So far, CVB3 infection is among the best-characterized models of cardiac infection[181]. CVB3 gains access to the host via the gastrointestinal or respiratory tracts and subsequently targets the heart and pancreas[182, 183]. The stage of CVB3 infection of the myocardium is artificially divided into three phases[184] **(Figure 4)**. In the acute phase (0-4 days post infection), CVB3 enters the myocardium via DAF and CAR, followed by initiation of its replication[99]. The myocardial injury in this phase is mainly caused by virus-mediated lytic processes[185]. In the subacute phase (5-14 days post infection), in response to viremia, innate immunity is activated by a profound infiltration of inflammatory cells within the myocardium. Natural killer (NK) cells, together with macrophages, exert viral clearance as part of host defense machinery by mediating cytolysis and phagocytosis of infected cells. At the same time, the newly synthesized pro-inflammatory cytokines sensitize host adaptive immunity executed by antigen-specific T-lymphocytes and antibody-producing B-lymphocytes, simultaneously leading to the elimination of CVB3-infected cells[186, 187]. During this period, virus-mediated direct damage to the

myocardium, the recruitment of NK cells, macrophages, and eventually T lymphocytes to the sites of infection, and the production of a variety of cytokines, including tumor necrosis factor, IL-1α, IL-1β, IL-2 and IFN-γ together with neutral antibodies contribute to myocardial injury and compromised heart function[187]. This phase usually lasts one to four weeks, and ventricular contractile function can finally be improved in most patients as virus titres decrease[186]. However, some patients will enter a chronic phase (15 days post infection onwards) with chronic inflammation, due to a delayed or ineffective viral clearance[188]. In this phase, cardiomyopathy becomes irreversible, and is manifested by myocyte degeneration, interstitial fibrosis, and cardiac dilation[189, 190]. Clinically, left ventricle ejection fraction gradually declines and eventually develops into chronic DCM and heart failure[191].

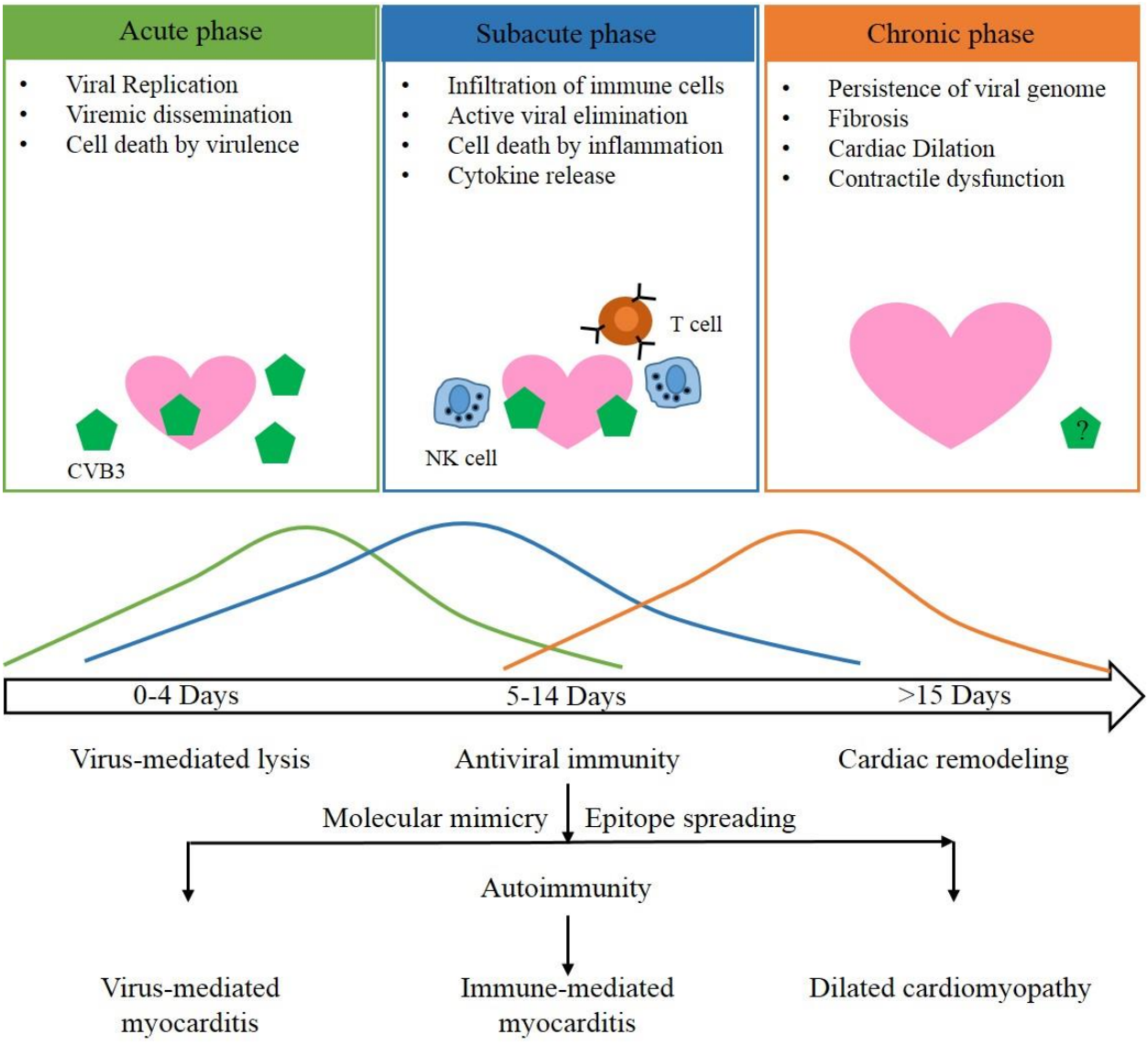


Figure 4: Three Phases of Viral Myocarditis

Viral myocarditis is a tri-phasic disease. In the acute phase, cardiomyocyte injury is mainly caused by viral replication. In the subacute phase, autoimmune injury is mediated by autoreactive T cells, auto-antibodies, and antiviral cytokines. In the chronic phase, impaired cardiomyocytes have limited ability for proliferation, leaving the region of damage replaced by fibroblasts and deposited collagen, contributing to the impairment of cardiac contractility, DCM and heart failure.

1.2.8 Host Signaling Determinants of CVB3 Pathogenesis

Intracellular signal transduction plays an unequivocal role in maintaining cell homeostasis that allows the host to monitor intracellular and extracellular changes and evoke an appropriate response in a time-dependent manner[192-195]. Dysregulation of such precise machinery is thought to be involved in various human diseases[196-198]. CVB3 is known to manipulate the host signaling machinery to regulate its replication and host responses[145, 199, 200]. It was first documented by Kandolf in 1997 that CVB3 can alter protein phosphorylation, and that such cellular phosphorylation events can subsequently enhance viral replication[201]. Our laboratory revealed that CVB3 infection induces a biphasic activation of ERK1/2, early transient activation versus late sustained activation, which are regulated by distinct mechanisms[202]. Infection by UV-irradiated CVB3 (which is capable of binding to receptors and endocytosis) triggers early ERK1/2 activation, but fails to induce late ERK1/2 activation[202]. It was further demonstrated that inhibition of mitogen activated protein kinase (MAPK) activity significantly decreases virus production and limits CVB3 progeny release, indicating a mechanism by which CVB3 subverts the host ERK1/2 signaling pathway to benefit its own replication[202]. Belonging to the same superfamily of ERK1/2, c-Jun NH_2-terminal kinase (JNK) and stress-activated protein kinase p38 MAPK were reported to play important roles in the course of CVB3 infection[203]. It was shown that CVB3 infection upregulates phosphorylated JNK and p38 MAPK, and that inhibition of p38 MAPK results in a significant reduction of viral progeny release and host cell apoptosis[203]. Thereafter, it was demonstrated that pretreatment of host cells with a PI3K inhibitor LY294002 blocks the activity of PKB or Akt, and significantly suppresses VP1 expression and subsequent virus release[204]. It is generally believed that the host responses to viral infection are tightly regulated by sophisticated

mechanisms, such that a single signaling pathway is not sufficient to determine "virus fate". Recent studies have proposed that viruses can drive signaling networks rather than an individual pathway to support their life cycles. Thus, understanding the mechanisms by which CVB3 integrates individual signaling pathways into a dedicate network that accurately reflects the consequence of CVB3 infection may provide us with a potential target for developing antiviral agents.

1.3 Scaffolding Adaptor Protein

1.3.1 Functional Roles of Scaffolding Adaptor Proteins in Signaling Transduction

The binding of an extracellular ligand with an associated receptor tyrosine kinase controls a variety of biological processes, such as cell cycle progression, cell proliferation, migration, and apoptosis[205, 206]. The process by which extracellular stimuli are transduced from the plasma membrane to the nucleus is an essential aspect of cellular regulation[207]. Many protein kinases and protein phosphatases are involved in this process by altering the phosphorylation status of tyrosine, serine, or threonine residues of specific signaling proteins[208, 209]. The assembly of signaling proteins into an intracellular signaling network is achieved by the association of autophosphorylated receptor tyrosine kinases with scaffolding adaptor proteins[210, 211]. The scaffolding adaptor proteins are a group of signaling molecules that work as a platform for the assembly of an intracellular signaling system. Moreover, evidence showed that scaffolding adaptor proteins are also involved in the regulation of intracellular signaling network, providing specificity[212-214].

1.3.2 The Scaffolding Adaptor Protein Grb2 Associated Binding (GAB) Family

The GAB proteins are members of the insulin receptor substrate 1 (IRS1)-like multi-substrate docking protein family, which includes GAB1, GAB2 and GAB3 in *mammals*, as well as daughter of sevenless (DOS) and suppressor of clear 1 (SOC1) in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively[215-217]. In humans, GAB1 and GAB2 are ubiquitously expressed, while GAB3 is highly expressed in lymphoid tissues[218, 219]. All GAB proteins share a common structure consisting of a highly conserved pleckstrin homology (PH) domain, a central proline-rich domain and multiple phosphorylated tyrosine residues[220]. GAB proteins integrate and amplify signals from a variety of extracellular stimuli including a diversity of growth factors, cytokines and antigen receptors[221-223]. Further, they also contribute to signal diversification by directing signals into different intracellular pathways with distinct biological functions[219].

1.3.3 Signaling From GAB Proteins

There are two distinct mechanisms by which GAB proteins are recruited to an activated receptor of tyrosine kinase (RTK) **(Figure 5)**. The direct mechanism involves the interaction between GAB1 and c-Met (a receptor for hepatocyte growth factor)[224-226]. GAB1 interacts with tyrosine-phosphorylated c-Met via a Met-binding domain that is absent in both GAB2 and GAB3[227]. Indirect binding represents a dominant type of RTKs-mediated GAB recruitment and is achieved via the association of GAB with other adaptor proteins[228]. For example, GAB proteins recruit growth factor receptor bound protein 2 (Grb2) through the binding of Grb2 Src homology-3 (SH3) domains with their proline-rich motifs, while the Src homology-2 (SH2) domain on Grb2 directs the Grb-GAB complex to the RTKs that contain Grb2 SH2 domain binding sites[229]. As bona fide signal transducers, GAB proteins are not only recruited by membrane receptors, but also

recruit downstream signaling molecules for signal transduction[219]. Once GAB proteins are associated with RTKs, they become auto-phosphorylated at multiple sites and then interact with the specific SH2 domains of signaling proteins that include SH2-containing protein tyrosine phosphatase 2 (SHP2), PI3K regulatory subunit p85, phospholipase C γ (PLC γ), and Crk[230-233]. Associations of GAB proteins with SHP2 and the p85 unit of PI3K are the best-characterized and considered as two major effector arms of GAB proteins[234]. SHP2 interacts with all GAB proteins, suggesting a conserved feature of GAB proteins[219]. Accumulating evidence demonstrated that GAB proteins recruit SHP2 phosphatase, which in turn activates MAPK signaling[235]. A study of chimeric receptors revealed that mutant GAB1 lacking SHP2 binding sites fails to activate MAPK signaling[224]. Moreover, overexpression of such mutants also renders GAB1 incapable of potentiating MAPK activation, suggesting that GAB1-SHP2 complex formation is essential for the activation of MAPK signaling pathway[236-238]. Similarly, the association of GAB1 with p85 plays a crucial role in mediating PI3K/Akt signaling upon a variety of stimuli[236, 239].

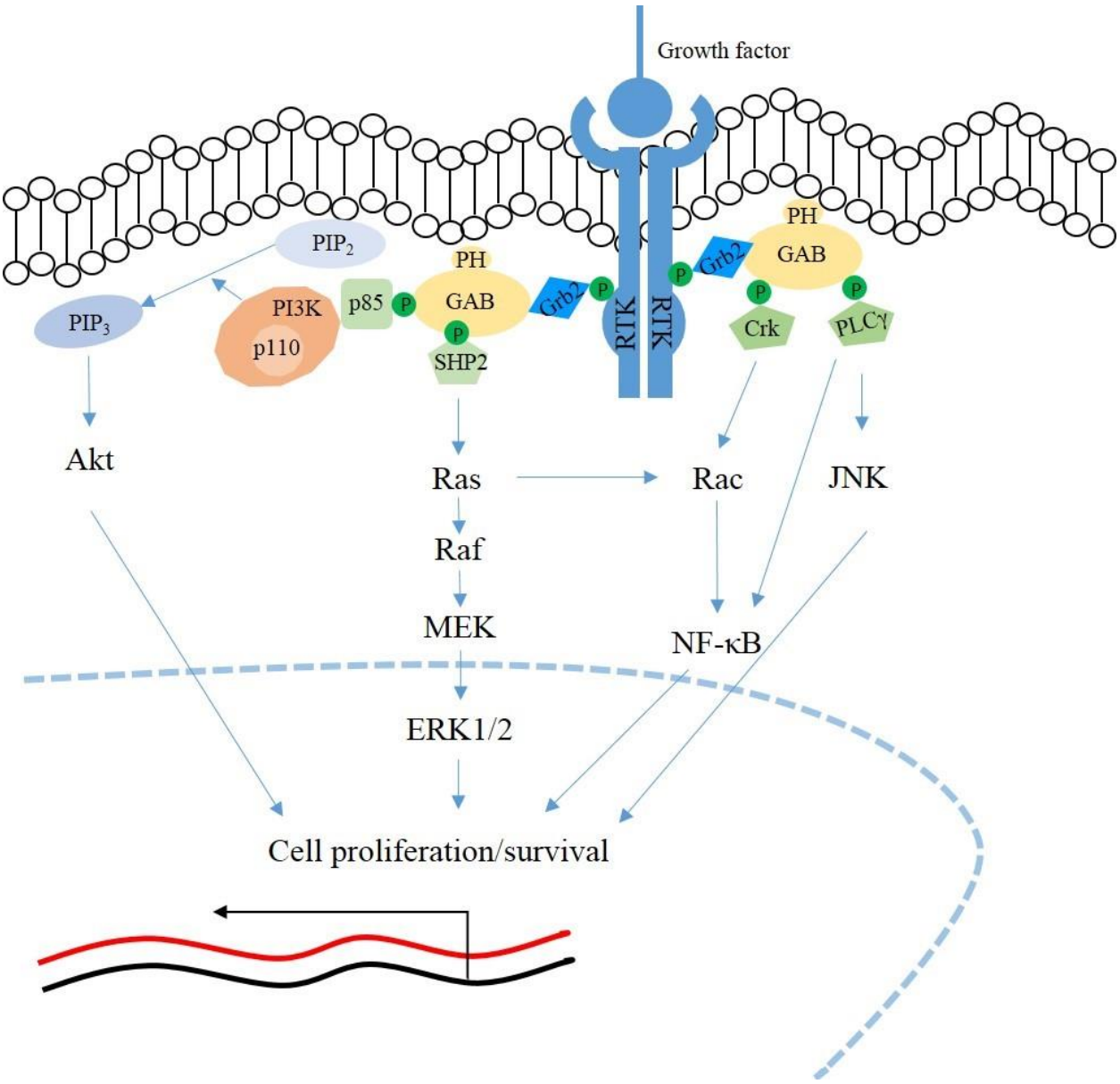


Figure 5: The Functional Roles of GABs in Intracellular Signaling

GAB family proteins serve as scaffolding adaptor proteins responsible for the assembly of intracellular signaling molecules to coordinate the signaling cascades of growth factors, antigens, cytokines and numerous other molecules. Phosphorylation of tyrosine residues of GAB proteins provides docking sites for SH2-containing molecules. The engagement of GAB proteins to tyrosine phosphatase SHP2 and the p85 regulatory subunit of PI3K results in the activation of

ERK1/2 and Akt, respectively. PIP_2: phosphotidylinositol 4,5-bisphosphate; PIP_3: phosphotidylinositol 3,4,5-bisphosphate; NF-κB: nuclear factor kappa beta

1.3.4 Distinctive Physiological Roles of Each GAB Protein

Most mammalian cells express more than one GAB family member. Specific knockout of different GAB protein leads to different outcomes, suggesting that a functional redundancy among different GAB proteins is absent[240]. Previous studies found that GAB1 deficiency results in embryonic lethality due to severe defects in heart, placenta, liver, skin, and muscle development[241]. GAB1 knock-in mice carrying a mutation in an SHP2 binding site demonstrate a phenotype of defects in muscle and placental development, indicating a central role of the GAB1-SHP2 complex in the migration of muscle progenitor cells[228]. Moreover, GAB1 knock-in mice with mutations in p85 binding sites exhibit defects in embryonic eyelid closure and keratinocyte migration[242]. Together, these results suggest that GAB1 regulates distinctive biological responses via the recruitment of different downstream effectors. Recently, we found that mice with cardiac-specific GAB1 knockout develop DCM in hemodynamic stress- and age-dependent manners, suggesting a key role for GAB1 in the maintenance of heart function[243]. Furthermore, it was also reported a crucial role for GAB1 in regulating postnatal angiogenesis using endothelial cell specific knockout mice and hindlimb ischemia models[244].

Although GAB2 knock-out mice are not embryonic lethal, GAB2 deficiency is associated with reduced responsiveness of hematopoietic progenitors to early-acting cytokines, impaired allergic reaction caused by decreased degranulation and cytokine production, and defective osteoclast differentiation[245-248]. Knock-in mice expressing either mutant Δp85 GAB2 or ΔSHP2

GAB2 display degranulation and anaphylaxis responses, suggesting that both arms contribute to the pathogenesis of inflammation and allergy[249].

To date, due to the fact that genetic GAB3 knockout mice manifest a healthy phenotype, the specific role of GAB3 remains to be elucidated[250].

1.3.5 Human Diseases Associated with GAB Deficiency

Given the demonstrated roles for GAB proteins in the regulation of fundamental cellular processes, GAB deficiency is believed to be associated with a series of human diseases[251]. For example, aberrant GAB-mediated signaling attributes to various forms of neoplasia[242]. Clinical studies found that GAB2 gene is amplified in breast cancer, ovarian cancer, gastric cancer, chronic myeloid leukemia, as well as metastatic melanomas[252-256]. The progression of these cancers is mainly dependent on the activation of GAB2-mediated downstream signaling effectors, SHP2 and PI3K. Similarly, GAB1 is also associated with tumorigenesis implied by its strong relationship with c-Met signaling, which is constitutively activated in a wide range of cancers[257, 258]. Further, as a downstream of EGFR, GAB1 also plays an important role in the pathogenesis of glioblastomas and intestinal adenomas[259, 260].

Since GAB proteins play essential roles in the pathogenesis of various cancers, allergic and cardiovascular diseases, the original definition of GAB protein as a scaffolding adaptor protein may require modification. A better understanding of the complexity of GAB-mediated signaling and the ensuing consequences may provide us with a novel strategy for therapeutic development.

1.4 Rationale

The term of 'OVs' applies to the viruses that are able to selectively replicate within tumor cells, thus this property has become a prerequisite for the development of novel virotherapies[261, 262]. Theoretically, tumor-selective viruses could exclusively target cancerous cells by exploiting the very same cellular aberrations that occur in tumor cells, while keeping normal cells uninfected[87]. However, existing preclinical trials demonstrated that the insufficient selectivity of viruses for tumor cells, low oncolytic potency, inability to penetrate and spread in tumor tissues, premature clearance of viruses, and poor induction of antitumor immunity, remain critical challenges that need to be addressed[263-265].

Due to its highly lytic nature, CVB3 usually causes overwhelming cytotoxicity and subsequent cell death of infected cells, which makes CVB3 a promising candidate for oncolytic virotherapy[266]. In addition, given the importance of ERK1/2 signaling in regulating CVB3 replication and the fact that constitutive ERK1/2 activation is widely observed in *KRAS*^{mut} NSCLCs, it is speculated that CVB3 selectively replicates within NSCLCs that display constitutively activated ERK1/2 signaling[267-270]. CVB3 also possesses several advantages that make it an excellent candidate for oncolytic virotherapy. First, CVB3 avoids genotoxicity caused by the integration of viral genome into the host DNA because its replication cycle does not include DNA intermediates. Second, due to a relatively small genome of ~7.4kb, CVB3 can be easily genetically manipulated by reverse genetics systems. Third, a recent large-scale screening of 28 enterovirus strains has identified that CVB3 is one of the most potent OVs against all human cancer cell lines tested, including NSCLC cell lines[94]. Finally, although CVB3 infection is associated with a high incidence of myocarditis in children and young adults, infection in adults is generally asymptomatic or causes mild flu-like symptoms [271].

Clinical studies reported that CVB3 genome was detected in 10.4% biopsy samples of patients with histologically proven myocarditis[272, 273]. Thus, CVB3-induced cardiomyopathy has become a major concern when developing CVB3 as an oncolytic agent. Viral myocarditis is a refractory disease without effective therapeutic approaches[274]. Although the pathogenesis of this disease has been well elucidated in regards to the interplay between viral processes and host immune responses, the detailed mechanisms remain to be addressed[274]. Recently, we found that the expression level of a scaffolding adaptor protein, GAB1, is significantly downregulated in the heart of a mouse model of CVB3-induced myocarditis and DCM[243]. Furthermore, *in vivo* studies demonstrated that GAB1-mediated ERK1/2 and p38 signaling are involved in the maintenance of cardiomyocyte survival[243]. Disruption of these two signaling pathways by knockout of cardiac GAB1 impairs mitochondrial function and activates caspase-mediated cell apoptosis, leading to the onset of DCM and heart failure[243]. These results indicate that GAB1 is a target of CVB3 and loss of GAB1 plays a key role in the pathogenesis of CVB3-induced cardiomyopathy. As alluded to above, ERK1/2 signaling is essential for both the host and CVB3 to obtain a survival benefit. Upstream molecules of this pathway are therefore speculated to be the potential targets of CVB3, contributing to viral pathogenesis in the heart.

In this dissertation, my overarching hypothesis is that CVB3 is a potent OV for NSCLC therapy, and elimination of CVB3 replication within the heart can further improve the safety of CVB3-based virotherapy. My **specific aims** are to:

1. Investigate the safety and efficacy of CVB3 as an OV for the treatment of NSCLC;
2. Explore the molecular mechanism by which CVB3 selectively kills NSCLCs;
3. Determine GAB-mediated pathogenesis of viral myocarditis;

Chapter 2: Materials and Methods

2.1 Cell Culture

The cell lines used in this work include: a HeLa cell line derived from human cervical cancer cells (American Type Culture Collection (ATCC)® CCL-2™, Manassas, VA, USA), which was maintained in Dulbecco's modified Eagle medium (DMEM, #SH30243.01, Thermo, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, #12483-020, Life Technologies, Waltham, MA, USA). In addition, three *KRAS*^{mut} (A549, H2030, and H23), four *EGFR*^{mut} (H1975, PC-9, HCC4006, and H3255) NSCLC cell lines and three normal lung epithelial cells (1HAEo, BEAS-2B, and HPL1D) were used in this study: an A549 cell line derived from adenocarcinomic human alveolar basal epithelial cells (ATCC® CCL-185™, Manassas, VA, USA), an H2030 cell line derived from metastatic lymph node of stage III lung adenocarcinoma (ATCC® CRL-5914™, Manassas, VA, USA), an H23 cell line derived from lung adenocarcinoma of epithelial origin (ATCC® CRL-5800™, Manassas, VA, USA), an H1975 cell line derived from lung adenocarcinoma of epithelial origin (ATCC® CRL-5908™, Manassas, VA, USA), a PC-9 cell line derived from an undifferentiated type of lung adenocarcinoma (#90071810, Sigma-Aldrich, St. Louis, MO, USA), an HCC4006 cell line derived from a metastatic pleural effusion of lung adenocarcinoma (ATCC® CRL-2871™, Manassas, VA, USA), an H3255 cell line derived from a metastatic pleural effusion of lung adenocarcinoma (ATCC® CRL-2882™, Manassas, VA, USA), 1HAEo, a post-crisis SV-40 T antigen transformed epithelial cell line (obtained from Dr. Dieter Gruenert, California Pacific Medical Center, University of California San Francisco)[275], a BEAS-2B cell line expressing keratins and an SV40 T antigen derived from normal human bronchial epithelium (ATCC® CRL-9609™, Manassas, VA, USA), and an HPL1D cell line expressing SV40 T antigen derived from normal human small airway epithelium (originally generated by

Takashi Takahashi from Nagoya University, Japan). All cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (#11875093, Thermo, Waltham, MA, USA) supplemented with 10% FBS. The stable cell lines of tetracycline-inducible HPL1D-GFP, HPL1D-*KRAS*^{G12V} and HPL1D-*EGFR*^{L858R} were generated as previously described[276] and maintained in RPMI 1640 medium supplemented with 10% FBS. To induce transgene expression, doxycycline hyclate (#D9891, Sigma-Aldrich, St. Louis, MO, USA) was added at 100ng/ml at the time of cell seeding for 48 or 72 hrs as indicated.

2.2 Animal Protocol

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (also known as NOD SCID gamma (NSG)) and NOD.CB17-*Prkdc*^{scid}/J (also known as NOD-SCID) immunocompromised mice were purchased from the Jackson Laboratory and bred at the Animal Resource Centre of the BC Cancer Research Centre. All animal experiments were performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of British Columbia (A15-0015). Patient-derived *KRAS*^{mut} H2030 cells and *EGFR*^{mut} H1975 cells were used to establish NSCLC xenograft mouse models. Briefly, H2030 or H1975 cells (5×10^6 cells) were injected subcutaneously into the left flank of male NSG mice or both flanks of male NOD-SCID mice. When tumors reached a palpable size (30~60mm^3), mice were intratumorally injected with a single dose of either wild-type (WT) or UV-inactivated CVB3 (5×10^4 plaque forming unit (PFU)). Mice were monitored daily for general appearance, behavior, weight, and any sign of infection at the injection site. Tumor size was measured every three days and tumor volume was calculated as

length × width × width/2. Mice were euthanized when they manifested severe symptoms related to CVB3 infection or the tumor diameter exceeded 2.0 cm.

2.3 Viral Infection

CVB3 infection was performed by incubating an individual cell line with CVB3 (Kandolf strain) at different multiplicities of infection (MOI) as indicated. Cells with CVB3 were kept in serum free medium for 1 hr and then the medium containing virus particles was replaced with fresh medium supplemented with 10% FBS for the remaining time points. Sham-infected cells (negative control) were treated with equal volumes of phosphate-buffered saline (PBS). UV irradiation was performed using UV Stratalinker 1800 (Stratagene) for 4 hrs with the virus container kept 5 cm from the UV bulb.

2.4 Inhibitor Treatment

For drug treatment experiments, Z-VAD-FMK, a pan caspase inhibitor (50 μM, #550377, BD Biosciences, San Jose, CA, USA) was applied for the general caspase inactivation. When virus-containing medium was replaced by fresh medium 1 hr post-infection, Z-VAD-FMK was added into the medium for 6 hrs. For experiments involving ERK inhibition or p38 inhibition, cells cultured in serum-free medium were incubated with MEK inhibitor, U0126 (20 μM, #9903; Cell Signaling, Beverly, MA, USA) or p38 inhibitor, SB203580 (50 μM, #S8307, Sigma-Aldrich, St. Louis, MO, USA), starting 30 mins prior to infection and until the end of experiments.

2.5 Plasmid, siRNA and Transfections

The 3×Flag-GAB1^{G175E} and 3×Flag-GAB1^{G436E} mutants were established by replacing the glycine (G) of WT-GAB1 at amino acids 175 and 436 with glutamic acid (E), respectively. The 3×Flag-GAB1^{WT} was used as a template to generate 2 truncated fragments of GAB1 (3×Flag-GAB1-$N_{1–174}$ and 3×Flag-GAB1-$C_{175–694}$). Hemagglutinin (HA)-tagged WT-GAB2 (GAB2^{WT}) was a generous gift from Dr. Roger Daly at Monash University (Melbourne, Australia). The HA-tagged GAB2^{G238E} was established by replacing the glycine (G) residue at amino acid 238 of WT-GAB2 with glutamic acid (E). The WT-GAB2 was used as a template to generate two truncated forms of GAB2. The resulting fragments were cloned into a vector expressing 3 × Flag at its N-terminus (3 × Flag-GAB2-$N_{1–237}$ and 3 × Flag-GAB2-$C_{238–676}$). The small interfering RNAs (siRNAs) against human GAB1 and human GAB2 were purchased from Dharmacon (#L-012455 , #L-131213; Dharmacon, Ottawa, ON, Canada).

Transfection of plasmids and siRNAs was performed according to manufacturer's instructions using Lipofectamine® 2000 (#11668019, Invitrogen, Burlington, ON, Canada) for plasmid transfection or Oligofectamine® (#12252-011, Invitrogen, Burlington, ON, Canada) for siRNA transfection. Briefly, plasmid transfection was performed on HeLa cells at a confluence of ~90% for 24hrs, while siRNA transfection was performed on HeLa cells at a confluence of ~30% for 48hrs.

2.6 Western Blot Analysis

Cells were harvested using modified oncogene science lysis buffer (250 mM NaCl, pH 7.2, 50 mM Tris-HCl, 0.1% NP-40, 2 mM EDTA, and 10% glycerol) supplemented with protease inhibitors. Briefly, equal amounts of proteins were subjected to SDS-PAGE gel electrophoresis

and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk solution containing 0.1% Tween-20 for 1 hr, the membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase–conjugated secondary antibodies at room temperature for 1 hr. The immunoreactive bands were visualized by enhanced chemiluminescence. Primary antibodies used in this study were 1) anti-human GAB1 (#3232; Cell Signaling, Beverly, MA, USA); 2) anti-human GAB2 (#3239, Cell signaling, Beverly, MA, USA); 3) anti-Flag (sc-807; Santa Cruz, Dallas, TX, USA); 4) anti-viral capsid protein VP1 (NCL-ENTERO; Leica Biosystems, Concord, ON, Canada); 5) anti-phospho-ERK1/2 (#4370; Cell Signaling, Beverly, MA, USA); 6) anti-cleaved caspase-3 (#9661; Cell Signaling, Beverly, MA, USA); 7) anti-β-actin (#2228; Sigma-Aldrich, St. Louis, MO, USA); 8) anti-low-density lipoprotein receptor-related protein 6 (LRP6) (#2560; Cell Signaling, Beverly, MA, USA); 9) anti-HA (sc-805, Santa Cruz, Dallas, TX, USA); 10) anti-phospho-p38 MAPK (#4511, Cell signaling, Beverly, MA, USA); 11) anti-phospho-SAPK/JNK (#4668, Cell signaling, Beverly, MA, USA); 12) anti-phospho-HSP27 (sc-81498, Santa Cruz, Dallas, TX, USA). 13) anti-RAS (#3965, Cell signaling, Beverly, MA, USA); 14) anti-EGFR (#4267, Cell signaling, Beverly, MA, USA); 15) anti-CAR (#16984, Cell signaling, Beverly, MA, USA); 16) anti-phospho-Akt (sc-52940, Santa Cruz, Dallas, TX, USA); 17) anti-phospho-signal transducer and activator of transcription 1 (STAT1) (#9167, Cell signaling, Beverly, MA, USA);

2.7 Plaque Assay

The viral titres in CVB3-infected cells or mouse organs were evaluated by plaque assay as previously described[277]. Briefly, culture media collected from CVB3-infected cells or homogenized tissue supernatants were serially diluted and overlaid on a monolayer of HeLa cells.

After 1 hr incubation, the medium was replaced by complete DMEM containing 0.75% agar. After 3-days incubation, cells were fixed with Carnoy's fixative (75% ethanol and 25% acetic acid) for 30 mins, followed by crystal violet staining. The plaques were counted and the viral titres subsequently calculated and represented as PFU per milliliter or per gram.

2.8 Crystal Violet Staining

CVB3-induced cytotoxicity was evaluated by crystal violet staining as previously described[278]. Briefly, after washing with PBS, viable cells attached to the bottom of the plates were fixed and stained with 0.4% crystal violet solution in methanol for 30 mins.

2.9 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Cell viability was determined using a cell titer 96 aqueous non-radioactive cell proliferation assay kit (#G5421, Promega, Madison, WI, USA) according to the manufacturer's protocols. Briefly, 20µl of combined MTS/ phenazine methosulfate (PMS) solution was added into each well of a 96 well assay plate containing ~1×10^5 cells/well in a final volume of 100µl culture medium, and the plate was incubated at 37 ºC for 4 hrs. Subsequently, the absorbance at 490nm was recorded on a microplate reader. The absorbance of sham-infected cells was defined as a value of 1. Cell viability of CVB3-treated cells is presented as the ratio of treated to sham-infected cells.

2.10 Hematoxylin and Eosin (H&E) Staining and Immunohistochemical (IHC) Staining

Tissues were harvested and fixed in 10% formalin, followed by embedding in paraffin and sectioning for standard H&E staining. IHC was conducted using the Vectastain Elite ABC kit

(#PK6100, Vector Laboratories, Burlingame, CA, USA) as previously described[279]. Briefly, 6-mm thick frozen tumor sections were fixed in 1:1 cold acetone/methanol solution. After blocking with serum, the sections were incubated with primary antibodies: anti-cleaved caspase 3 and anti-ki67 (sc-15402, Santa Cruz, Dallas, TX, USA) in a 1:100 dilution overnight at 4 ºC. 3, 3'-diaminobenzidine tetrahydrochloride (#SK4100, Vector Laboratories, Burlingame, CA, USA), chromagen, and nickel enhancement were utilized to visualize the location of individual protein in the tissue sections. Finally, the slides were counterstained by hematoxylin and mounted in an aqueous medium.

2.11 Cell Fractionation

Cell fractionation was performed as previously described[280, 281]. Briefly, HeLa cells were incubated with hypotonic lysis buffer (20 mM HEPES, pH7.4, 10mM KCl with phosphatase and protease inhibitors) for 20 mins on ice. Cell lysates were collected and homogenized with 30 strokes in a Dounce homogenizer, followed by centrifugation at 3,000rpm for 5 mins to remove nuclei and unbroken cells. The supernatant was further centrifuged at 55,000rpm for 1 hr to separate the cytoplasm from the membrane fraction. The purity of the membrane fraction was verified by the absence of the intracellular protein β-actin and the presence of the membrane protein LRP6 using western blotting.

2.12 *In Vitro* Cleavage Assay

Purified viral protease $2A^{pro}$ and catalytically inactive $2A^{pro}$ were generous gifts from Dr. Eric Jan at the University of British Columbia. *In-vitro* cleavage assay was conducted as previously

described[140, 141]. In brief, HeLa cell lysates (50µg) were incubated with either viral protease $2A^{pro}$ or catalytically inactive $2A^{pro}$ in cleavage reaction buffers (20 mM HEPES-pH 7.4, 150 mM KOAc and 1 mM DTT) at 37˚C for overnight. The reaction was stopped by adding 6 × sample buffers. Then the cell lysates were processed for the determination of GAB1&GAB2 cleavage by western blotting.

2.13 Immunocytochemical Staining and Confocal Laser Scanning Microscopy

The immunocytochemical staining was performed as previously described[137]. Briefly, cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 and blocked with 5% bovine serum album plus Tween 20. Coverslips were then incubated with primary antibodies at 4˚C for overnight, followed by the incubation with secondary antibodies at room temperature for 1 hr. After washing with PBS, the coverslips were counterstained with 4' 6-diamidino-2-phenylindole (DAPI) (#H1200, Vector Laboratories, Burlington, ON, Canada). Images were visualized by using a Leica SP2 AOBS inverted confocal laser scanning microscope (Leica, Wetzlar, Hesse, Germany).

2.14 Statistical Analysis

All results presented are representative of at least 3 independent experiments. Results generated from *in vitro* experiments are expressed as mean ± SDs and results from *in vivo* murine studies are presented as mean ± SEMs. Statistical analysis was conducted using the unpaired Student's *t* test. Values of $p<0.05$ were considered to be statistically significant.

Chapter 3: Coxsackievirus Type B3 Is a Potent Oncolytic Virus against *KRAS*-Mutant Non-Small-Cell Lung Cancer

3.1 Background

Lung cancer is one of the leading causes of cancer-related death in both sexes in North America and worldwide and confers a substantial burden of suffering on patients and their families[282-284]. Currently, most patients with lung cancer are diagnosed at an advanced stage when curative treatment is no longer possible[2]. NSCLC is the most common type of lung cancer, accounting for almost 85% of all lung cancers[2]. Recently, further subcategorization has been fueled following evidence that NSCLCs can be defined by various molecular criteria, such as specific driver mutations in genes that encode signaling proteins crucial for cellular proliferation and survival[285, 286]. Somatic mutations in *EGFR* have been identified in ~15% of all patients with NSCLCs, with the proportion increasing to 58% in patients of never smoker[287]. Although, patients with *EGFR*-mutant (*$EGFR^{mut}$*) tumors have increased sensitivity to TKIs, primary and acquired resistance towards these agents remains a major clinical obstacle[288]. Conversely, *KRAS* mutations are more common in patients who have a history of cigarette smoking, which accounts for ~30% of all cases of NSCLC[289]. However, these patients have a poor prognosis due to the lack of survival benefit from adjuvant chemotherapy and resistance to erlotinib and gefitinib, two anti-EGFR TKIs[290-294]. Therefore, there is an urgent need to develop new therapeutic approaches.

An OV is clinically defined as a virus that is capable of inducing lysis of malignant cells through its self-replication process without causing damage to normal tissues[295, 296]. Within the past decades, a better understanding of tumor biology and the molecular mechanisms of viral

cytotoxicity has provided scientific rationale to develop more efficient OVs as potent, self-amplifying cancer therapeutics[295]. As a result, several viruses, including adenovirus, HSV-1, MV, and reovirus have demonstrated varying degrees of success in clinical trials, while a modified HSV-1 has been approved by the US Food and Drug Administration in October 2015 for the treatment of melanoma[64-66, 69, 297]. On the other hand, there are still several disadvantages to be overcome, which includes poor tropism for targeted organs and pre-existing immunity in adults against OV replication[298].

CVB3, a non-enveloped, human-pathogenic enterovirus from the *picornaviridae* family, encompasses a 7.4kb single-stranded, positive-sense RNA genome[277]. Although CVB3 infection is associated with high incidence of myocarditis and pancreatitis in children and young adults, infection in elders is generally asymptomatic or causes mild flu-like symptoms[99, 299]. Recently, a large-scale screening of 28 enterovirus strains identified CVB3 as one of the most potent OVs against a panel of different human cancer cells, including NSCLC cells[94]. In addition to its natural tropism for NSCLC cells, CVB3 also possesses two features that make it an excellent candidate for oncolytic virotherapy. *First*, CVB3 preferentially infects and lyses actively dividing cells rather than quiescent cells due to the fact that activation of oncogenic signaling pathways within tumor cells creates a favorable microenvironment for virus replication [300]. *Second*, CVB3 infection is profoundly inhibited by type-I interferon; as a result, normal cell lines with intact interferon signaling are more resistant to CVB3 infection than tumor cells that often display impaired interferon signaling[97, 98].

Given the strong rationale for developing CVB3 as an OV, we investigate the safety and efficacy of WT-CVB3 in the treatment of NSCLC.

3.2 Specific Aims

The OBJECTIVE of this chapter is to determine the safety and efficacy of WT-CVB3 as a novel OV against NSCLC.

The SPECIFIC AIMS include:

Aim1: To examine the selectivity and cytotoxicity of CVB3 against NSCLC in *vitro*;

Aim2: To determine the safety and efficacy of CVB3 in human xenograft of NSCLC;

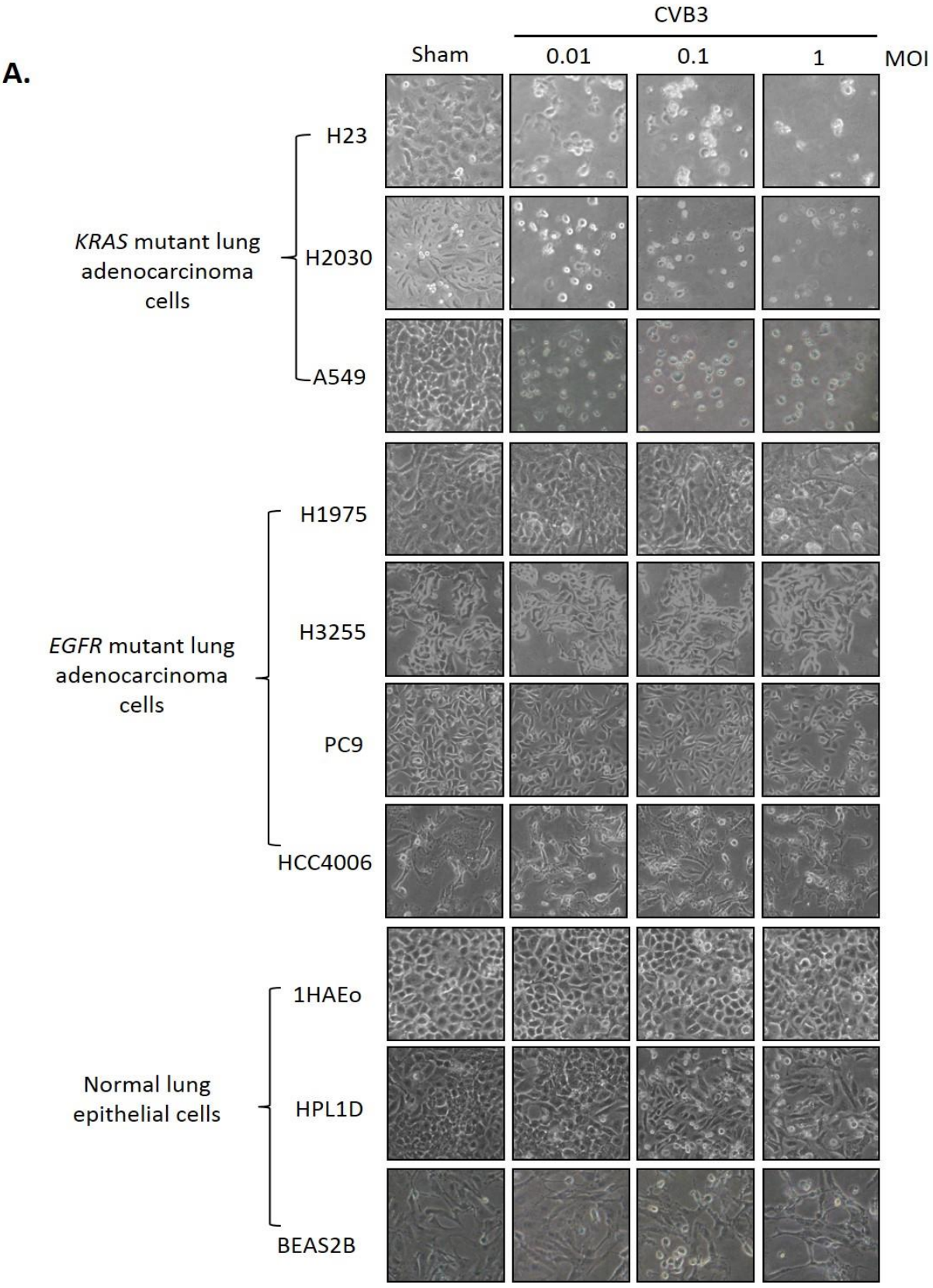
Aim3: To study the mechanisms by which CVB3 selectively targets $KRAS^{mut}$ NSCLC;

3.3 Results

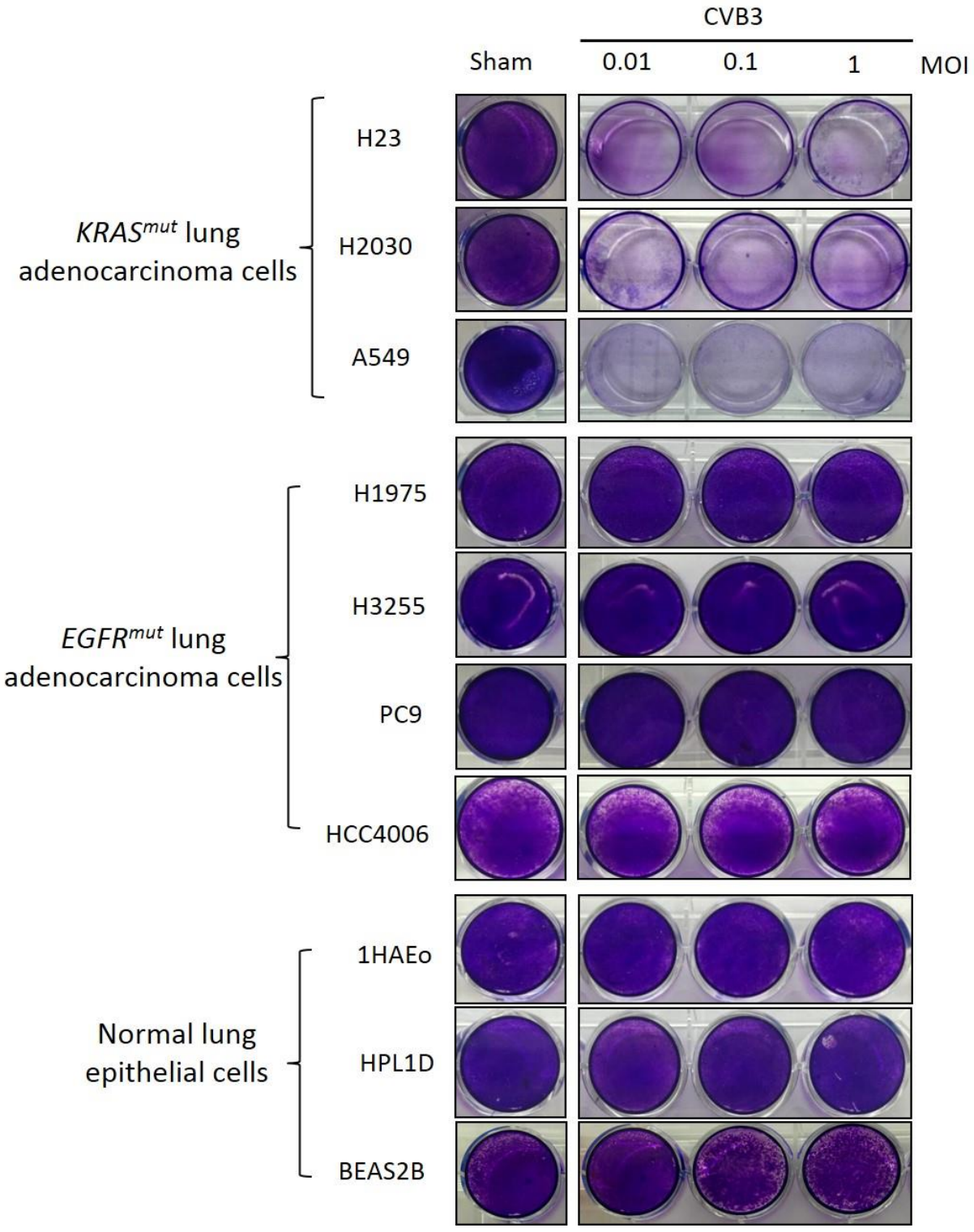
3.3.1 CVB3 Specifically Infects and Lyses $KRAS^{mut}$ NSCLC Cells

The development of driver-oncogenic mutation-targeted therapies has led to a substantial benefit for NSCLC patients carrying *EGFR* mutations; however, $KRAS^{mut}$ patients can currently not be treated with drugs. This led us to question whether CVB3-based virotherapy could be used as a novel approach specifically targeting $KRAS^{mut}$ NSCLCs. To test our hypothesis, seven patient-derived NSCLC cell lines, including three $KRAS^{mut}$ (H23, H2030, and A549) and four $EGFR^{mut}$ (H1975, H3255, PC-9, and H4006) cell lines were selected to examine their sensitivities to CVB3 infection. We also chose three normal lung epithelial cell lines (1HAEo, HPL1D, and BEAS2B) to evaluate the safety of CVB3 treatment *in vitro*. As shown in **Figure 6A & B**, CVB3 exhibited powerful cytotoxic activities against $KRAS^{mut}$ NSCLC cells in a dose-dependent manner. However, $EGFR^{mut}$ NSCLC cells and normal lung epithelial cells displayed only minimally cytopathic effects after 48-hr infection with CVB3 even at an MOI of 1. Cell viability assays further validated that CVB3 infection resulted in a profound reduction (~85%) of cell survival in $KRAS^{mut}$ NSCLC cells (**Figure 6C**). No significant cell death in $EGFR^{mut}$ NSCLC cells and a slight decrease of cell

survival in normal lung epithelial cells were observed upon CVB3 infection, especially at the lower dose of CVB3 (**Figure 6C**). Moreover, we examined the replication ability of CVB3 in NSCLCs and normal lung epithelial cells by plaque assay. As shown in **Figure 6D**, the virus titres in the supernatant of CVB3-infected *KRAS*^{mut} NSCLC cells were significantly higher than those from *EGFR*^{mut} NSCLC and normal lung epithelial cells, suggesting that CVB3-mediated oncolytic effect is highly associated with its replicative capacity. Together, these results indicate that CVB3 specifically targets *KRAS*^{mut} NSCLCs to exert its oncolytic effect by self-replication.


A.
CVB3
Sham
0.01
0.1
1
MOI
H23
H2030
A549
KRAS mutant lung adenocarcinoma cells
H1975
H3255
PC9
HCC4006
EGFR mutant lung adenocarcinoma cells
1HAEo
HPL1D
BEAS2B
Normal lung epithelial cells

B.


CVB3
Sham
0.01
0.1
1
MOI
H23
H2030
A549
KRASmut lung adenocarcinoma cells
H1975
H3255
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EGFRmut lung adenocarcinoma cells
1HAEo
HPL1D
BEAS2B
Normal lung epithelial cells

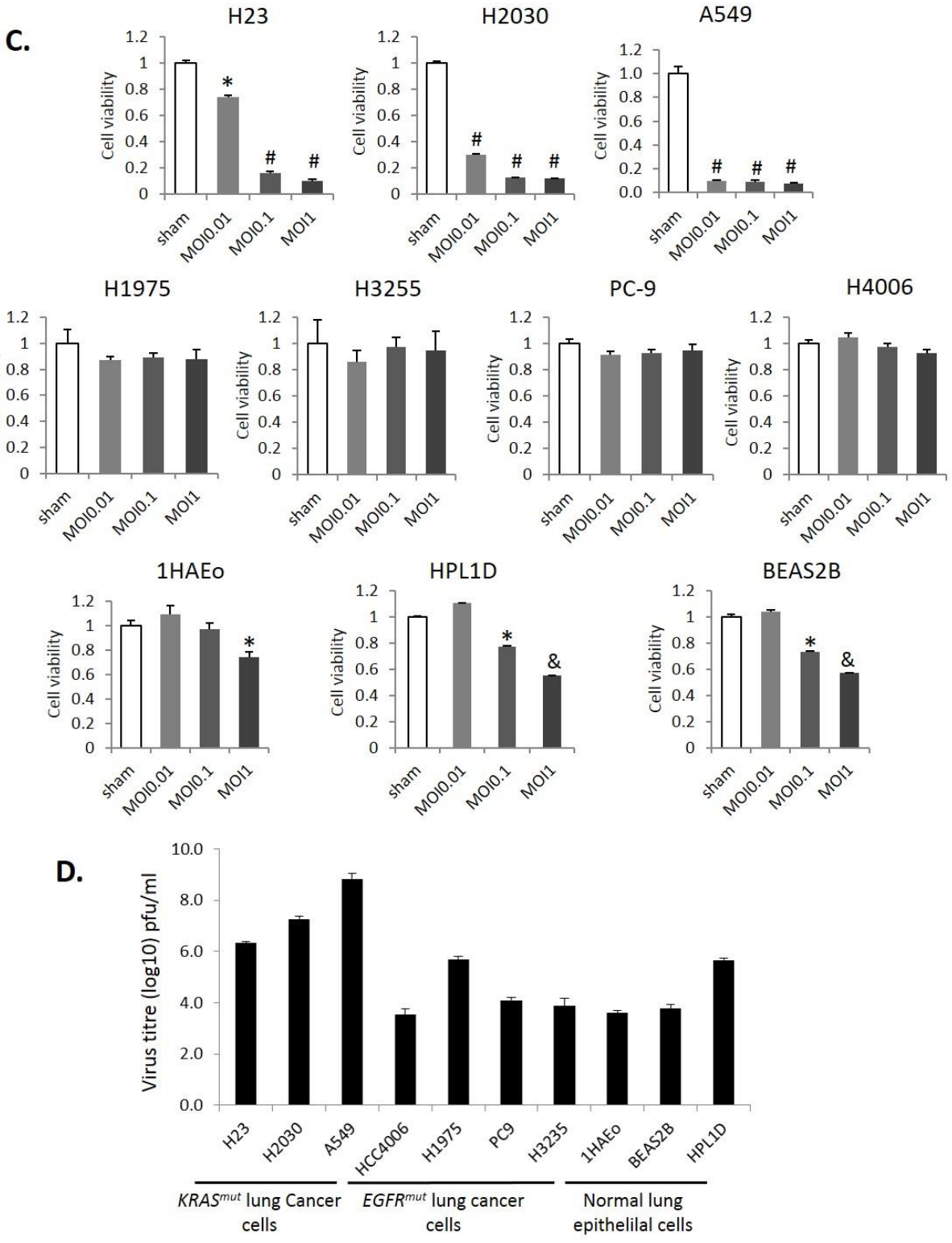
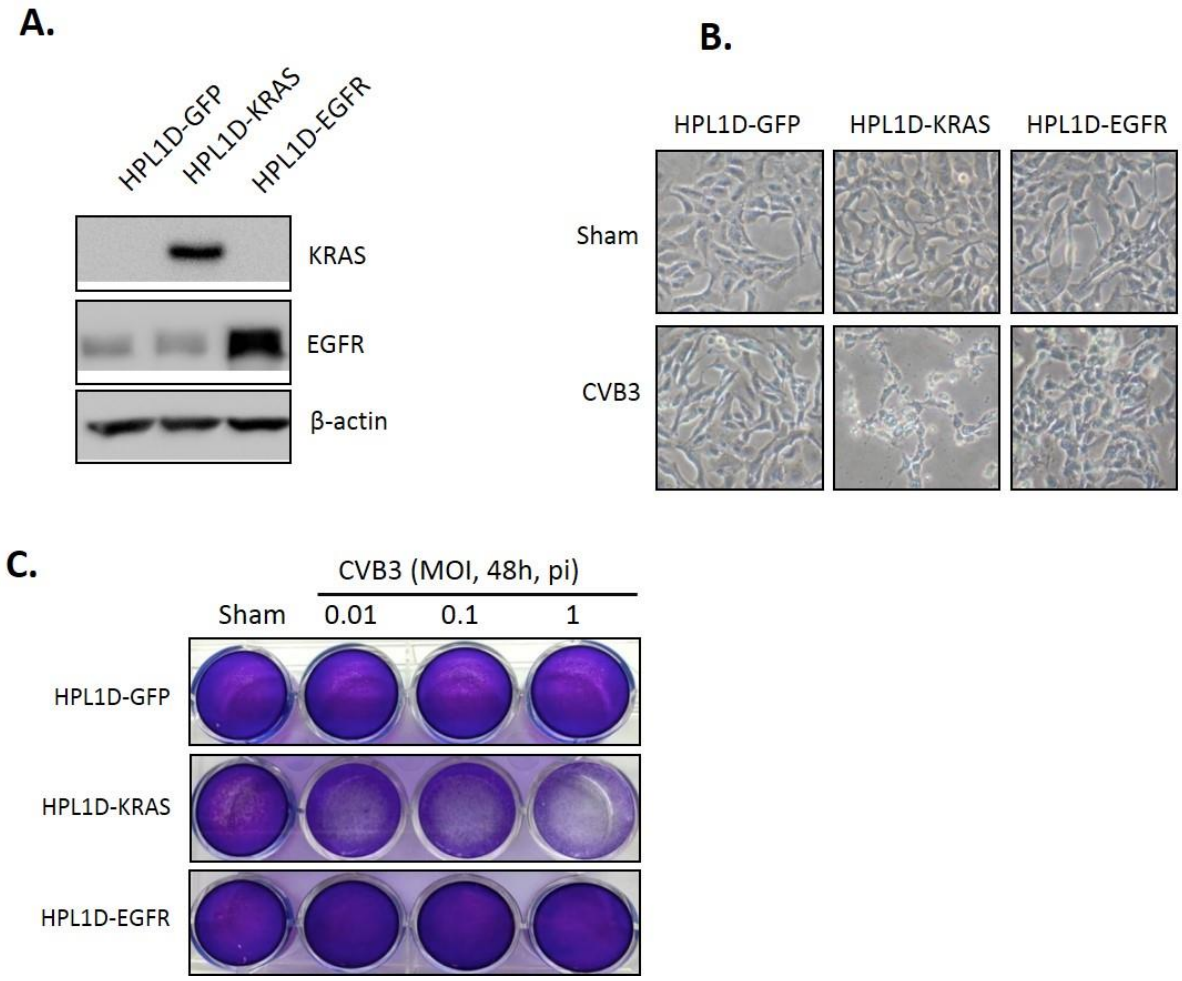

C.
H23
H2030
A549
H1975
H3255
PC-9
H4006
1HAEo
HPL1D
BEAS2B
Cell viability
sham
MOI0.01
MOI0.1
MOI1
D.
Virus titre (log10) pfu/ml
H23
H2030
A549
HCC4006
H1975
PC9
H3235
1HAEo
BEAS2B
HPL1D
KRASmut lung Cancer cells
EGFRmut lung cancer cells
Normal lung epithelilal cells

Figure 6. CVB3 Selectively Infects and Lyses *KRAS^{mut}* NSCLC Cells. Various NSCLC cell lines, including patient-derived *KRAS^{mut}* (H23, H2030, and A549), *EGFR^{mut}* (H1975, H3255, PC-9, and H4006), and normal lung epithelial cell lines (1HAEo, HPL1D, and BEAS2B) were sham- or CVB3-infected at different MOIs as indicated for 48 hrs. **(A)** Cell morphology was recorded by light microscopy (10×). **(B)** Cytotoxicity was evaluated by crystal violet staining. **(C)** Cell viability was determined by the MTS assay. Each value of CVB3-infected cells was normalized to that of sham-infected cells (arbitrarily set at a value of 1) and expressed as mean ± SD (n=3). #, $P<0.001$; &, $P<0.005$; *, $P<0.01$ compared to sham infection. **(D)** Virus titres in the supernatant of cells infected with CVB3 at an MOI of 0.1 for 24 hrs were measured by plaque assay. The results are presented as means ± SD (n=3).

3.3.2 *KRAS* Mutation Is a Determinant of NSCLC Susceptibility to CVB3

It has become evident that NSCLC is a heterogeneous disease marked with a high rate of somatic mutations, including mutations of *EGFR*, *KRAS*, echinoderm microtubule associated protein-like protein 4 fused with anaplastic lymphoma kinase, *MET*, c-ros oncogene 1, *RET*, v-Raf murine sarcoma viral oncogene homologue B1, and tumor suppressor protein 53[301-305]. Since each NSCLC cell line may harbor more than one somatic mutation, causing a potential synergistic role in supporting viral replication, to specifically determine the role of *KRAS* or *EGFR* mutation in CVB3 tropism we generated HPL1D-based stable cell lines expressing single driver mutation of either *KRAS mutant* (*KRAS^{G12V}*) or *EGFR* mutant (*EGFR^{L858R}*). HPL1D cells expressing GFP were used as a negative control. Western blot analysis verified overexpression of KRAS or EGFR in these cell lines (**Figure 7A**). As shown in **Figure 7B-D**, CVB3 specifically targeted and killed

HPL1D-*KRAS*^{G12V} cells with very minimal harm to HPL1D-*EGFR*^{L858R} and normal cells, indicating that *KRAS* mutation is a determinant of viral sensitivity.



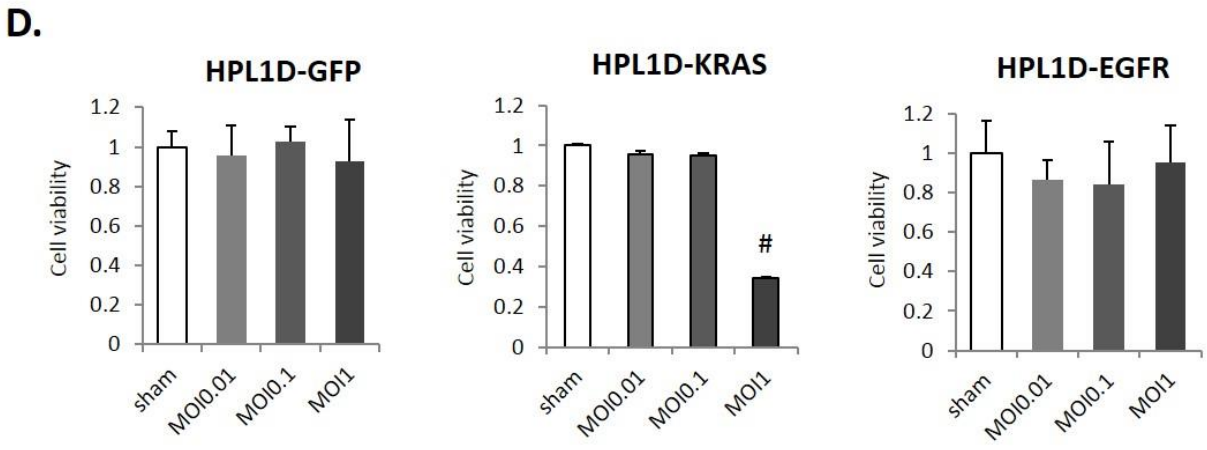
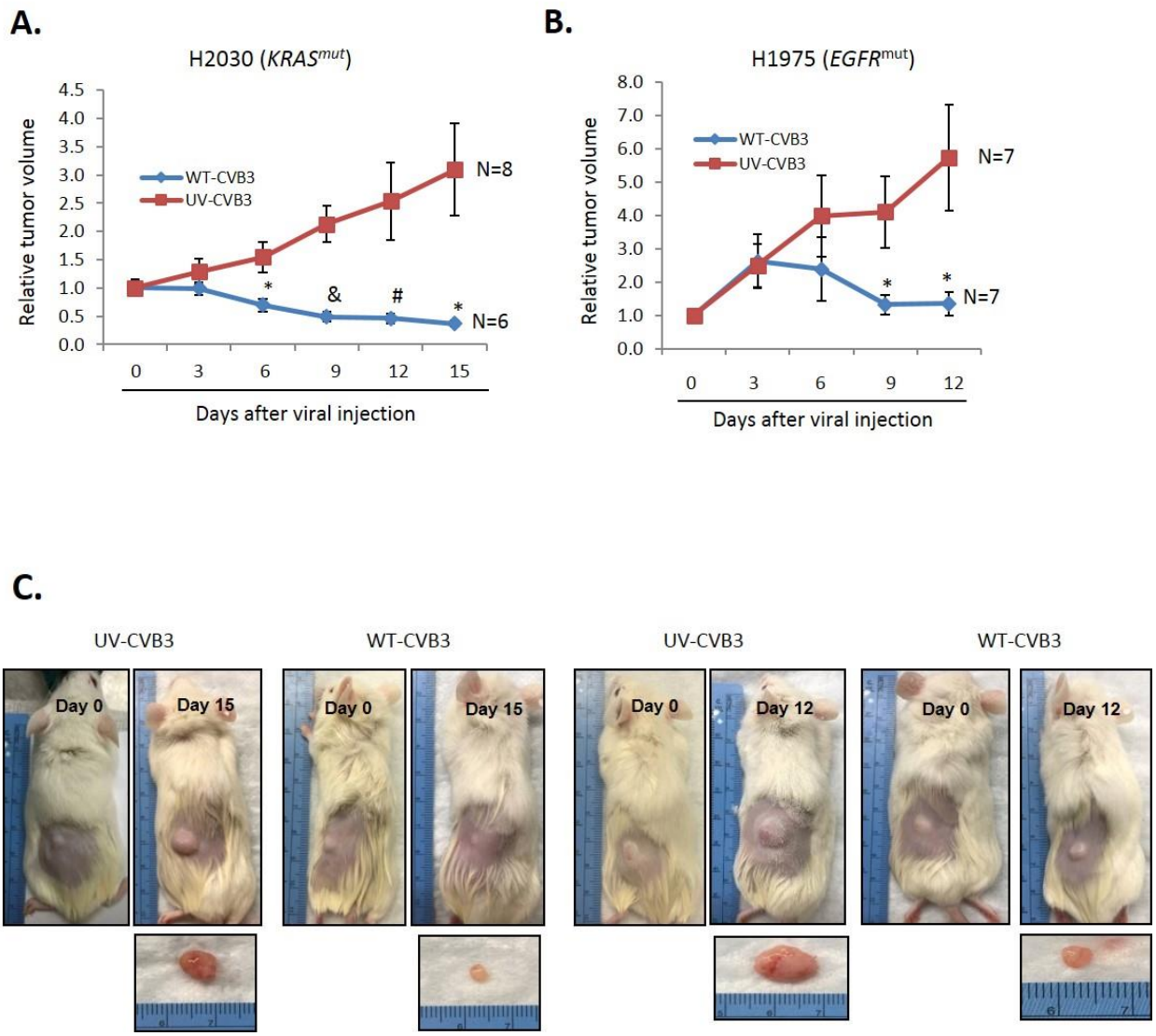


Figure 7. CVB3 Specifically Infects and Kills Lung Epithelial Cells Stably Expressing *KRAS^{G12V}*. (A) HPL1D cell lines stably expressing *GFP* (control), *KRAS^{G12V}*, or *EGFR^{L858R}* were harvested and protein expression of RAS and EGFR was validated by Western blot analysis. **(B)** Various HPL1D stable cells were sham- or CVB3-infected (MOI=1) for 48 hrs. Cell morphology was examined by light microscopy (10×). **(C & D)** Various HPL1D stable cells were sham- or CVB3-infected at different MOIs as indicated for 48 hrs. Cytotoxicity was examined by crystal violet staining (C). Cell viability was measured by MTS assay (D). Each value of CVB3-infected cells was normalized to that of sham-infected cells, which was arbitrarily set a value of 1, and presented as the mean ± SD (n=3). #, P<0.001 compared to sham infection.

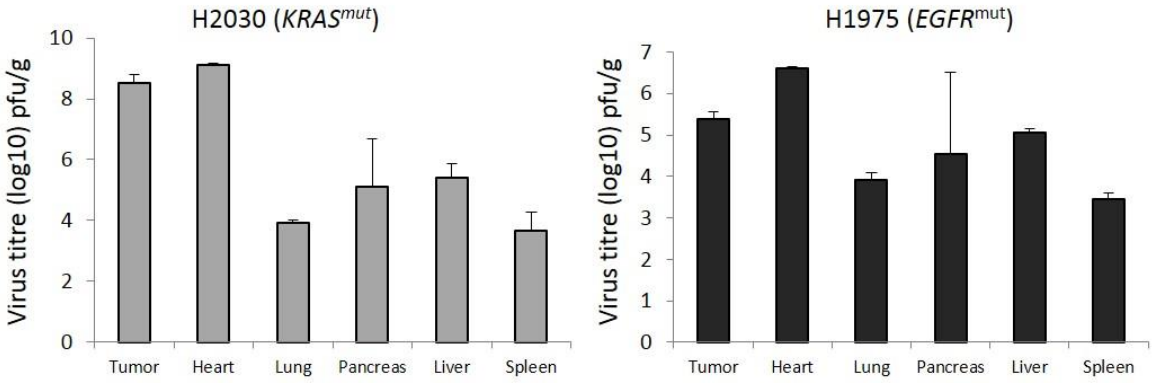
3.3.3 Intratumoral Injection of CVB3 Leads to A Significant Regression of *KRAS^{mut}* Xenograft NSCLC in An NSG Mouse Model

We next conducted patient-derived xenograft animal experiments to determine the anti-tumor effects of CVB3 *in vivo*. We first used the NSG immunodeficient mice whose immunity is severely restricted due to the lack of mature T cells, B cells, and functional natural killer (NK) cells[84, 306-312]. **Figure 8A & C** showed that intratumoral injection of WT-CVB3 resulted in a

dramatic reduction in *KRAS*^{mut} xenograft tumor volumes (~60% smaller on day 15 than on day 0), while tumor sizes continued to increase with the UV-CVB3 treatment. The tumor volume of mice exposed to WT-CVB3 on day 15 was ~12 fold smaller than that of mice exposed to UV-CVB3, suggesting that CVB3 potently kills *KRAS*^{mut} NSCLC *in vivo*. Similar to UV-CVB3-treated *KRAS*^{mut} xenografts, *EGFR*^{mut} xenograft tumors kept growing after UV-CVB3 injection (**Figure 8B & C**). The tumor size of *EGFR*^{mut} xenografts increased following WT-CVB3 inoculation on day 3 as compared to day 0, and then gradually reduced (**Figure 8B & C**). The difference in *EGFR*^{mut} tumor volume between UV-CVB3 and WT-CVB3 groups was significant (~4 fold difference on day 12), indicating that WT-CVB3 is also able to limit *EGFR*^{mut} tumor growth although its magnitude is much smaller than that observed in *KRAS*^{mut} tumors. Despite significant regression of both the *KRAS*^{mut} and *EGFR*^{mut} xenograft tumors, mice obtained no survival benefit after WT-CVB3 treatment and all mice died after day 15. Next, we compared virus loads in the xenograft tumors and different organs. **Figure 8D** showed that virus titre in *KRAS*^{mut} tumors was ~1000 fold higher than that in *EGFR*^{mut} tumors. Viral replication was also detected in various mouse organs, in particular the heart, suggesting an active systemic viral infection following intratumoral injection of WT-CVB3. Finally, we examined possible tissue damage caused by CVB3. As shown in **Figure 8E**, the heart and pancreas displayed significant cytotoxicity as characterized by myocardial injury and inflammatory infiltration, and destruction of acinar cells of the pancreas. Minimal pathological changes were observed in the lung, liver, and spleen.


A.
H2030 (KRASmut)
Relative tumor volume
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT-CVB3
UV-CVB3
N=8
N=6
*
&
#
*
0
3
6
9
12
15
Days after viral injection
B.
H1975 (EGFRmut)
Relative tumor volume
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
WT-CVB3
UV-CVB3
N=7
N=7
*
*
0
3
6
9
12
Days after viral injection
C.
UV-CVB3
WT-CVB3
UV-CVB3
WT-CVB3
Day 0
Day 15
Day 0
Day 15
Day 0
Day 12
Day 0
Day 12

D.


H2030 (KRASmut)
Virus titre (log10) pfu/g
10
8
6
4
2
0
Tumor
Heart
Lung
Pancreas
Liver
Spleen
H1975 (EGFRmut)
Virus titre (log10) pfu/g
7
6
5
4
3
2
1
0
Tumor
Heart
Lung
Pancreas
Liver
Spleen

E.

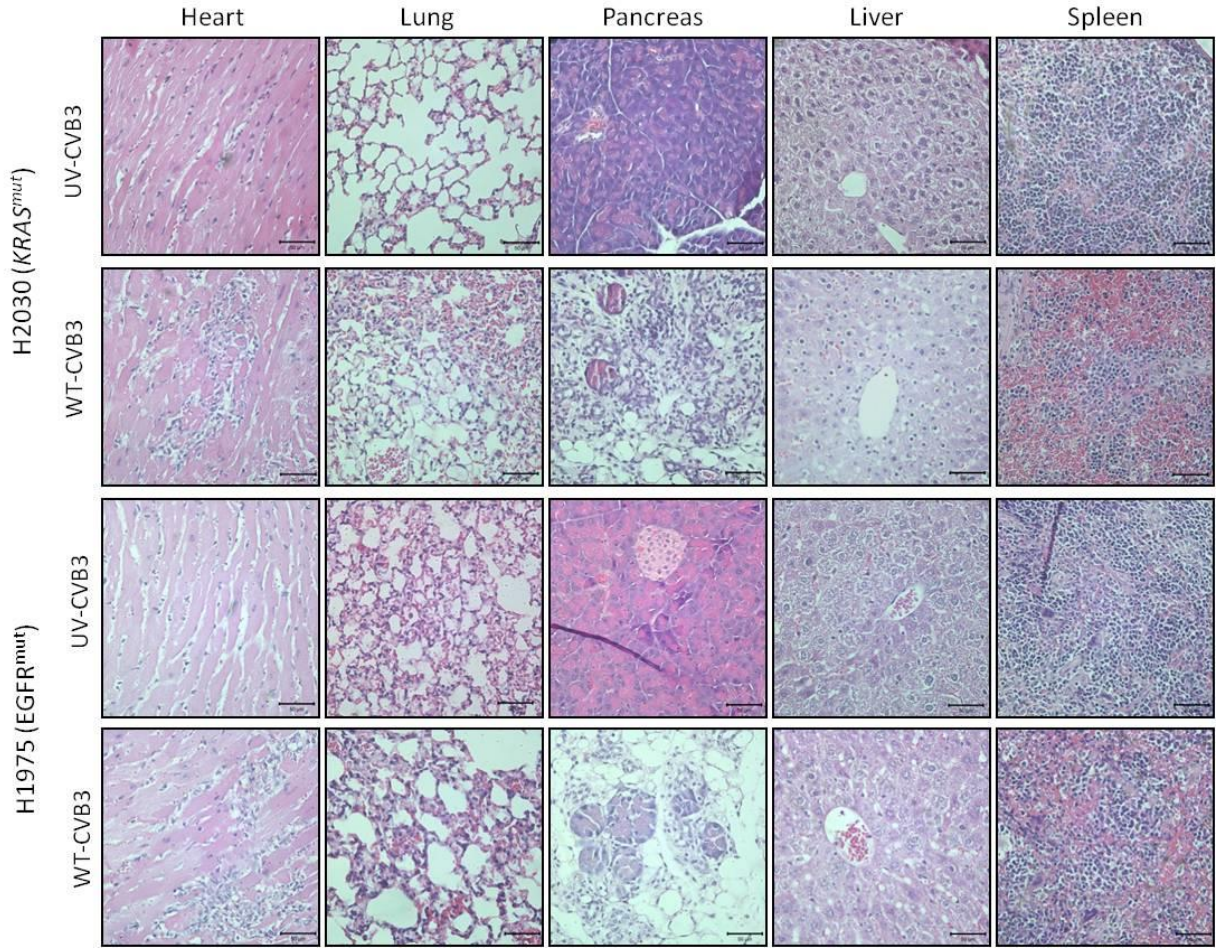

Heart
Lung
Pancreas
Liver
Spleen
H2030 (KRASmut)
UV-CVB3
WT-CVB3
H1975 (EGFRmut)
UV-CVB3
WT-CVB3

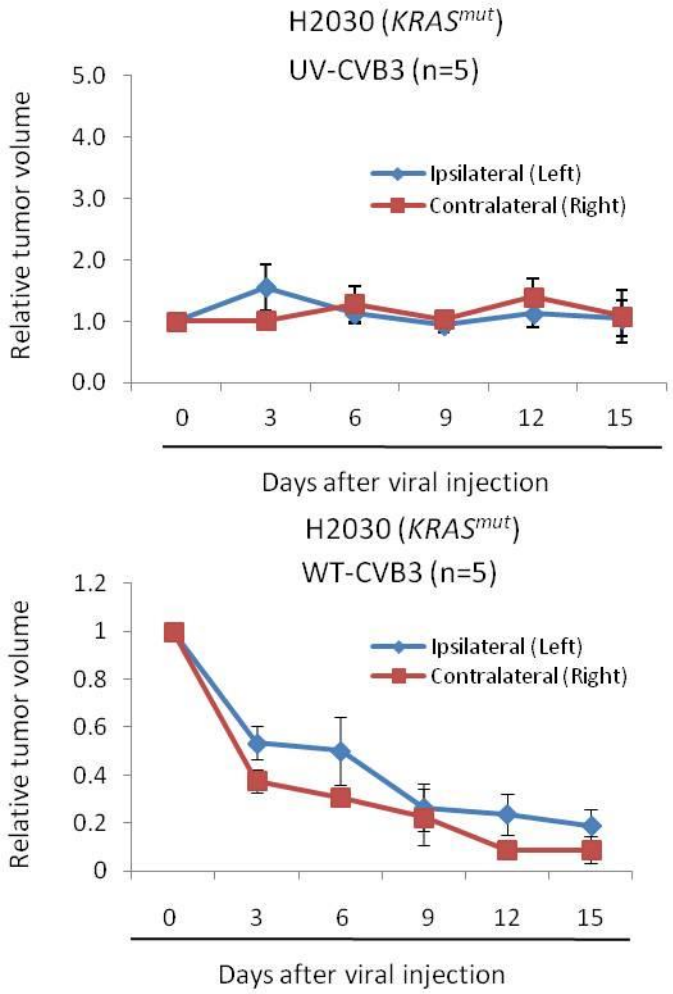
Figure 8. Intratumoral Injection of CVB3 Leads to a Significant Regression of *KRAS^{mut}* Xenograft NSCLC in NSG Immunodeficient Mice. (A & B) Patient-derived *KRAS^{mut}* H2030 or *EGFR^{mut}* H1975 cells (5×10^6 cells) were injected subcutaneously into the left flank of NSG immunodeficient mice. When tumors reached a palpable size, mice were intratumorally injected with a single dose of WT-CVB3 (5×10^4 PFU) or UV-inactivated CVB3 in the left flank. UV-CVB3 was given as a negative control. Tumor volumes of *KRAS^{mut}* (A) or *EGFR^{mut}* (B) xenografts were measured every 3 days and expressed as means ± SEM, *, $P<0.05$; &, $P<0.005$; #, $P<0.001$ as compared to UV-CVB3 controls. **(C)** Representative images of mice with *KRAS^{mut}* or *EGFR^{mut}* xenograft tumors treated with UV- or WT-CVB3 for different days as indicated. **(D)** Virus titres in the different organs collected from *KRAS^{mut}* or *EGFR^{mut}* xenograft mice treated with WT-CVB3 at the end of experiment. Results are presented as means ± SD (n=3). **(E)** H&E staining of different organs harvested from *KRAS^{mut}* or *EGFR^{mut}* xenograft mice. Images were taken using the SPOT Insight camera and Nikon ECLIPSE E600 microscope at × 40 magnification. Scale bar=50μm.

3.3.4 Intratumoral Injection of CVB3 Results in a Significant Reduction in *KRAS^{mut}* Tumor Size with Attenuated Damage to Normal Tissues in NOD-SCID Immunocompromised Mice

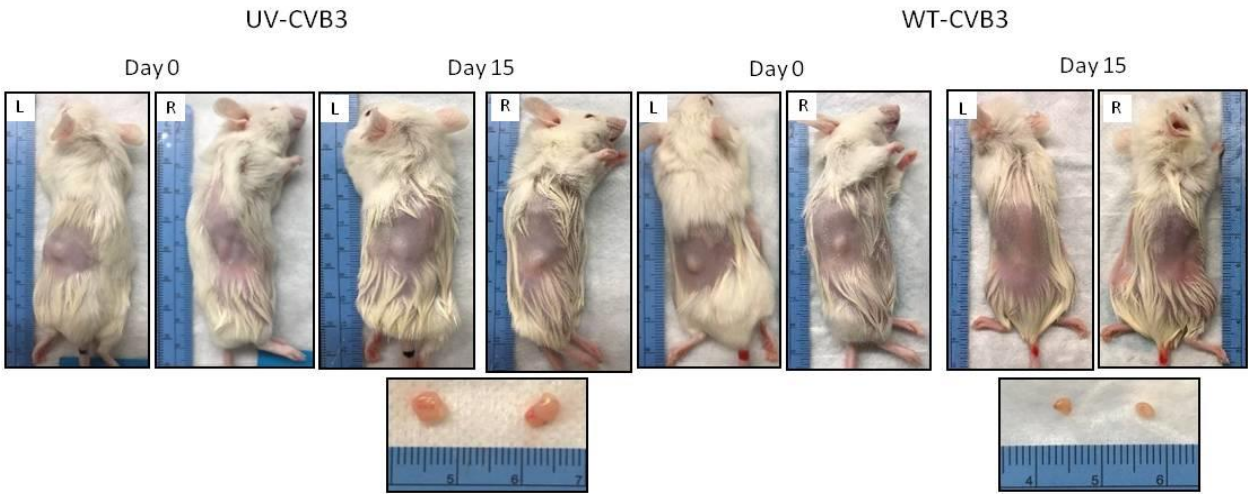
It is well known that the host innate immune response plays a crucial role in limiting viral spread. To determine whether partial recovery of innate immunity can attenuate CVB3-induced tissue injury, we carried out the xenograft experiments using NOD-SCID mice, which have residual innate immunity including defective NK cells, macrophages, granulocytes, and complement[313]. In order to investigate both local and systemic oncolytic effects of CVB3, NSCLC cells were injected subcutaneously into the bilateral flanks of the mice. We showed that the tumor

volumes of *KRAS*^{mut} xenografts on both sides significantly decreased after left tumor injection of one dose of WT-CVB3, whereas the tumor sizes remained unchanged after UV-CVB3 inoculation (**Figure 9A & B).** In the *EGFR*^{mut} xenograft model, upon UV-CVB3 injection, tumors continued to grow, while addition of WT-CVB3 led to a slight increase in contralateral and no changes in ipsilateral tumor volumes (**Figure 9C & D**). Together, our results indicate that innate immunity has no major impact on CVB3-mediated regression of *KRAS*^{mut} tumors and *EGFR*^{mut} xenografts remain more resistant to CVB3 than *KRAS*^{mut} xenografts in NOD-SCID mice. We further evaluated the potential cytotoxicity of CVB3 by histological analysis. H&E staining showed that CVB3-induced injury to the pancreas was markedly reduced in NOD-SCID mice as compared to in NSG mice; however, myocardial damage still occurred (**Figure 9E**). Finally, immunohistochemical staining was conducted to assess cell proliferation and apoptosis in xenografts. Since *KRAS*^{mut} xenograft tumors exposed to WT-CVB3 almost disappeared at the end of the experiment, here we focused on *EGFR*^{mut} xenograft tumors. **Figure 9F** demonstrated the presence of positive ki67 cells, suggesting active tumor cell proliferation. Positive immunoreactivity for cleaved caspase-3 was detected in WT-CVB3-treated tumors, but not in the UV-CVB3 group, indicative of apoptosis induced by CVB3.

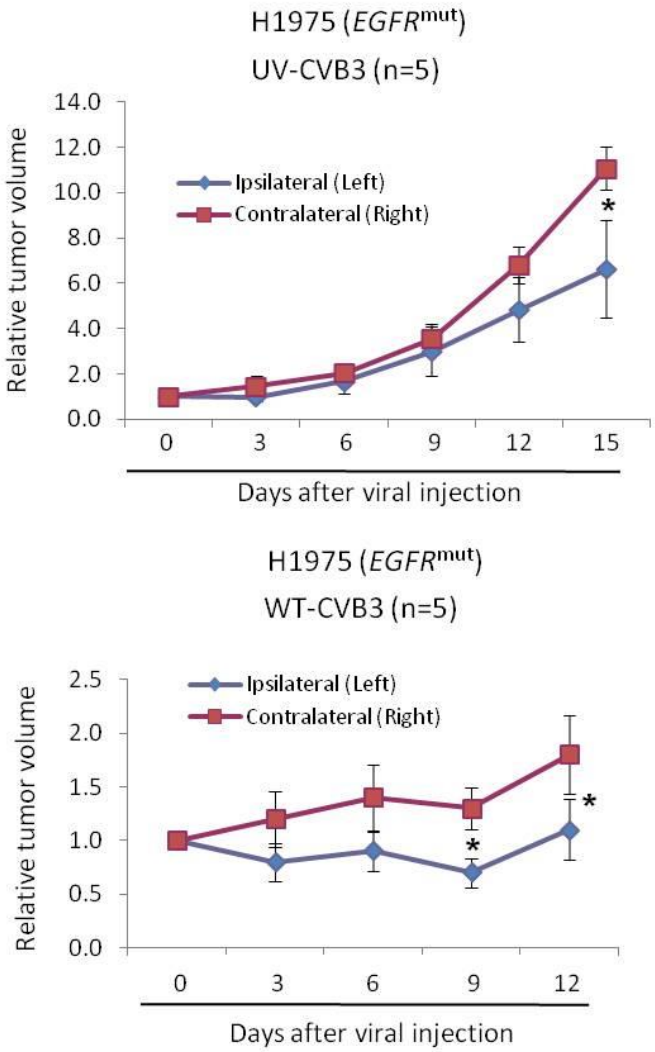
A.


H2030 (KRASmut)
UV-CVB3 (n=5)
Relative tumor volume
5.0
4.0
3.0
2.0
1.0
0.0
Ipsilateral (Left)
Contralateral (Right)
0
3
6
9
12
15
Days after viral injection
H2030 (KRASmut)
WT-CVB3 (n=5)
Relative tumor volume
1.2
1
0.8
0.6
0.4
0.2
0
Ipsilateral (Left)
Contralateral (Right)
0
3
6
9
12
15
Days after viral injection

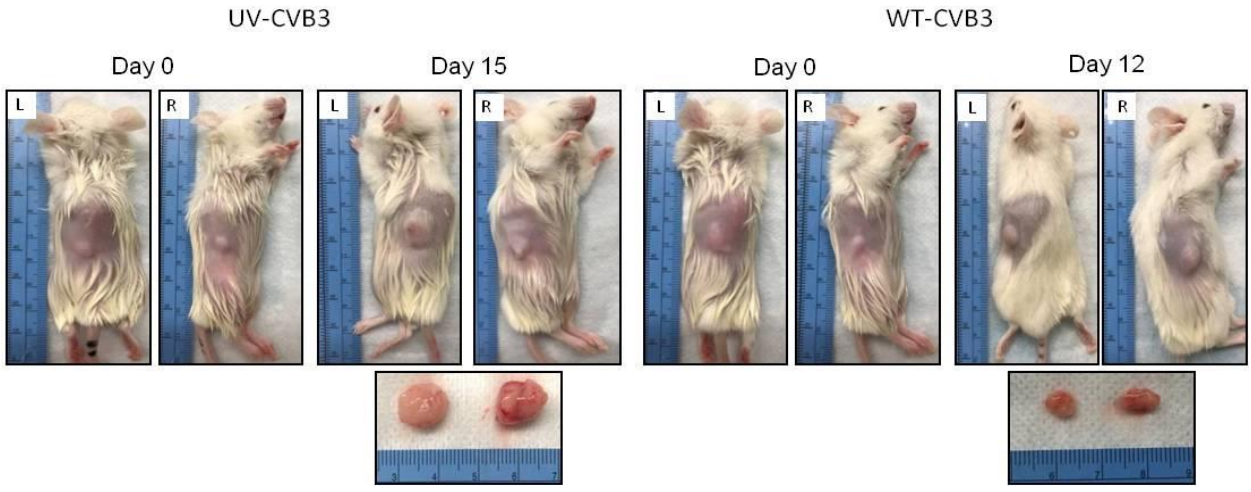
B.


UV-CVB3
WT-CVB3
Day 0
Day 15
Day 0
Day 15
L
R
L
R
L
R
L
R

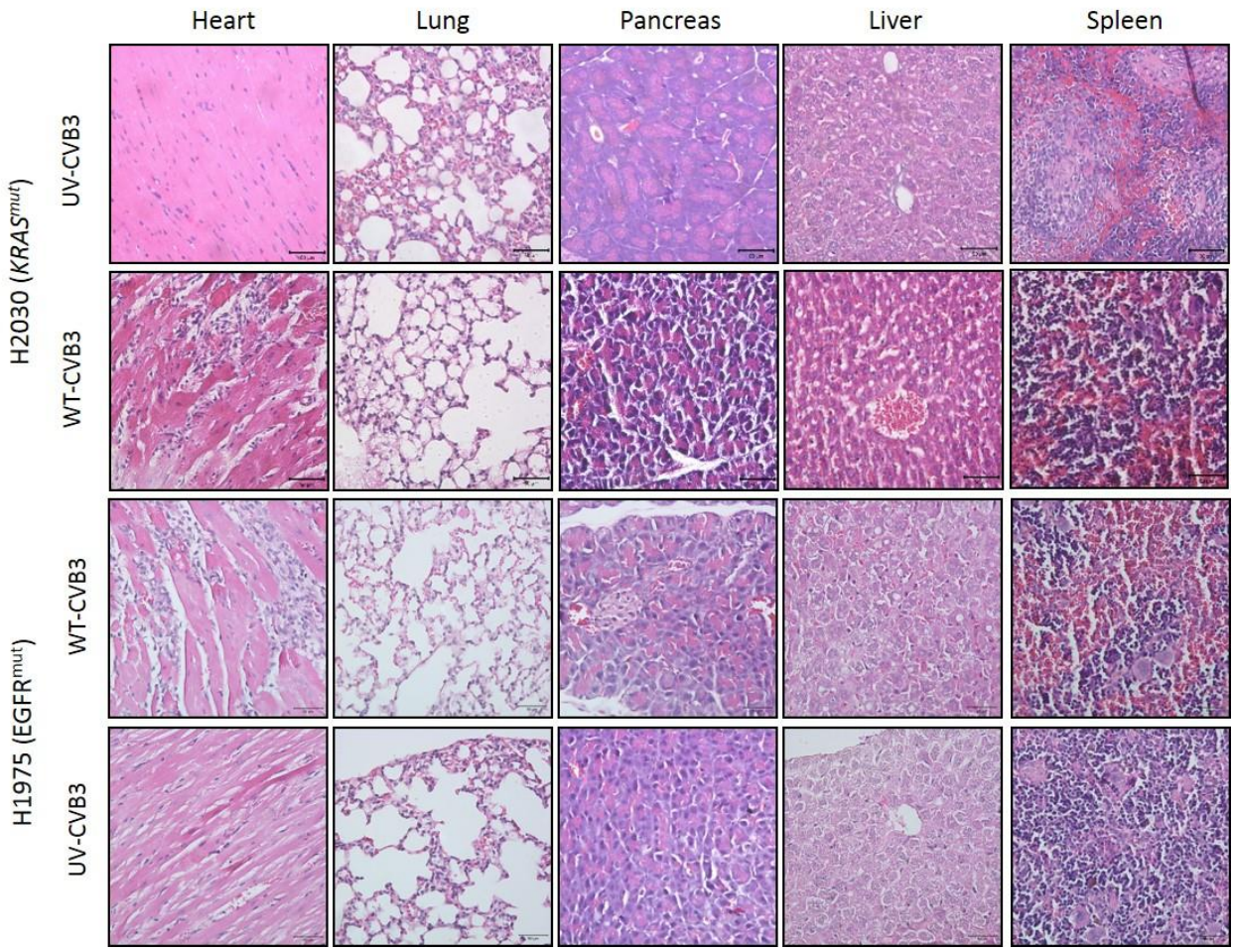
C.


H1975 (*EGFR*^{mut})
UV-CVB3 (n=5)
Relative tumor volume
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Ipsilateral (Left)
Contralateral (Right)
*
0
3
6
9
12
15
Days after viral injection
H1975 (*EGFR*^{mut})
WT-CVB3 (n=5)
Relative tumor volume
2.5
2.0
1.5
1.0
0.5
0.0
Ipsilateral (Left)
Contralateral (Right)
*
*
0
3
6
9
12
Days after viral injection

D.


UV-CVB3
WT-CVB3
Day 0
Day 15
Day 0
Day 12
L
R
L
R
L
R
L
R

E.


Heart
Lung
Pancreas
Liver
Spleen
H2030 (KRAS^mut)
UV-CVB3
WT-CVB3
H1975 (EGFR^mut)
WT-CVB3
UV-CVB3

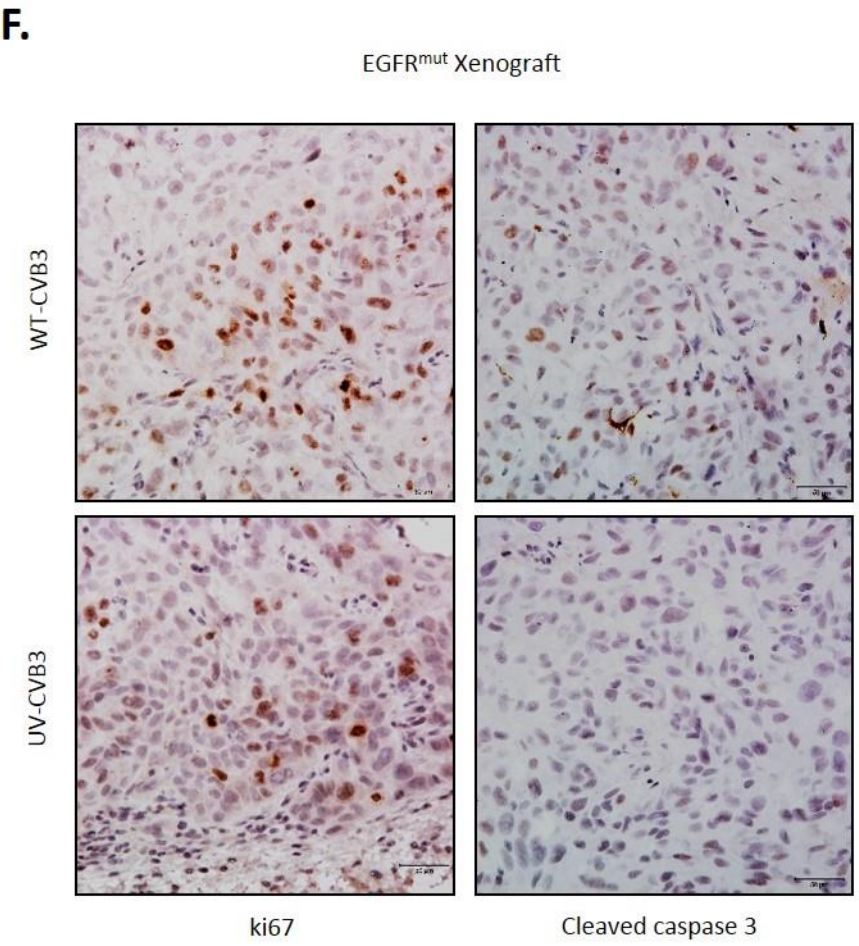


Figure 9. Intratumoral injection of CVB3 results in a significant reduction in *KRAS^{mut}* tumor size with attenuated damage to normal tissues in NOD-SCID immunocompromised mice. (A & C) Patient-derived *KRAS^{mut}* H2030 or *EGFR^{mut}* H1975 cells (5×10^6 cells) were injected subcutaneously into both flanks of NOD-SCID mice. When tumors reached a palpable size, the mouse was intratumorally injected with a single dose of WT-CVB3 (5×10^4 PFU) or UV-CVB3 in the left flank as indicated. Tumor volumes of *KRAS^{mut}* (A) or *EGFR^{mut}* (C) xenografts were measured every 3 days and expressed as means ± SEM. *, $P<0.05$ as compared to UV-CVB3 controls. **(B & D)** Representative images of animals with *KRAS^{mut}* (B) or *EGFR^{mut}* (D) xenograft tumors treated with UV- or WT-CVB3 for different days as indicated. **(E)** H&E staining of

different organs harvested from *KRAS*^{mut} or *EGFR*^{mut} xenograft mice at the end of the experiment. Scale bar=50μm. **(F)** IHC staining of *EGFR*^{mut} xenograft tumors for Ki67 and cleaved caspase-3. Scale bar=50μm.

3.3.5 Enhanced Expression of CAR in *KRAS*^{mut} Cells Is Associated with Increased Susceptibility of NSCLCs to CVB3

Lastly, we investigated the potential mechanism by which CVB3 preferentially replicates in *KRAS*^{mut} NSCLCs. CAR is the primary receptor responsible for CVB3 internalization[314]. A previous study has shown that deletion of CAR in the adult heart can significantly eliminate cardiac CVB3 infection, indicating an intimate link between the levels of CAR and the cell permissiveness to CVB3[315]. Here we questioned whether the differential susceptibilities of *KRAS*^{mut} and *EGFR*^{mut} NSCLC cells and normal lung epithelial cells to CVB3-induced cytotoxicity are due to the difference of the expression levels of CAR. We first compared CAR protein levels among nine different cell lines. As shown in **Figure 10A**, protein levels of CAR were noticeably higher in *KRAS*^{mut} NSCLC cells as compared to *EGFR*^{mut} NSCLC cells and normal lung epithelial cells. We then examined the CAR expression in HPL1D stable cells and demonstrated that CAR was upregulated in HPL1D-*KRAS*^{mut} cells compared with HPL1D-*GFP* and HPL1D-*EGFR*^{mut} cells (**Figure 10B**). Finally, we showed that CAR expression was significantly higher in *KRAS*^{mut} than in *EGFR*^{mut} xenografts (**Figure 10C**). Collectively, our data suggest that CAR expression promotes the susceptibility of tumor cells to CVB3.

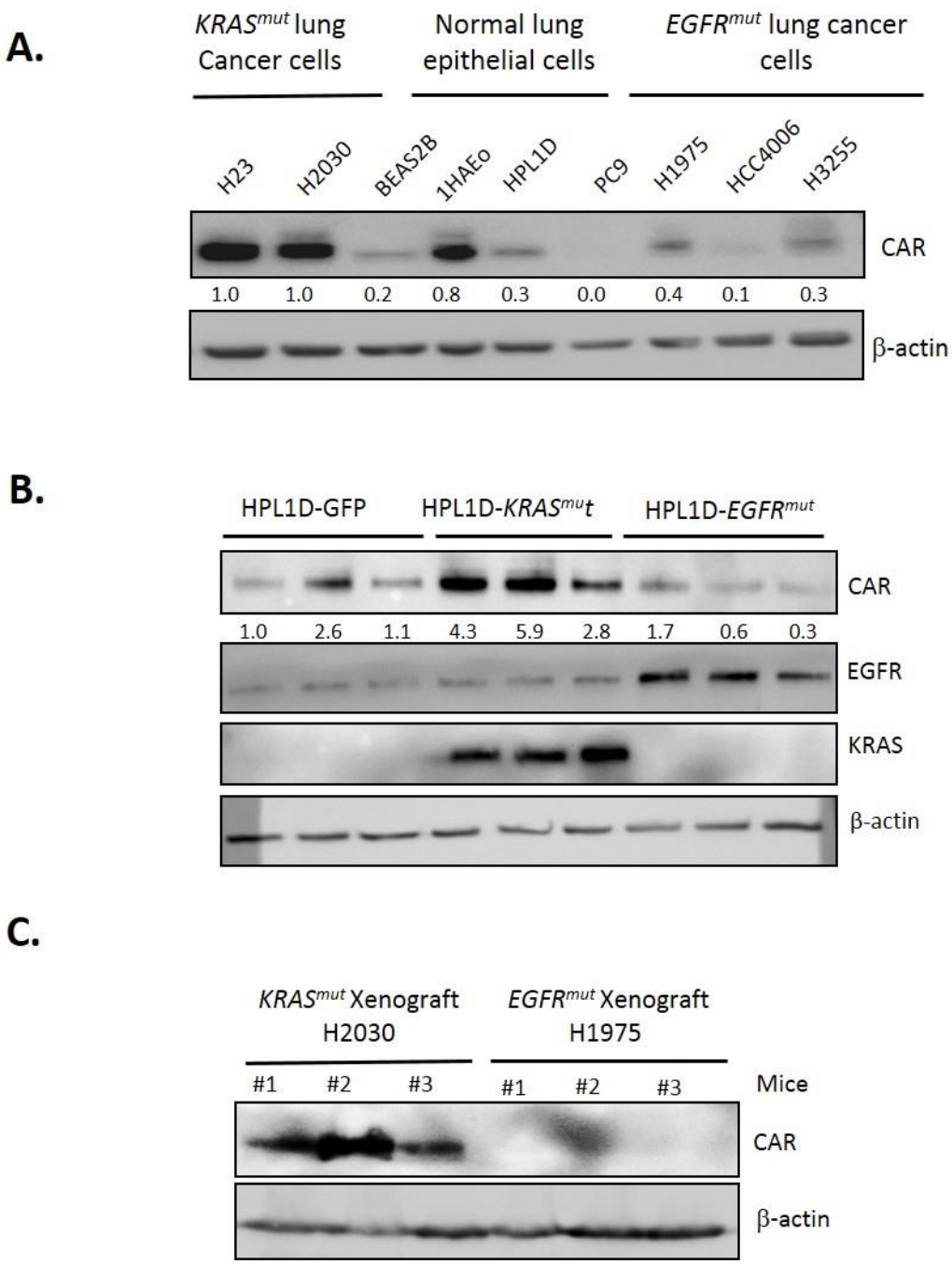


Figure 10. Enhanced Expression of CAR in *KRAS^{mut}* Cells Is Associated with Increased Susceptibility of NSCLCs to CVB3. (A-C) Protein levels of CAR in various NSCLCs and normal lung epithelial cells (A), in HPL1D cells stably expressing *GFP* (control), *KRAS^{G12V}*, or *EGFR^{L858R}*

(B), or in *KRAS*^{mut} and *EGFR*^{mut} xenograft tumors (C) by western blot analysis. CAR expression was quantitated by densitometry analysis using NIH ImageJ, normalized to β-actin and presented as fold change compared to the first lane that is arbitrarily set a value of 1.

3.3.6 Aberrant Activation of ERK1/2 Signaling in *KRAS*^{mut} NSCLC Cells Enhances CVB3 Replication

Previous *in vitro* and *in vivo* evidence demonstrated that CVB3 replication relies largely on the activation of oncogenic signaling pathways, among which ERK1/2 signaling is the best-characterized and proven to be the most-important signaling pathway hijacked by CVB3 for its effective replication[145, 267]. To determine the potential contribution of ERK1/2 activation in cell permissiveness, we examined ERK1/2 activation/phosphorylation status in different NSCLC cells. We found that the ERK1/2 was increasingly activated/phosphorylated in *KRAS*^{mut} cells as compared to *EGFR*^{mut} and normal lung epithelial cells (**Figure 11A & B**). Furthermore, we showed that inhibition of ERK1/2 using a MEK1/2 inhibitor U0126 dose-dependently decreased viral protein synthesis and virus titres in both patient-derived *KRAS*^{mut} H2030 and HPL1D-*KRAS*^{G12V} cells (**Figure 11C-F**). Taken together, our data suggest that increased CAR expression and sustained ERK1/2 activation contribute, at least in part, to the susceptibility of *KRAS*^{mut} NSCLC cells to CVB3-induced cytotoxicity.

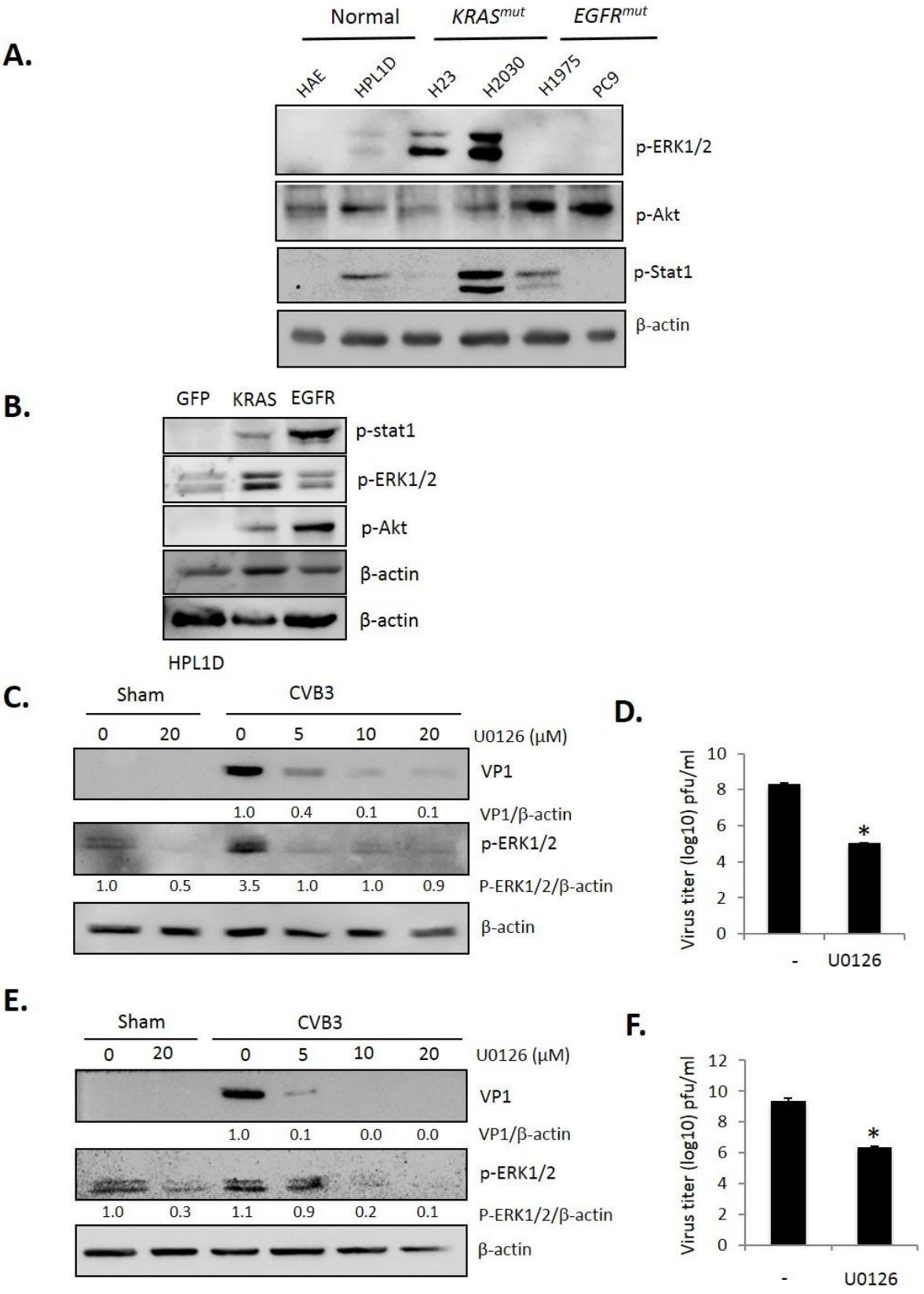

A.
Normal
KRASmut
EGFRmut
HAE
HPL1D
H23
H2030
H1975
PC9
p-ERK1/2
p-Akt
p-Stat1
β-actin
B.
GFP
KRAS
EGFR
p-stat1
p-ERK1/2
p-Akt
β-actin
β-actin
HPL1D
C.
Sham
CVB3
0
20
0
5
10
20
U0126 (μM)
VP1
1.0
0.4
0.1
0.1
VP1/β-actin
p-ERK1/2
1.0
0.5
3.5
1.0
1.0
0.9
P-ERK1/2/β-actin
β-actin
D.
Virus titer (log10) pfu/ml
10
8
6
4
2
0
*
-
U0126
E.
Sham
CVB3
0
20
0
5
10
20
U0126 (μM)
VP1
1.0
0.1
0.0
0.0
VP1/β-actin
p-ERK1/2
1.0
0.3
1.1
0.9
0.2
0.1
P-ERK1/2/β-actin
β-actin
F.
Virus titer (log10) pfu/ml
12
10
8
6
4
2
0
*
-
U0126

Figure 11. Aberrant Activation of ERK1/2 Signaling in *KRAS^{mut}* NSCLC Cells Promotes CVB3 Replication. (A & B) Levels of p-ERK1/2, p-Akt, and p-Stat1 in various NSCLC and normal lung epithelial cells (A) or in HPL1D cells stably expressing GFP (control), *KRAS^{G12V}*, or *EGFR^{L858R}* (B). **(C-F)** Inhibition of ERK1/2 activation blocks CVB3 replication in *KRAS^{mut}* cells. H2030 (C & D) and HPL1D-*KRAS^{mut}* cells (E & F) were infected with CVB3 (MOI=10) in the presence or absence of different concentrations of MEK inhibitor U0126 as indicated for 7 hrs. Cells were harvested and protein levels of p-ERK1/2, VP1 and β-actin were examined by western blotting (C & E). Densitometry analysis was performed as described in Figure 10. Culture medium was collected for plaque assay (D & F) and the results are presented as means ± SD (n=3). *, $P<0.01$ as compared to vehicle-treated controls "-".

3.4 Discussion

Emerging evidence has indicated that *KRAS* mutations are negative predictors of benefit from either adjuvant chemotherapy or TKI therapy and so far there is no effective therapy available for *KRAS^{mut}* NSCLCs[316, 317]. Because of this, *KRAS* mutations as a drug target for anti-tumor therapy have gained considerable attention in recent years. However, little progress has been made to develop specific RAS inhibitors. Here we reported that CVB3 as a natural agent can specifically target *KRAS^{mut}* NSCLCs, leading to a significant tumor regression *in vivo*. Our findings highlight that CVB3 could be an excellent candidate for further development as a novel oncolytic virus for *KRAS^{mut}* NSCLC therapy.

CVB3 is known to subvert the host signaling pathways to facilitate its own replication[127, 204, 267]. Among these pathways, MAPK module, which consists of RAF, MEK1/2, and ERK1/2, plays a central role[100, 267, 318]. As the upstream activator of the RAF/MEK1/2/ERK1/2 cascade,

small GTP-binding protein RAS activates the ERK1/2 pathway by binding RAF and anchoring it at the cell membrane, where it is activated by other kinases[319]. In NSCLCs, KRAS protein acquires an impaired GTPase activity as a result of point mutation in the gene, leading to a constitutive activation of ERK1/2 signaling[320]. In the present study, we found that specific inhibition of the ERK1/2 activation by MEK1/2 inhibitor U0126 results in a significant attenuation of virion production in $KRAS^{mut}$ NSCLC cells, suggesting that viral replication within $KRAS^{mut}$ NSCLC cells is predominantly dependent on host ERK1/2 signaling. Although EGFR is also an upstream activator of the ERK1/2 signaling pathway, we showed that the extent of ERK1/2 phosphorylation is much lower in $EGFR^{mut}$ than in $KRAS^{mut}$ NSCLCs, similar to previous reports showing that constitutive EGFR activation in $EGFR^{mut}$ NSCLCs selectively activates the Akt and/or STAT signaling pathways to promote cell survival and invasion, but has less effect on the ERK1/2 pathway that is generally associated with cell proliferation and survival[321, 322]. Thus, ERK1/2 signaling appears to be preferentially activated by $KRAS^{mut}$ rather than $EGFR^{mut}$, and the relative resistance of $EGFR^{mut}$ NSCLCs to CVB3 is likely due to latent activation of the ERK1/2 signaling pathway.

Viral entry into the cytoplasm of host cells is the first step of a successful viral life-cycle. CAR is a transmembrane receptor responsible for CVB3 entry and is hence a determinant of virus tropism[323, 324]. In cancerous cells, CAR expression is frequently lost, resulting in a significant reduction of CVB3 uptake. For example, reduced CAR expression level has been reported in prostate cancer, bladder cancer, glioma, and gastrointestinal cancer, which are refractory to oncolytic therapy[325-327]. In the current study, we found that the protein levels of CAR are markedly higher in $KRAS^{mut}$ NSCLCs than in normal lung epithelial cells and $EGFR^{mut}$ NSCLCs. We also found that upregulation of CAR is highly associated with viral tropism. The exact mechanism by

which CAR is regulated by mutated *KRAS* remains unclear. It was reported that altered CAR expression in tumor cells can be caused by multiple mechanisms, including transforming growth factor-β signaling cascade, epithelial–mesenchymal differentiation, histone deacetylation of the CAR gene promoter, hypoxia-inducible factor-1α-dependent hypoxia, and the MAPK signaling pathways[328, 329]. Given that *KRAS* activation plays distinct roles in the regulation of individual intracellular signaling cascades, leading to different biological outcomes, we speculate that multiple mechanisms are involved in the upregulation of CAR expression in $KRAS^{mut}$ cells.

It is well documented that the host immune system plays a dual role in oncolytic virotherapy[330, 331]. On the one hand, early innate immune responses to viruses result in rapid viral clearance; on the other hand, viral infection elicits a significant anti-tumor immune response that breaks immune tolerance and allows for long-term cancer destruction. Both direct oncolysis and anti-tumor immunity triggered by virus infection are believed to contribute to the efficacy of cancer virotherapy [331-333]. Thus, maintaining a delicate balance between the anti-viral response and the anti-tumor immunity is crucial to mediate successful anti-cancer virotherapy. In this study, we showed that the cytotoxicity caused by CVB3, especially to the pancreas, is greatly attenuated in $KRAS^{mut}$ NOD-SCID mice as compared to $KRAS^{mut}$ NSG mice, suggesting a protective function of the host innate immunity in limiting viral spread and replication. Meanwhile, we found that CVB3 injection causes a similar rate of tumor regression in both NOD-SCID and NSG mice, indicating that CVB3-mediated direct oncolytic lysis plays a predominant role in tumor reduction. It is noteworthy that CVB3 inoculation into one side of the bilateral $KRAS^{mut}$ xenografts results in significant tumor regression on both sides, suggesting a potential application of CVB3 in patients with metastatic tumors.

Our study provides the first evidence that CVB3 is a potent oncolytic virus against $KRAS^{mut}$

NSCLCs; however, several limitations have to be taken into account: 1) our *in vivo* mouse model is immunocompromised, which limits the generalizability of our findings; 2) ectopic xenograft fails to mimic the tumor microenvironment, which may result in an exaggeration of CVB3 efficiency; 3) cardiotoxicity of CVB3 causes unsatisfactory survival rates. Hence, in the future we will develop a non-cardiovirulent CVB3 to reduce potential cardiotoxicity and use an immunocompetent orthotropic animal model to investigate whether host immunity plays a synergetic role with direct oncolysis in killing tumors.

In conclusion, our study suggests that CVB3 selectively kills $KRAS^{mut}$ NSCLCs mainly via the virus self-replication process. The potential application of CVB3 as an oncolytic therapy may provide a new direction for refractory $KRAS^{mut}$ NSCLC.

Chapter 4: Enhanced Enteroviral Infectivity via Viral-Protease-Mediated Cleavage of GAB1

4.1 Background

CVB3-induced cardiac damage has become an obstacle for developing CVB3 as an oncolytic virus as no effective therapy is available for the treatment of myocarditis and DCM. Although it is widely accepted that myocytolysis caused by replicating viruses is a major part of virus-mediated damage to the heart, the underlying molecular mechanisms by which cardiotropic viruses cause myocarditis and DCM remain unclear. The generation of cardiac-specific knockout mice provides a more definitive clue regarding the pathogenesis of myocarditis and DCM. Our *in vivo* study showed that deletion of cardiac GAB1 is associated with DCM in both human and mouse hearts, shedding light on a potential crucial role for GAB1 in maintaining heart function[243].

GAB1 is a scaffolding adaptor protein belonging to the family of insulin receptor substrate 1-like multi-substrate proteins[244]. Emerging evidence has suggested that signaling mediated through GAB1 plays a critical role in the regulation of a variety of cellular processes, including cell proliferation, cell differentiation, apoptosis, and stress responses [234]. GAB1 deficiency results in embryonic lethality due to severe defects in heart, placenta, liver, and spleen development[241]. Disruption of GAB1-mediated signaling has been associated with multiple human diseases, including tumor, cardiovascular disease, and inflammation [242]. Functionally, GAB1 serves as a platform for assembling multiple intracellular signaling pathways evoked by various extracellular stimuli via its multiple functional domains, including a highly conserved PH domain at its N-terminal, a specific c-Met binding domain (MBD), proline-rich regions, and multiple tyrosine and serine/threonine phosphorylation residues [225, 234, 334, 335]. Upon activation, GAB1 translocates from

cytoplasm to the cellular membrane where it promotes signaling amplification and transduction by tyrosine phosphorylation and recruitment of downstream proteins, such as SHP2, p85, Crk, and PLC γ, which further contributes to the activation of ERK1/2, PI3K, JNK, and transducer and activator of transcription 5 (STAT5) signaling pathways, respectively [334, 336, 337].

Similar to many other viruses, CVB3 has evolved diverse mechanisms to modulate the host signaling machinery to ensure successful viral infection[100, 271, 338]. We have previously revealed an important role for the ERK1/2 signaling pathway in regulating viral replication[267]. Nevertheless, despite the profound effects of the ERK1/2 signaling pathway on virus propagation, the upstream factors that regulate its activation remain poorly understood.

Given the important function of GAB1 in transducing signals from extracellular cues, we questioned whether CVB3 could manipulate GAB1 in order to gain advantage on viral replication.

4.2 Specific Aims

The OBJECTIVE of this chapter is to elucidate the mechanism by which CVB3 manipulates GAB1-mediated signaling pathway to gain survival benefit.

The SPECIFIC AIMS include:

Aim1: To examine the protein expression level of GAB1 following CVB3 infection;

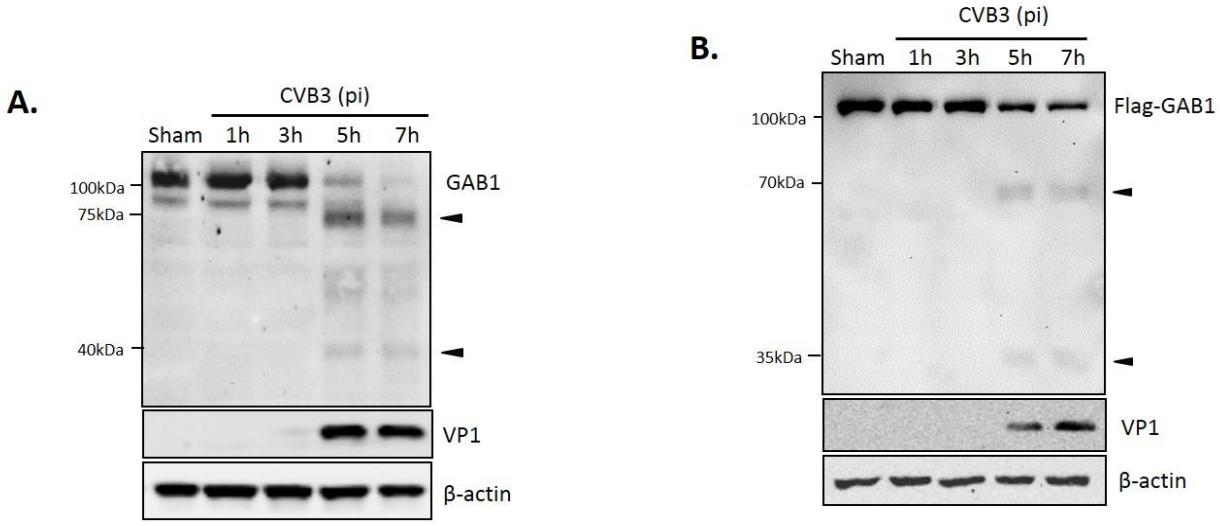
Aim2: To elucidate the underlying mechanism by which CVB3 regulates GAB1 expression;

Aim3: To study the functional consequences of GAB1 dysregulation in virus replication;

4.3 Results

4.3.1 GAB1 Is Cleaved during CVB3 Infection

Given the significance of GAB1 in the activation of the MAPK/ERK pathway, we first determined the effect of CVB3 infection on the protein expression of GAB1. We demonstrated that the protein level of GAB1 decreased following CVB3 infection, accompanied by the manifestation of two additional bands (~75kDa and ~40kDa, respectively) using an anti-GAB1 antibody that targets residues surrounding Tyr472 of human GAB1 (**Figure 12A**). To verify whether the production of these extra bands is a result of GAB1 cleavage, we transiently transfected a plasmid expressing N-terminal Flag-tagged GAB1 into HeLa cells, then infected cells with CVB3. Western blotting using an anti-Flag antibody that targets the N-terminal region of GAB1 detected two cleavage fragments of GAB1 (~70kDa and ~35kDa, respectively), corresponding well with the findings of endogenous GAB1 (**Figure 12B**). The structure of full-length GAB1 with various functional domains, the resulting cleavage fragments, and the regions that individual antibodies detected are illustrated in **Figure 12C**. Together, our results suggest that GAB1 is cleaved during CVB3 infection.



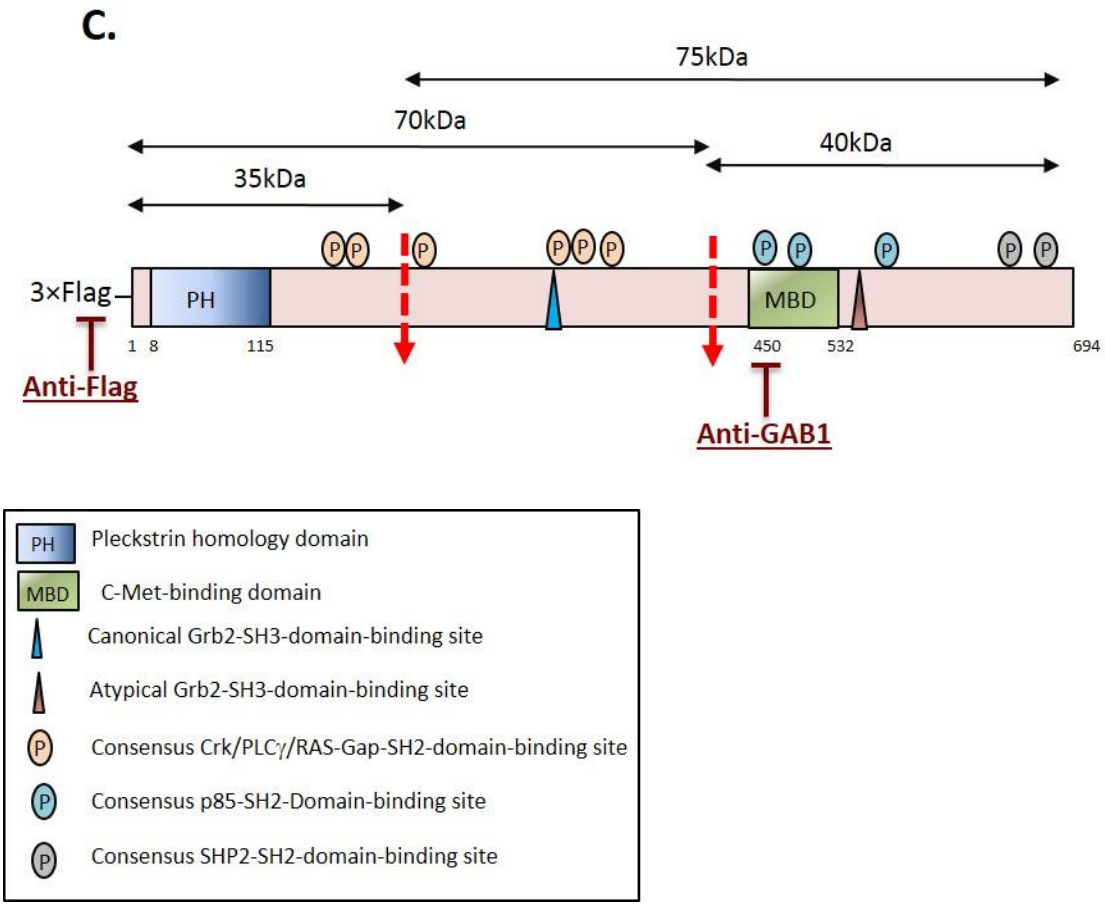


Figure 12. GAB1 Is Cleaved during CVB3 Infection.

(A) Cleavage of endogenous GAB1 following CVB3 infection. HeLa cells were sham- or CVB3-infected at MOI 10 for various time points as indicated. Cell lysates were collected and processed for western blotting for detection of viral capsid protein VP1, and GAB1 protein expression (using an anti-GAB1 antibody targeting residues surrounding Tyr472 of human GAB1). The protein level of β-actin was examined as a loading control. (B) Cleavage of exogenous GAB1 following CVB3 infection. HeLa cells were transiently transfected with a plasmid expressing N-terminal Flag-tagged GAB1 (3×Flag-GAB1) for 24 hrs, followed by CVB3 infection for different time points as indicated. Western blotting was performed to assess the protein levels of exogenous

GAB1 (using anti-Flag antibody), VP1, and β-actin. Arrowheads indicate CVB3-induced GAB1 cleavage fragments. (C) Schematic diagram of full-length GAB1 with various functional domains, the resulting cleavage fragments, and the regions that individual antibodies detected. Red arrows indicate two potential cleavage sites.

4.3.2 GAB1 Is Cleaved by Viral Protease $2A^{pro}$

To further determine the potential mechanisms by which CVB3 infection results in the cleavage of GAB1, we utilized an ultraviolet (UV)-irradiated CVB3 (UV-CVB3) that is unable to self-replicate but maintains virus-host receptor binding. Similar to the result shown in Figure 12, infection with WT-CVB3 led to the formation of the 75kDa and 40kDa cleavage fragments, while these proteolytic products were not detected in UV-CVB3-infected cells (**Figure 13A**), indicating that GAB1 cleavage is associated with CVB3 replication. We then questioned whether this cleavage is mediated through the function of virus-encoded proteases. *In vitro* cleavage assay showed that incubation with WT-viral protease $2A^{pro}$, but not catalytic inactive $2A^{pro}$ (**Figure 13B**) nor viral protease $3C^{pro}$ (data not shown), induced the production of cleaved GAB1 fragments, suggesting that cleavage of GAB1 is triggered by viral protease $2A^{pro}$. It was previously reported that GAB1 can be cleaved by caspases in cells undergoing apoptosis. Caspase activation is a late cellular event compared with accumulation of viral proteases during CVB3 infection. However, to further eliminate the possibility of caspase-induced cleavage of GAB1, HeLa cells were treated with z-VAD-fmk, a pan-caspase inhibitor. As shown in **Figure 13C**, caspase inhibition did not block the cleavage of GAB1. Collectively, our results suggest that CVB3-induced GAB1 cleavage is an outcome of viral replication, relying on the function of viral protease $2A^{pro}$, but independent of caspase activities.

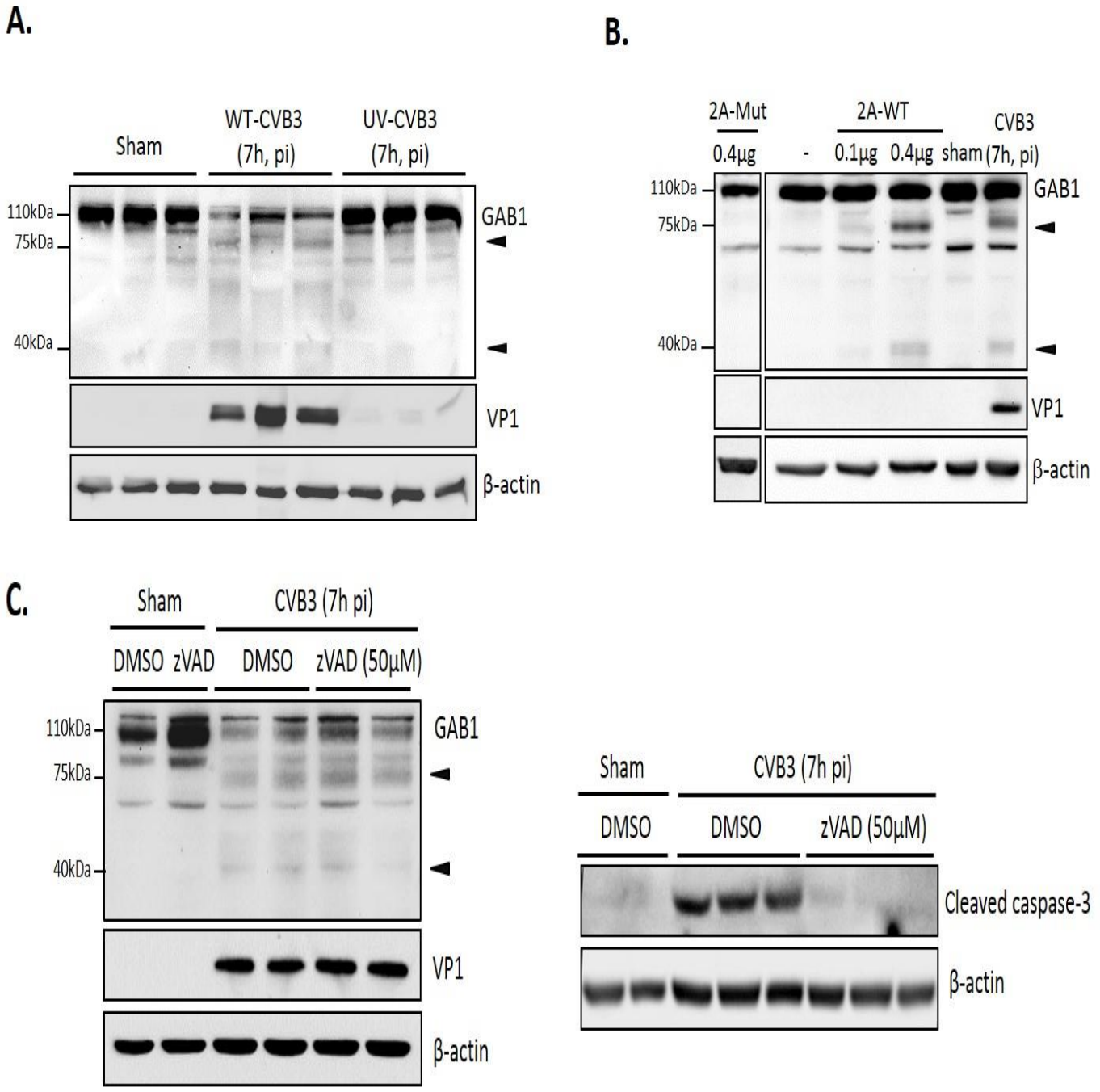


Figure 13. GAB1 Is Cleaved by Viral Protease 2A^{pro}.

(A) CVB3-induced cleavage of GAB1 is dependent on viral protein production. HeLa cells infected with either WT-CVB3 or UV-CVB3 were harvested at 7 hrs post-infection (pi). Cell

lysates were processed for western blotting to determine the protein expression levels of GAB1 (using anti-GAB1 antibody), VP1 and β-actin. (B) Cleavage of GAB1 is mediated by viral protease $2A^{pro}$. Cell lysates (50μg) from HeLa cells transfected with Flag-GAB1 were incubated with either purified viral protease $2A^{pro}$ (0.1 or 0.4μg) or catalytically inactive mutant $2A^{pro}$ (0.4μg) for overnight and *in vitro* cleavage assay was conducted as described in the "Materials and Methods". Protein levels of GAB1 (using anti-GAB1 antibody), VP1 and β-actin were examined by western blotting. CVB3-infected HeLa cell lysates (7 hrs pi) were loaded (right lane) as a positive control for GAB1 cleavage. (C) Cleavage of GAB1 following CVB3 infection is independent of caspase activation. HeLa cells were infected with CVB3 in the presence or absence of a pan-caspase inhibitor, z-VAD-fmk (50μM), for 7 hrs. Protein levels of GAB1 (using anti-GAB1 antibody), VP1, cleaved caspase-3, and β-actin were examined by western blotting. Arrowheads indicate CVB3-induced GAB1 cleavage fragments.

4.3.3 Viral Protease $2A^{pro}$ Cleaves GAB1 at G436 and G175

To identify the potential cleavage sites on GAB1 by viral protease $2A^{pro}$, amino acid sequence of human GAB1 was analyzed and two potential cleavage sites (glycine175 and glycine436) were identified based on the consensus cleavage motif by $2A^{pro}$ (The cleavage recognition site by $2A^{pro}$ protease usually contains a T (threonine), S (serine), or N (asparagine) at position P2 and an L (leucine), I (isoleucine), or M (methionine) at position P4. A G (glycine) residue at the P1' C-terminal side of the cleavage site takes place in all known substrates of $2A^{pro}$ [133]) and the size of the cleavage products. Two GAB1 mutants were then established by site-directed mutagenesis through replacing the glycine (G) at amino acid 175 and 436 with glutamic acid (E), respectively. Using anti-GAB1 antibody, we demonstrated that CVB3 infection failed to

induce the generation of the 75kDa band in cells expressing 3×Flag-GAB1^{G175E} (**Figure 14A**) and the formation of the 40kDa products in cells expressing 3×Flag-GAB1^{G436E} (**Figure 14B**) as compared to WT-GAB1 control. These results indicate that G175 and G436 are targeted during CVB3 infection by 2A^{pro}, generating the 75kDa and 40kDa products, respectively (**Figure 14C**).

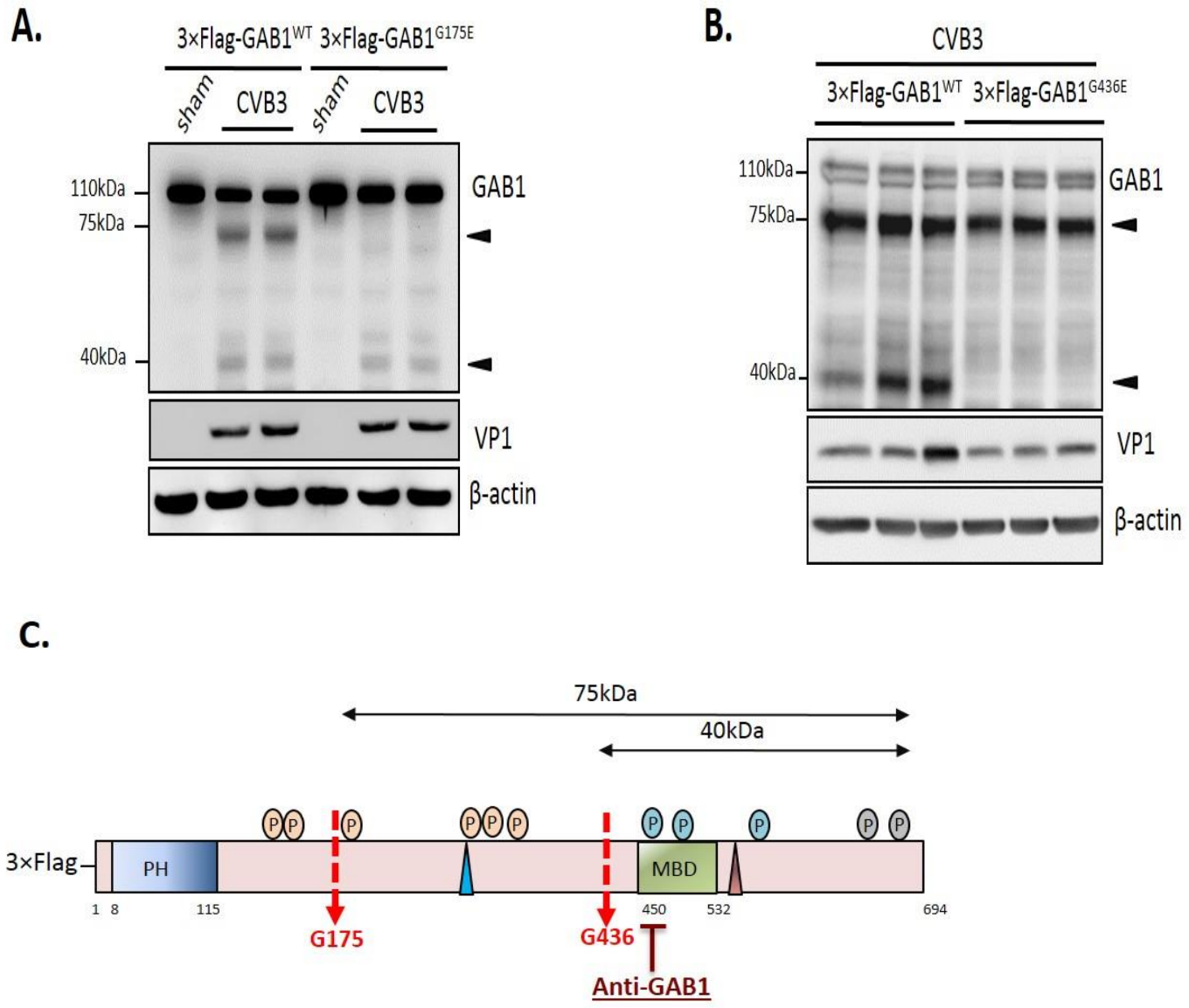


Figure 14. GAB1 Is Cleaved at G175 and G436 during CVB3 Infection.

(A) GAB1^{G175E} mutant blocks the generation of the 75kDa cleavage product triggered by CVB3 infection. HeLa cells were transiently transfected with either 3×Flag-GAB1^{WT} or 3×Flag-GAB1^{G175E} for 24 hrs, followed by sham or CVB3-infection for 7 hrs. Cell lysates were harvested for western blot analysis to detect the cleaved fragments of GAB1 using anti-GAB1 antibody. VP1 and β-actin were determined as an infection and a loading control, respectively. (B) GAB1^{G436E} mutant inhibits the production of the 40kDa product. The same protocol was performed as described above using either plasmid of 3×Flag-GAB1^{WT} or 3×Flag-GAB1^{G436E}. Arrowheads indicate CVB3-induced GAB1 cleavage fragments detected by anti-GAB1 antibody. (C) Schematic diagram of full-length GAB1 with two cleavage sites at amino acid G175 and G436, respectively.

4.3.4 Knockdown of GAB1 Inhibits Viral Replication

To determine the functional significance of GAB1 in the course of CVB3 infection, GAB1 was knocked down by siRNA in HeLa cells. We demonstrated that gene-silencing of GAB1 led to a marked reduction of viral protein expression and ERK1/2 phosphorylation (**Figure 15A**), as well as a significant decrease of virus titers (~2.9 fold) (**Figure 15C**). Our results indicate a pro-viral function of GAB1, probably through regulating the activation of the MAPK/ERK signaling pathway.

We also examined the effects of overexpression of GAB1 on viral replication. Interestingly, we found that forced expression of GAB1 did not further increase viral protein expression (**Figure 15B**) and virus titers (**Figure 15C**), suggesting that the level of endogenous GAB1 may be already high or saturated and exogenous addition of GAB1 fails to trigger enhanced viral replication.

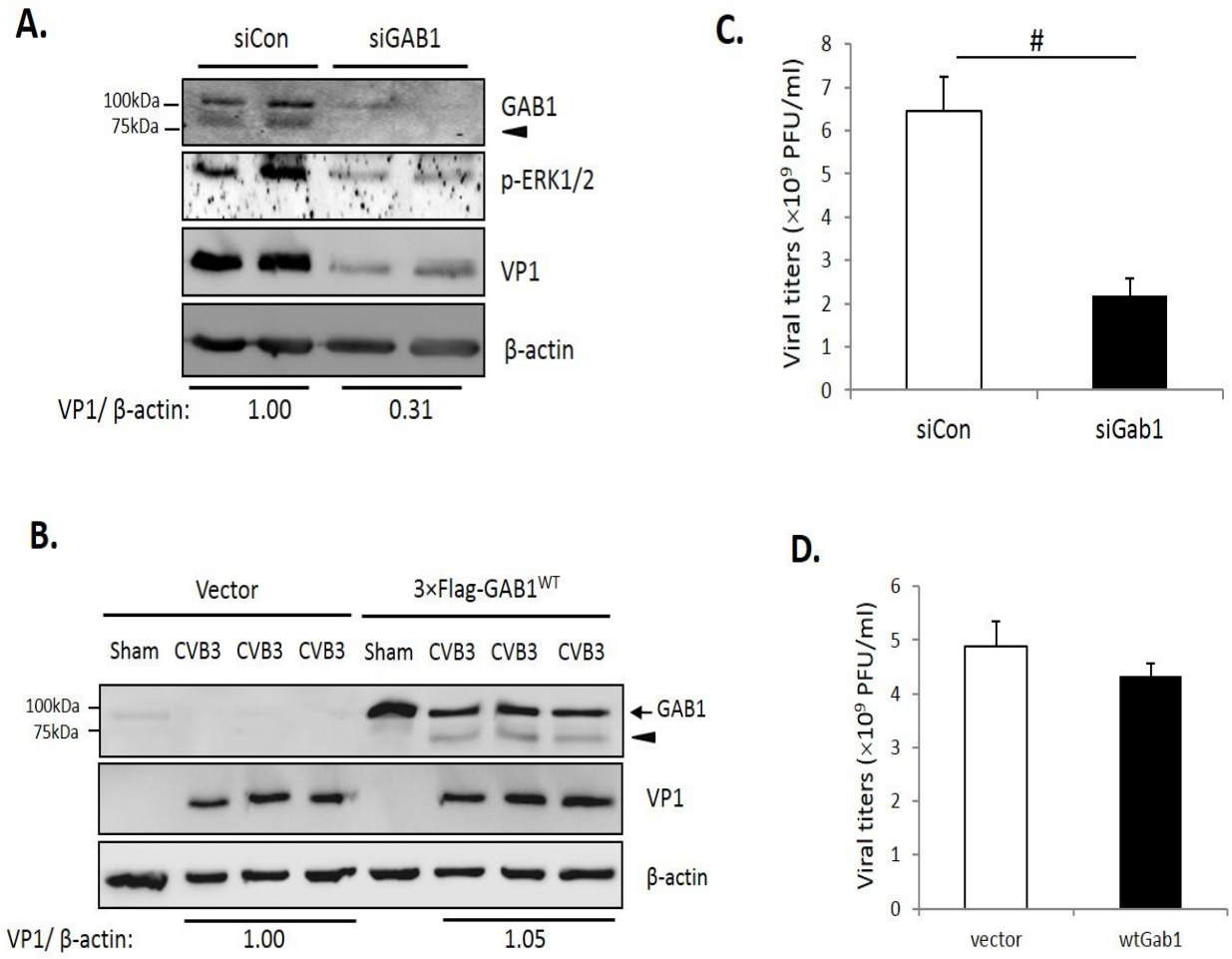


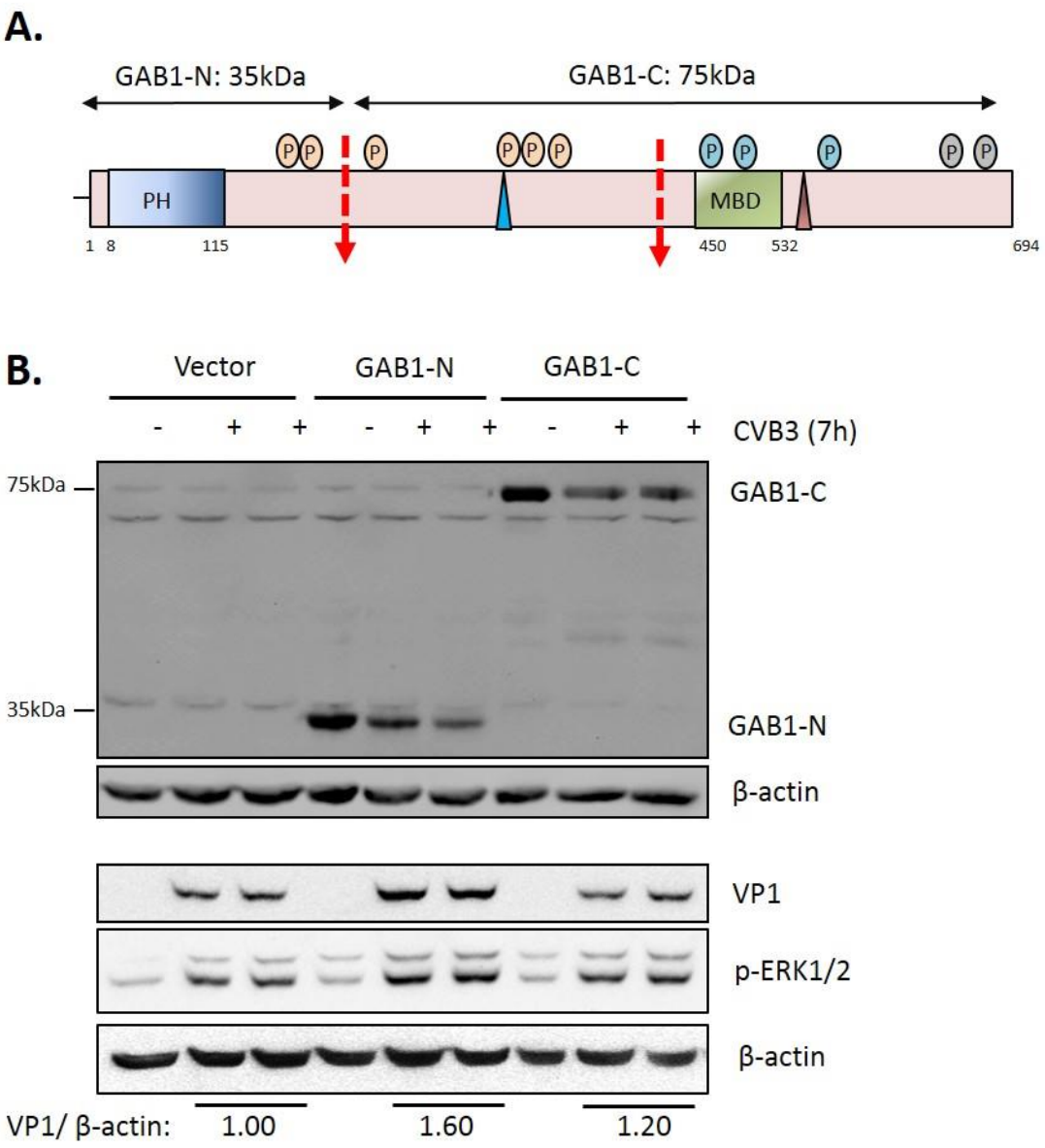
Figure 15. Knockdown of GAB1 Inhibits CVB3-Induced ERK Phosphorylation and Viral Replication. (A) HeLa cells were treated with either control siRNA (siCon) or GAB1-targeting siRNA (siGAB1) for 48 hrs, followed by CVB3 infection for 7 hrs. Cell lysates were harvested to examine the protein levels of GAB1 (using anti-GAB1 antibody), VP1, p-ERK1/2 and β-actin by western blotting. Protein levels of VP1 were quantitated by densitometric analysis using NIH ImageJ, normalized to β-actin, and presented underneath as fold changes compared to control group, which was arbitrarily set a value of 1. (B) HeLa cells were transiently transfected with 3×Flag-GAB1^{WT}, or corresponding vector control for 24 hrs, followed by CVB3 infection for 7 hrs. Western blotting was performed and analyzed as described above. (C, D) Supernatants were

collected from (A) and (B) for plaque assay and the results are presented as mean ± SD (n=4. #p<0.001).

4.3.5 The N-terminal Cleavage Fragment of GAB1 (GAB1-N) Promotes CVB3 Replication via Further Enhancing ERK1/2 Phosphorylation

Next, we asked whether CVB3-induced GAB1 cleavage results in a loss-of-function or a gain-of-function of GAB1 in the context of viral replication. The N-terminal (N1-174) and C-terminal (C175-694) fragments of GAB1 (**Figure 16A**), the predominant cleavage products observed during CVB3 infection, were sub-cloned into a Flag-tagged vector. The results showed that expression of GAB1-N1-174, but not GAB1-C175-694, further enhanced viral protein expression and ERK phosphorylation (**Figure 16B**). Viral plaque assay results also demonstrated a significantly increase in virus titers in the supernatants of cells expressing GAB1-N1-174 (**Figure 16D**). To further explore the role of ERK activation in GAB1-N1-174-induced viral replication, HeLa cells were treated with MEK inhibitor U0126. We demonstrated that inhibition of ERK phosphorylation attenuated GAB1-N1-174-induced augmentation of viral protein expression (**Figure 16C**) and virus titers (**Figure 16D**), indicating that GAB1-N1-174 promotes CVB3 replication, at least in part, via enhancing ERK1/2 phosphorylation.

The pro-viral role of the cleavage products of GAB1 in viral replication was further supported by the findings that expression of a non-cleavable GAB1 mutant ($GAB1^{G175E}$), which fails to produce GAB1-N1-174, resulted in decreased viral protein expression (**Figure 16E**) and reduced virus titers compared with $GAB1^{WT}$ control (**Figure 16F**).


A.
GAB1-N: 35kDa
GAB1-C: 75kDa
PH
MBD
1
8
115
450
532
694
B.
Vector
GAB1-N
GAB1-C
- + + - + + - + +
CVB3 (7h)
75kDa
35kDa
GAB1-C
GAB1-N
β-actin
VP1
p-ERK1/2
β-actin
VP1/ β-actin:
1.00
1.60
1.20

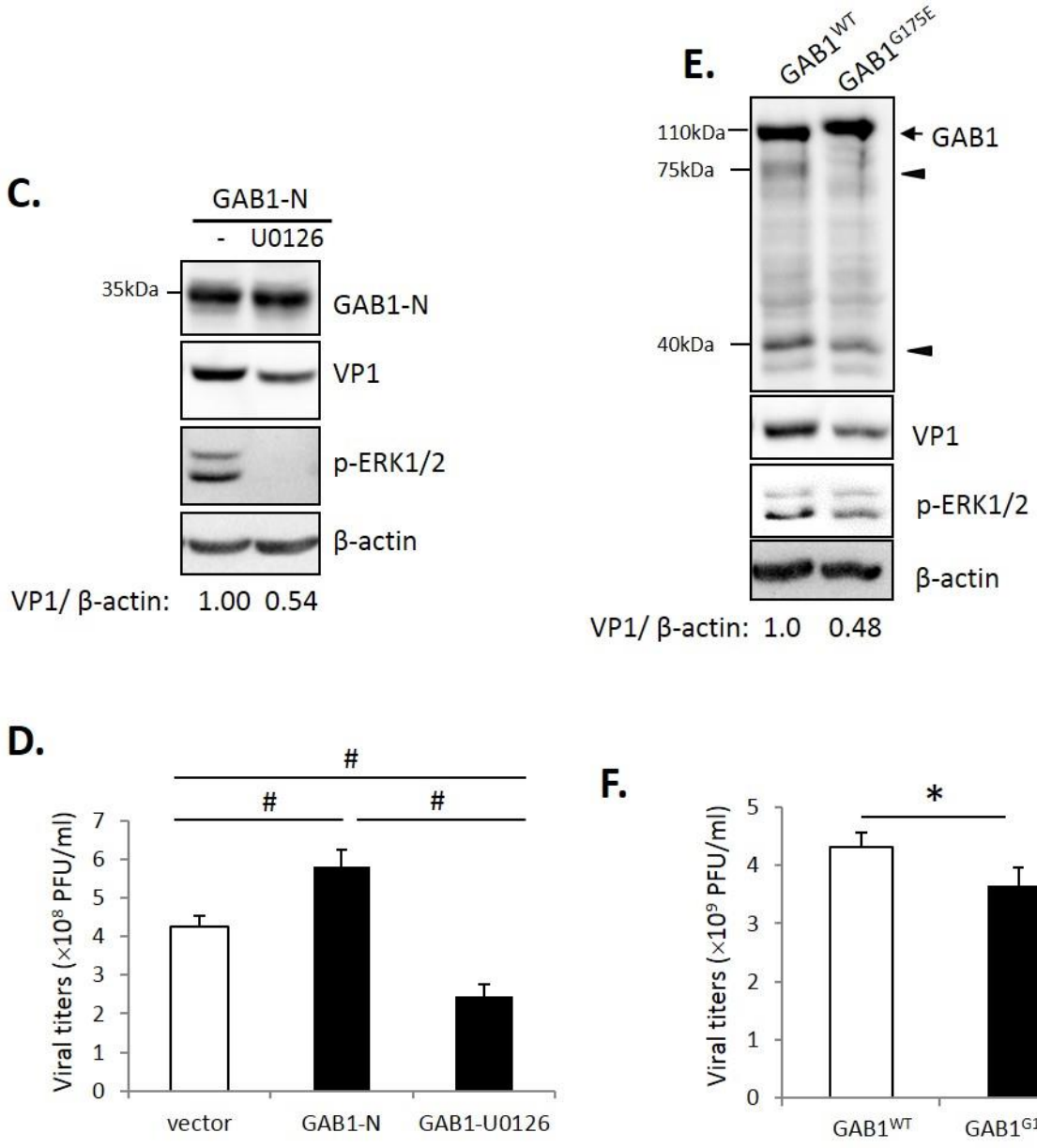


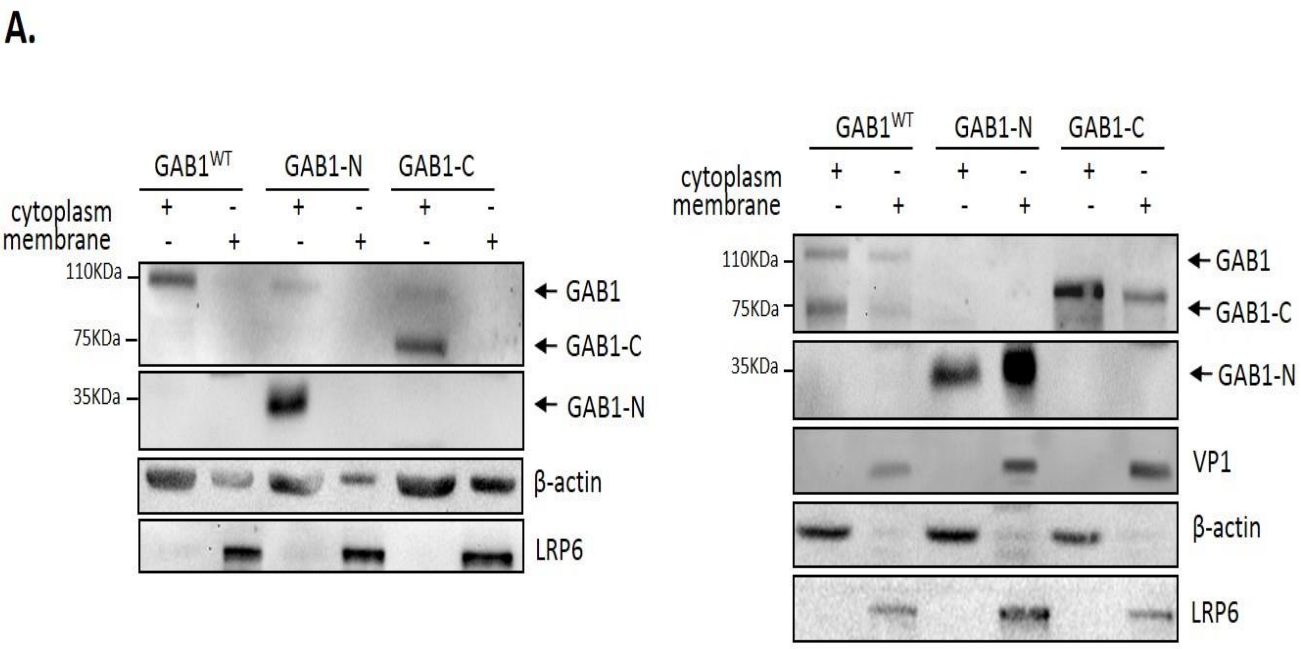
Figure 16. The N-terminal Cleavage Fragment of GAB1 Promotes CVB3 Replication by Further Enhancing ERK1/2 Phosphorylation. (A) Schematic diagram of the N- and C-terminal cleaved fragments of GAB1 used in this Figure and Figure 16 hereafter. (B) HeLa cells were transiently transfected with 3×Flag-GAB1-N, 3×Flag-GAB1-C, or corresponding empty vector

(3×Flag) for 24 hrs, followed by CVB3 infection for 7 hrs. Cell lysates were harvested to determine protein levels of VP1, GAB1-N or GAB1-C (using anti-Flag antibody), p-ERK1/2, and β-actin. Densitometric analysis was conducted as in Figure 15. (C, D) Inhibition of ERK1/2 activation attenuates CVB3 replication induced by GAB1-N. HeLa cells were transiently transfected with 3×Flag-GAB1-N for 24 hrs, followed by CVB3 infection in the presence or absence of MEK inhibitor U0126 (20μM). Protein levels of GAB1-N (using anti-Flag antibody), VP1, and β-actin were examined by western blotting (C). Supernatants were collected for plaque assay (mean ± SD, n=4). #$p<0.001$ (D). (E, F) HeLa cells were transiently transfected with 3×Flag-GAB1^{WT} or 3×Flag-GAB1^{G175E}, followed by viral infection as described above. Protein expression of GAB1 (using anti-GAB1 antibody) and VP1 was assessed by Western blotting (E). Plaque assay was conducted to determine virus titer in supernatants (F) (mean ± SD; n=4). *$p<0.05$.

4.3.6 The N-terminal Cleavage Fragment of GAB1 Is Constitutively Localized to the Cellular Membrane

Recruitment of GAB1 from the cytoplasm to the cellular membrane is a crucial step in the activation of the MAPK-ERK pathway. We then questioned whether the cleavage fragments of GAB1 could be recruited to the plasma membrane upon viral infection, contributing to enhanced ERK1/2 activation and increased viral replication. Cell fractionation was conducted and the results showed that majority of GAB1^{WT} and GAB1-C were present in the cytoplasmic fractions either under baseline condition (**Figure 17A**, left panel) or upon viral infection (**Figure 17A**, right panel), while GAB1-N was detected mainly in cytoplamic fractions in sham-infected cells (**Figure 17A**, left panel) and translocation of GAB1-N from cytoplasm to membrane was increased following CVB3 infection (**Figure 17A**, right panel). Consistent with previous studies [339], GAB1^{WT} was

found to translocate to the membrane fraction in cells treated with growth factors (**Figure 17B**). Confocal microscopy analysis further confirmed that GAB1-N was predominantly localized to the cellular membranes, while GAB1^{WT} and GAB1-C largely retained in the cytoplasm of CVB3-infected cells (**Figure 17C**). Taken together, our finding suggests that the pro-viral activity of GAB1-N may be related to its constitutive presence in cellular membrane fraction and preference to translocate to cellular membrane upon stimulation, and subsequently activation of the MAPK-ERK pathway that favors viral replication.



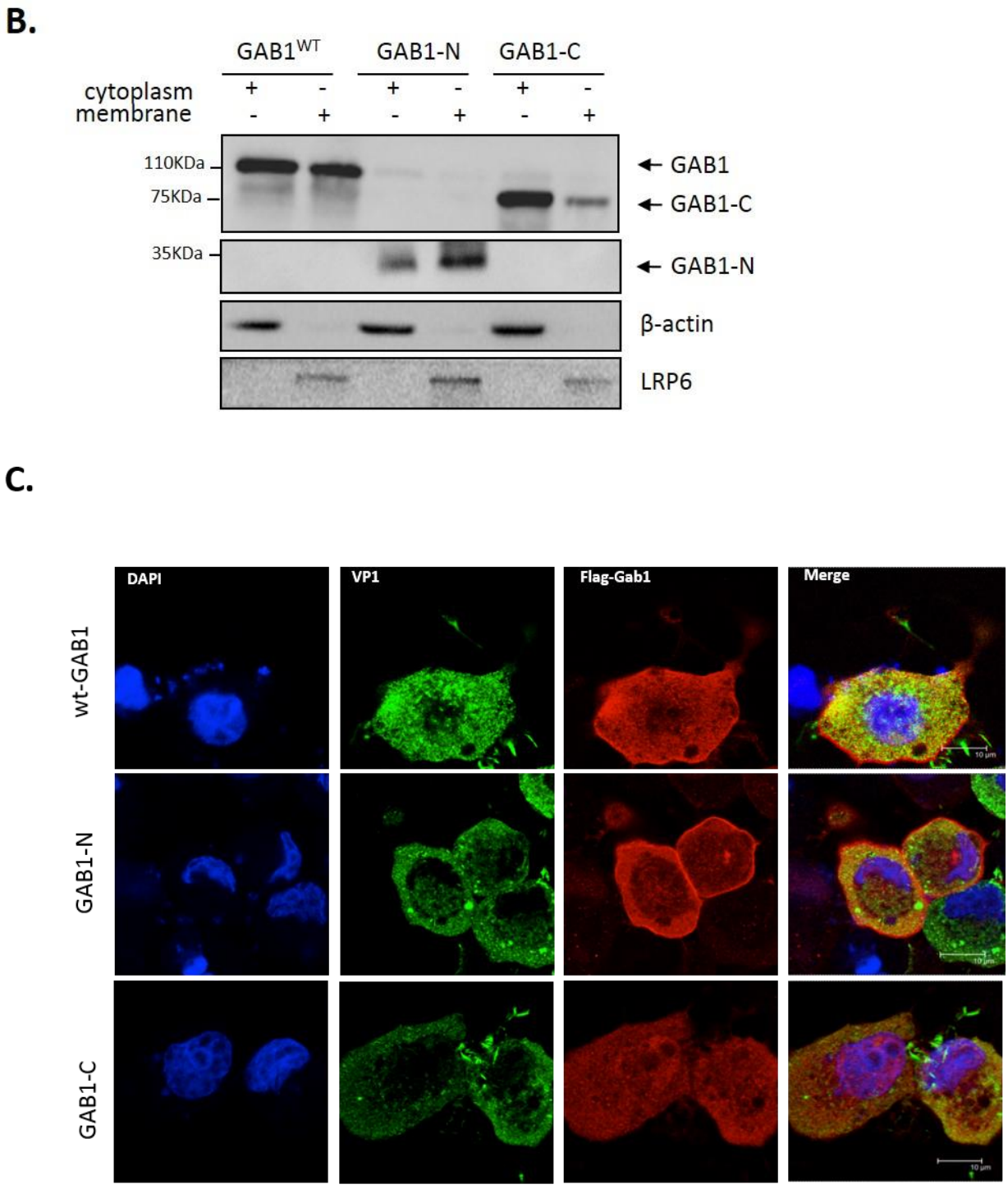


Figure 17. The N-terminal Cleavage Fragment of GAB1 Is Constitutively Localized in the Cell Plasma Membrane. (A, B) HeLa cells were transiently transfected with 3×Flag-GAB1^{WT},

3×Flag-GAB1-N or 3×Flag-GAB1-C for 24 hrs, followed by sham (A, left panel), or CVB3 infection (A, right panel), or treatment with 10% FBS (B). Cells were collected for cell fractionation. Plasma membrane and cytoplasm fractions were subjected to western blot analysis of protein levels of WT-GAB1 and GAB1-C (using anti-GAB1 antibody), GAB1-N (using anti-Flag antibody), VP1, LRP6 and β-actin. (C) Cells were transfected and infected as above and then immunocytochemical staining was conducted using anti-Flag (red) and anti-VP1 antibody (green). Nuclei were counterstained by DAPI (blue). Bar=10μM.

4.4 Discussion

The importance of CVB3 as a human pathogen and the emerging prospect of developing CVB3 as a potential oncolytic agent have necessitated a thorough investigation into the molecular basis of host-cell permissiveness to CVB3[340]. Although the presence of CAR on the cell membrane remains a major determining factor for the susceptibility of cancerous cells to CVB3 infection, emerging evidence showed that the intracellular signaling plays a crucial role in dictating the outcome of viral tropism[341].

Our findings in this study reveal a novel mechanism by which CVB3 employs to trigger ERK1/2 activation and promote consequent viral replication. Enteroviral protease $2A^{pro}$ plays an essential role in ensuring successful completion of viral life-cycle, through direct processing viral polyprotein, and by targeting host proteins for proteolytic degradation to create a favorable microenvironment for viral growth [271, 342, 343]. Several mechanisms have been proposed with regard to the latter pro-viral strategy. For example, it was well documented that $2A^{pro}$ cleaves host eIF4γ and PABP, resulting in the shutoff of host protein synthesis to benefit viral mRNA translation[344, 345]. In addition, it was reported that enteroviral protease $2A^{pro}$ mediates the cleavage of MDA5 and

MAVS, two critical regulators in type I interferon responses, to escape host antiviral immune surveillance[143, 346]. Our results in this study suggest, for the first time, that $2A^{pro}$ can also support enteroviral infection by manipulating and usurping the host signaling machinery. We demonstrated that scaffolding adaptor protein GAB1 is proteolytically cleaved by enteroviral protease $2A^{pro}$ during CVB3 infection, which leads to the release of the N-terminal PH domain-containing fragment that facilitates viral infectivity.

We have previously demonstrated that CVB3 infection mediates a late, persistent ERK1/2 activation that depends on viral protein production[267]. The present study identified the cleavage of GAB1 as a mechanism triggering the late phase activation of ERK1/2 during CVB3 infection. We reported that expression of the N-terminal cleavage truncation of GAB1 induces ERK1/2 activation, which appears to be related to its constitutive membrane association. Unlike stimulation by growth factors, such as hepatocyte growth factor, we found that upon CVB3 infection, $GAB1^{WT}$ fails to translocate from the cytoplasm to the cellular membrane, whereas the GAB1-N is constantly detected in the membrane fractions. GAB1-N contains several potential phosphorylation sites and the PH domain that is known to bind with the plasma membrane enriched in phosphatidylinositol lipids (Figure 12C). The exact mechanism by which GAB1-N promotes ERK1/2 activation is currently unclear. We hypothesize that GAB1-N preoccupies the PH-domain binding sites that are necessary for recruiting the upstream inhibitory modulators of the MAPK/ERK signaling pathway, which results in sustained activation of ERK1/2. For instance, SAPK-interacting protein 1 and Dok (for downstream of tyrosine kinases) were reported to contain PH domain and membrane binding is required for their function in inhibiting the activities of small GTPase Ras and protein kinases upstream of ERK1/2 [347, 348]. Thus, incapable of being recruited to lipid enriched membrane due to preoccupation of these regions by GAB1-N may result in the relief

of their inhibitory effects on ERK1/2 activation. Further studies are needed to define more specifically the mechanism of enhanced ERK1/2 activation and viral replication by cleaved GAB1 fragments.

The best characterized pathway for GAB1 activation of MAPK/ERK pathway is through interaction with SHP2 [238]. Although the C-terminal cleavage product of GAB1 contains the SHP2 binding site (Y627 and Y659), it lacks the PH domain and fails to be recruited to the plasma membrane. Previous studies have suggested an important role for PH domain in GAB1 function. It was reported that GAB1 mutant with PH domain deletion fails to be tyrosine phosphorylated upon growth hormone receptor stimulation, resulting in impaired ERK1/2 activation[349]. Thus we speculate that the inability of GAB1-C to function in activating ERK signaling may correlate with its failure to translocate to the plasma membrane.

In conclusion, our study demonstrated the cleavage of GAB1 by CVB3-encoded viral protease $2A^{pro}$ at G175 and G436, producing the predominant functional cleavage fragment GAB1-N. GAB1-N further enhances viral replication by upregulating host ERK1/2 signaling. These findings present a new mechanism by which CVB3 contributes to the pathogenesis of enterovirus infection. On the other hand, specific inactivation of the ERK1/2 signaling pathway within the heart offers a possibility of attenuating CVB3-induced cardiotoxicity, which may significantly enhance the safety of CVB3-based virotherapy.

Chapter 5: Cleavage of Grb2-Associated Binding Protein 2 by Viral Protease 2A^{pro} during Coxsackievirus Infection

5.1 Background

Recently, we reported that GAB1 (NCBI:NP_002030.2), a scaffolding adaptor protein that acts as a platform for intracellular signaling transduction and assembly, is cleaved upon CVB3 infection [145]. As a result, the generation of the N-terminal fragment of GAB1 further induces sustained activation of ERK1/2 MAPK and consequent enhancement of viral replication.

As a functional homologue of GAB1, GAB2 (NCBI:NP_536739.1) also belongs to the family of insulin receptor substrate 1-like multi-substrate proteins and serves as a platform for the assembly of signaling proteins [350-354]. Upon activation by receptor tyrosine kinases, GAB2 undergoes tyrosyl-phosphorylation, creating docking sites for downstream adaptor proteins that mediate further signal transduction. As such, GAB2 has also been considered as a major mediator of essential cellular processes, including proliferation, survival and differentiation. GAB2 is ubiquitously expressed in many organs and depletion of GAB2 has been associated with a severe defect in response to passive allergic challenge and a defective osteoclast differentiation [242]. Considering that depletion of GAB2 results in a different spectrum of human disorders from that of GAB1, we questioned whether each GAB protein has a functional redundancy in the intracellular signaling transduction.

It is observed that hepatocyte growth factor selectively activates GAB1 in epithelial cells that express both GAB1 and GAB2, partly due to the presence of the Met-binding domain in GAB1 but not in GAB2 [354], while Bcr-Abl oncoprotein preferentially utilizes GAB2 as its downstream signaling components in T cells [352]. Furthermore, signal transduction studies demonstrated that

GAB1 and GAB2 have non-redundant roles in vascular endothelial growth factor-mediated migration and survival of endothelial cells[240]. These finding indicates that host may execute signaling by utilizing different GAB protein to mediate specific downstream signaling event upon engagement of different extracellular stimuli, such as growth factors, cytokines and pathogen stress[355].

In this study, we further investigated whether GAB2 plays a synergistic role with GAB1 in benefiting CVB3 replication.

5.2 Specific Aims

The OBJECTIVE of this chapter is to determine whether GAB2 plays a synergistic role with GAB1 in regulating intracellular signaling during CVB3 infection.

The SPECIFIC AIMS include:

Aim1: To examine the protein expression level of GAB2 following CVB3 infection

Aim2: To elucidate the underlying mechanism by which CVB3 regulates GAB2 expression

Aim3: To study the functional consequences of GAB2 dysregulation in virus replication

5.3 Results

5.3.1 Proteolytic Process of GAB2 upon CVB3 Infection

To understand the possible role of GAB2 in CVB3 infection, we first examined the protein level of GAB2 upon viral infection. As shown in **Figure 18A**, protein level of GAB2 began to decrease at 5 hrs and disappeared at 7 hrs post-infection, accompanied by the generation of an additional band at around 60kDa using an antibody against the C-terminus of GAB2, suggesting a possible cleavage event. To verify this, a plasmid expressing C-terminal HA-tagged GAB2

(GAB2-HA) was utilized. HeLa cells transfected with GAB2-HA were infected with CVB3 for indicated hours and protein expression of GAB2 was examined. We found that, similar to the finding in Figure 18A, an extra band of exogenous GAB2 was detected at around 60kDa using an anti-HA antibody (**Figure 18B**), indicating that GAB2 is also cleaved after CVB3 infection. It is noted that the cleavage efficiency of exogenously transfected GAB2 is much lower than that of endogenous GAB2. We have previously reported that liposome-mediated cDNA transfection inhibits CVB3 attachment to the cells by disrupting membrane cholesterol[356]. Thus, the decreased cleavage efficiency is likely a result of reduced viral infectivity in transfected cells.

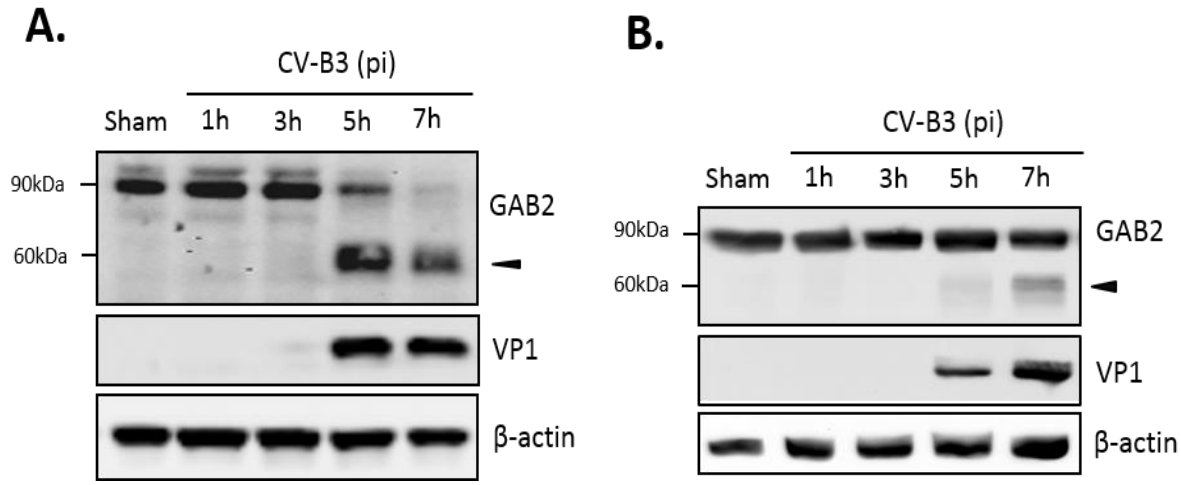


Figure 18. GAB2 Is Cleaved upon CVB3 Infection. (A) HeLa cells were inoculated with CVB3 at an MOI of 10 for indicated time. Western blotting was conducted to examine protein levels of GAB2 using an antibody that recognizes the C-terminus of GAB2. Viral capsid protein VP1 was probed as an evidence of viral infection and β-actin was examined as protein loading control. (B) HeLa cells were transiently transfected with a plasmid expressing WT-GAB2 with an epitope of HA at its C-terminus for 48 hrs, followed by CVB3 infection for various time points as indicated.

Western blot analysis was performed for detection of exogenous GAB2 (using anti-HA antibody), VP1, and β-actin.

5.3.2 CVB3 Protease $2A^{pro}$-Mediated Cleavage of GAB2

We then investigated the mechanism leading to GAB2 cleavage. We first tested whether viral replication is required for GAB2 cleavage. We utilized UV-irradiated viruses, which are capable of interacting with viral receptor and subsequent entering into cells, but unable to replicate. We showed that GAB2 was not cleaved in cells infected with UV-CVB3, suggesting that GAB2 cleavage is dependent on viral replication (**Figure 19A**).

CVB3 encodes two proteases, $2A^{pro}$ and $3C^{pro}$, which not only process viral polyprotein into individual structural and nonstructural protein, but also target cellular proteins to either facilitate infection or provoke host anti-viral machinery. *In vitro* cleavage assay was performed to determine whether viral proteases contribute to the cleavage of GAB2 upon CVB3 infection. As shown in **Figure 19B**, incubation with WT-$2A^{pro}$ ($2A^{pro\text{-}WT}$) led to the generation of a cleavage band at ~60kDa, corresponding to what was detected during CVB3 infection. However, the catalytic mutant of $2A^{pro}$ ($2A^{pro\text{-}mut}$) failed to cleave GAB2, suggesting that cleavage of GAB2 is mediated through the catalytic activation of $2A^{pro}$. *In vitro* cleavage assay was also conducted using purified $3C^{pro}$ protease. However, no cleavage products were detected (data not shown). Furthermore, to rule out the possible role of caspase activation in GAB2 cleavage, we treated cells with the general caspase inhibitor, z-VAD. We found that caspase inhibition did not prevent the cleavage of GAB2 (**Figure 19C**). The inhibition of caspase activity by z-VAD was confirmed by the blockage of caspase-3 cleavage (**Figure 19D**). Taken together, our results suggest that the

cleavage of GAB2 detected during CVB3 infection is mediated via the catalytic activity of viral protease $2A^{pro}$, independent of the activation of caspase.

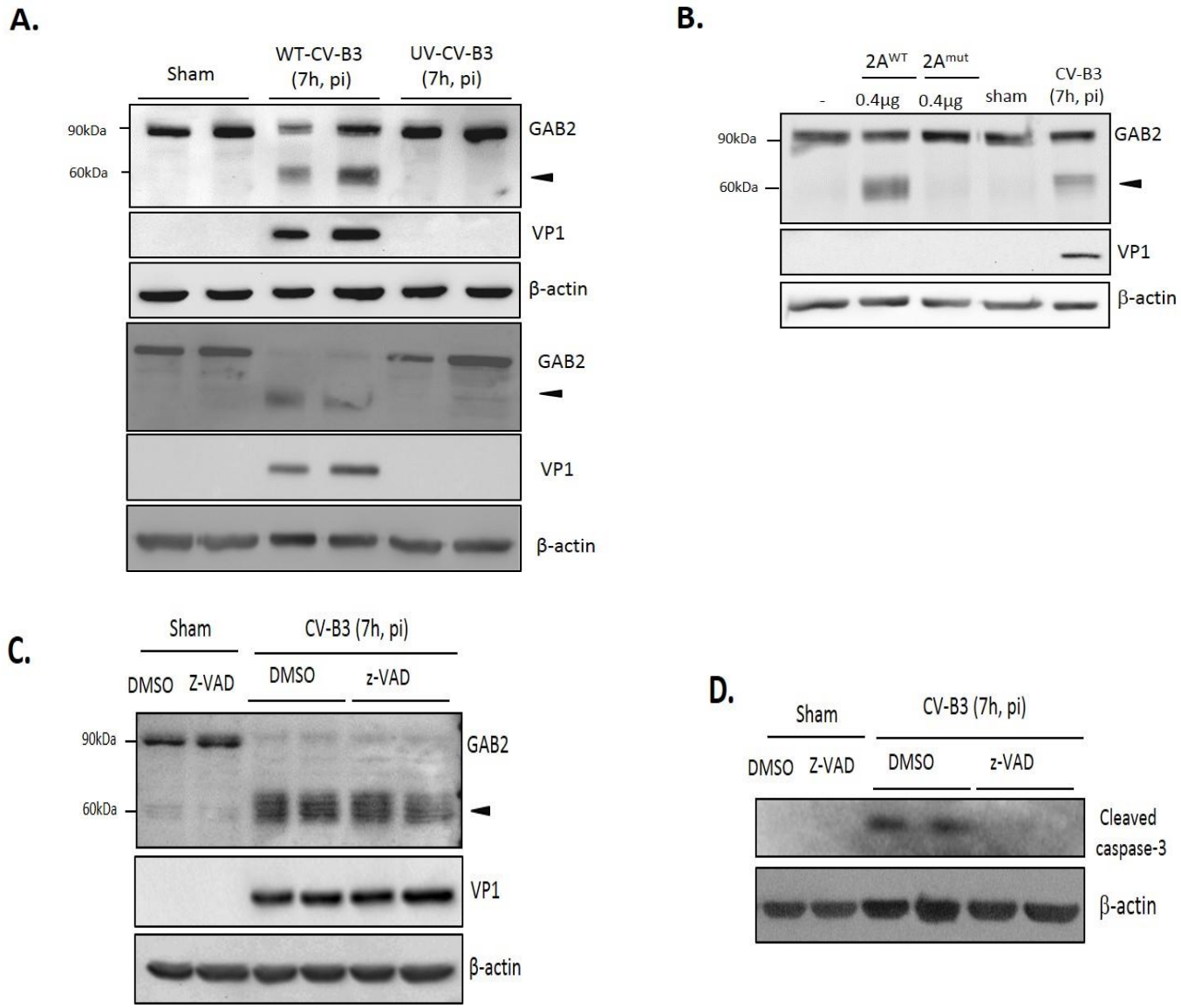


Figure 19. Cleavage of GAB2 Is Catalyzed by CVB3-Encoded Viral Protease $2A^{pro}$.

HeLa cells were sham-infected or infected with either WT or ultraviolet (UV)-irradiated CVB3 at an MOI of 10 for 7 hrs. Protein levels of GAB2, VP1, and β-actin were detected by Western blot analysis. (B) HeLa cells were transiently transfected with GAB2-hemagglutinin (HA) for 48 hrs. Fifty microgram of cell lysates were incubated with 0.4μg of purified CVB3 WT ($2A^{pro\text{-}WT}$) or

catalytic mutant ($2A^{pro\text{-}mut}$) of $2A^{pro}$ for 18 hrs. Western blotting was carried out to examine protein expression of GAB2 using an anti-HA antibody. Sham and CVB3-infected, GAB2-HA-transfected HeLa cell lysates were loaded as negative and positive control (right two lanes), respectively. (C) HeLa cells were sham or CVB3-infected in the presence of pan-caspase inhibitor, z-VAD (50μM), or vehicle (DMSO) for 7 hrs. Western blotting was performed and protein levels of GAB2, VP1, and β-actin were examined. (D) The inhibition of caspase activation by z-VAD was confirmed by the blockage of caspase-3 cleavage.

5.3.3 Cleavage of GAB2 between H237 and G238

Based on the reported cleavage consensus motif of protease $2A^{pro}$ (i.e., L/I/MxT/S/Nx//G, // indicates the scissile bond, P4 position – L (leucine), I (isoleucine) or M (methionine), P2 position –T (threonine), S (serine) or N (asparagine), P1' position is commonly G (glycine), x indicates any amino acid residues) [133], we found one potential cleavage sequence (234LASHG238) on GAB2. To determine whether GAB2 is cleaved at this site, we performed site-directed mutagenesis to replace G at position 238 with glutamic acid (E). As shown in **Figure 20A**, $GAB2^{G238E}$ mutant was uncleavable upon CVB3 infection, suggesting that G238 is the cleavage site. **Figure 20B** illustrates the functional domains, the cleavage site, and the resulting cleavage fragments of GAB2 following CVB3 infection.

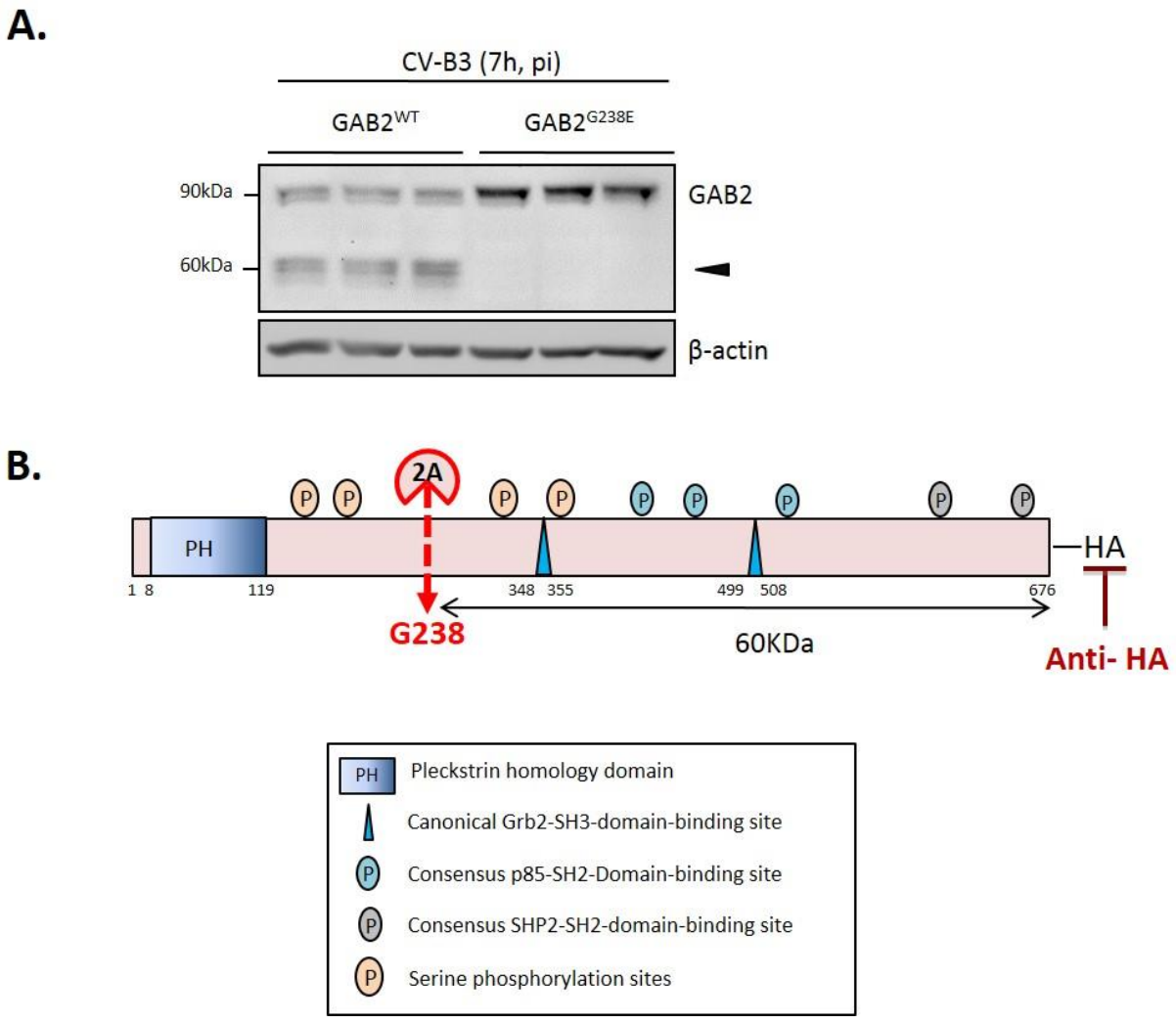


Figure 20. GAB2 Is Cleaved between H237 and G238 during CVB3 Infection. (A) HeLa cells were transiently transfected with either GAB2^{WT}-HA or GAB2^{G238E}-HA for 48 hrs, followed by CVB3 infection for 7 hrs. Cleavage of GAB2 was examined using anti-HA antibody by western blot analysis. Protein levels of β-actin were examined as loading control. (B) Schematic diagram of different function domains and cleavage site of GAB2.

5.3.4 Inhibition of Viral Replication and MAPK Signaling by Knocking Down GAB2

We have previously shown that knockdown of GAB1 inhibits CVB3 replication[145]. Here we questioned whether GAB2 plays a similar role in CVB3 infection. Figure 21 revealed that gene-silencing of GAB2 resulted in significant decreases in viral protein production (**Figure 21A,** left panel **& B**) and virus titers (**Figure 21C**), accompanied by a marked reduction of CVB3-induced phosphorylation of JNK and p38 (**Figure 21A,** left panel), suggesting a pro-viral function for GAB2. The importance of p38 activation in viral replication was further demonstrated using a selective p38 inhibitor (SB203580). **Figure 21A** (right panel) showed that treatment with SB203580 led to a marked reduction of viral protein expression. Inhibition of p38 activity by SB203580 was confirmed by reduced levels of phosphor-HSP27, a downstream target of p38. No significant effects of JNK inhibitor on viral replication were observed (data not shown). Interestingly, unlike deletion of GAB1 that caused a decrease in phosphorylated ERK1/2, knockdown of GAB2 had no effect on virus-mediated ERK1/2 phosphorylation (**Figure 21A**, left panel). We further examined the impacts of forced expression of exogenous GAB2 on viral replication. We found that viral protein levels and viral loads failed to further increase in cells overexpressing GAB2 (data not shown), indicating that the levels of endogenous GAB2 are already saturated for viral replication.

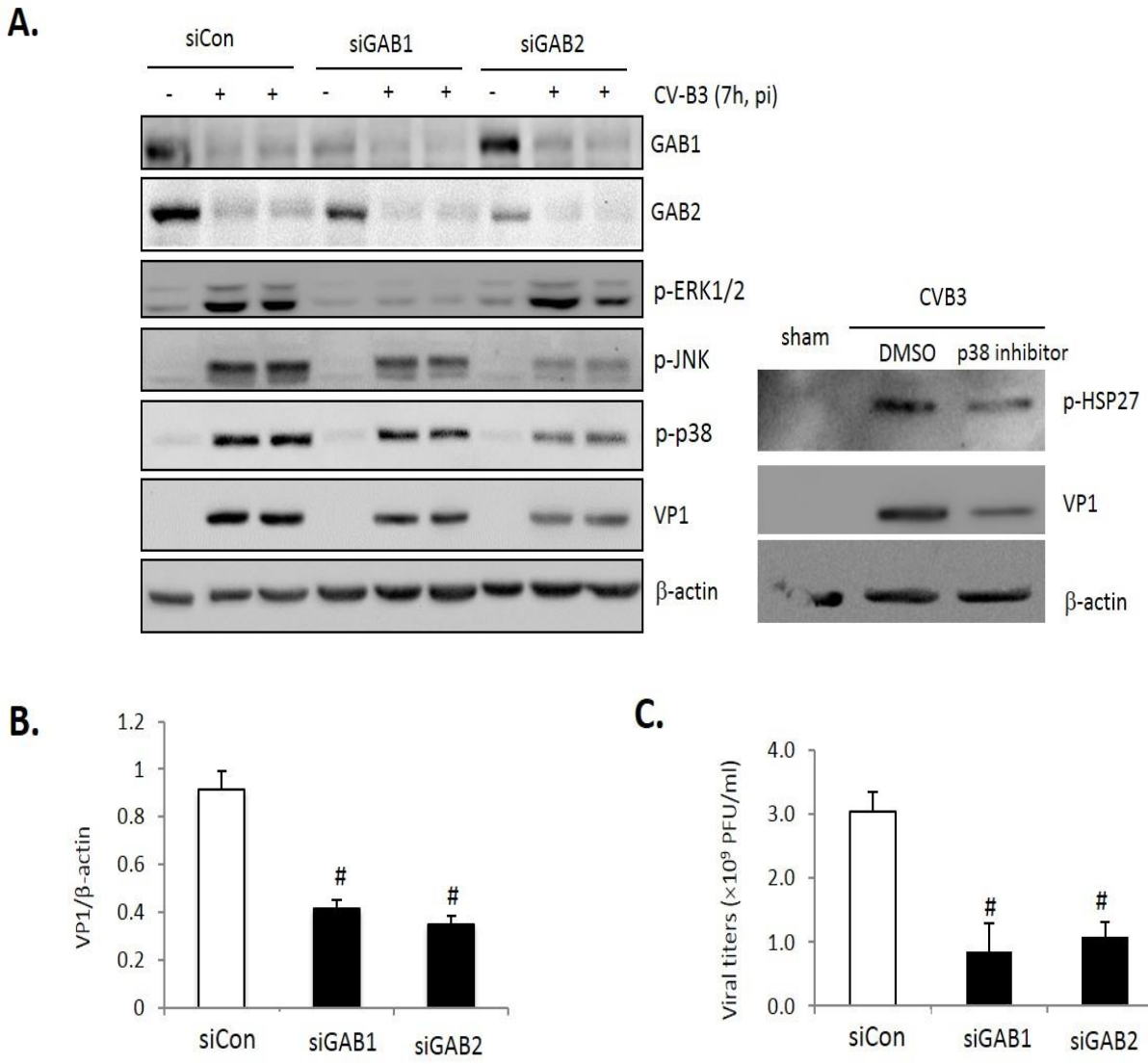


Figure 21. Knockdown of GAB2 Inhibits Viral Replication and MAPK Signaling. (A, left panel) HeLa cells were transiently transfected with control siRNA (siCon), GAB1- or GAB2-targeting siRNA (siGAB1 or siGAB2) as indicated for 48 hrs, followed by CVB3 infection for 7 hrs. Western blotting was performed to examine protein levels of GAB1, GAB2, VP1, p-ERK1/2, p-p38, p-JNK, and β-actin. (A, right panel) HeLa cells were sham or CVB3 infected in the presence of p38 inhibitor (SB203580, 50μM) or vehicle (DMSO) for 7 hrs. Western blotting was performed using anti-phospho-HSP27 and anti-VP1 antibodies. Anti-β-actin was used as a loading control.

(B) Protein levels of VP1 were quantitated by densitometric analysis, normalized to β-actin (mean ± SD, n=3). #, $p<0.001$ compared to siCon. (C) Virus titers in the supernatant collected from the experiments above were measured by plaque assay. The virus titers are presented as mean ± SD (n=4). #, $p<0.001$ compared to siCon.

5.3.5 Cleavage of GAB2 Does Not Further Increase Viral Replication

We next sought to determine the consequence of GAB2 cleavage during viral infection. HeLa cells were either transfected with $GAB2^{WT}$ or non-cleavable GAB2 ($GAB2^{G238E}$). We showed that the levels of viral protein (**Figure 22A & B**) and virus titers (**Figure 22C**) were comparable between cells expressing $GAB2^{WT}$ and $GAB2^{G238E}$, suggesting that cleavage of GAB2 has no direct benefits to virus replication. Moreover, we examined the influence of overexpression of either GAB2-N or GAB-C on viral replication. As shown in **Figure 22D, E, & F**, neither VP1 levels nor virus titers were significantly altered in cells expressing GAB2 cleavage fragments compared with empty vector control. Collectively, our results suggest that cleavage of GAB2 results in the loss of its function in promoting viral infection, rather than the gain of a pro-viral activity.

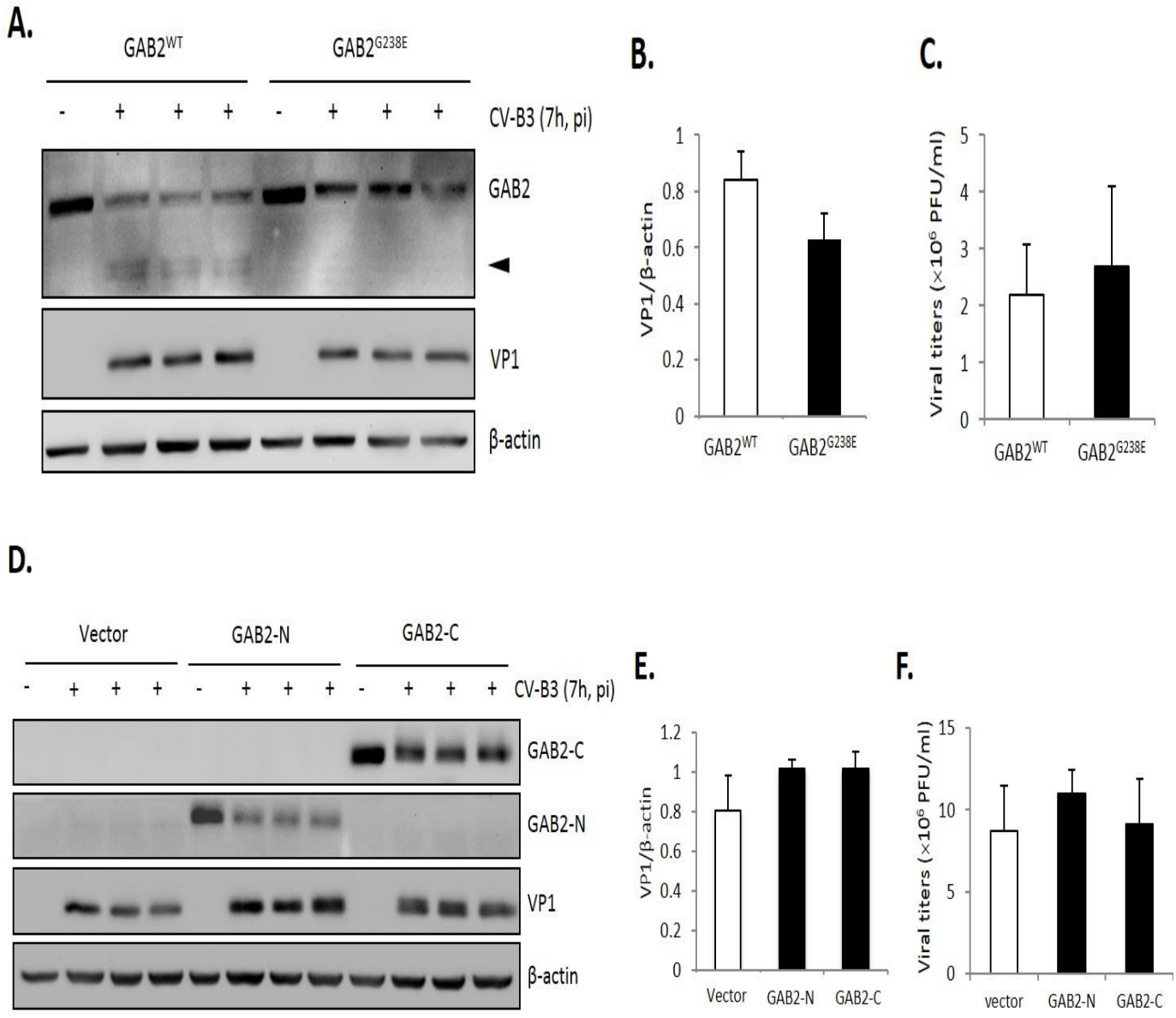


Figure 22. Cleavage of GAB2 Does Not Further Increase Viral Replication. HeLa cells were transiently transfected with (A) C-terminal HA-tagged GAB2^{WT} or non-cleavable GAB2 mutant (GAB2^{G238E}), or (D) empty vector, N-terminal Flag-tagged GAB2-N, or GAB2-C for 24 hrs, followed by CVB3 infection for 7 hrs. Cell lysates were collected for western blot analysis of protein levels of GAB2^{WT}–HA or GAB2^{G238E}-HA using anti-HA antibody, Flag-GAB2-N or Flag-GAB2-C using anti-Flag antibody. (B, E) Protein levels of VP1 in (A) and (D) were quantitated

by densitometric analysis, normalized to β-actin (mean ± SD, n=3). (C, F) The supernatant from above experiments was harvested for plaque assay, and the virus titers are presented as mean ± SD (n=3).

5.4 Discussion

GAB2 is a scaffolding adaptor protein that transduces cellular signals from the receptors to the intracellular downstream molecules. Upon activation by a variety of extracellular stimuli, such as growth factors and cytokines, GAB2 translocates from the cytoplasm to the plasma membrane, where it is tyrosine-phosphorylated by receptor tyrosine kinases and then recruits several SHP2, PI3K, and Crk, subsequently leading to the activation of multiple downstream signaling pathways (e.g., MAPKs and Akt) that are critical for cell proliferation, differentiation, apoptosis and survival [351-353].

It has been well-documented that activation of the ERK1/2 MAPK promotes CVB3 replication[267, 338, 357, 358]. Inhibition of this pathway by either chemical inhibitors or a dominant-negative construct significantly decreases the production of viral protein and progeny virion[267, 338, 357, 358]. We have previously revealed an important role for GAB1-mediated ERK1/2 activation in CVB3 infection[145]. However, in contrast to gene-silencing of GAB1, in this study we found that knockdown of GAB2 had no effect on ERK1/2 phosphorylation, suggesting a pro-viral activity for GAB2 independent of the ERK1/2 pathway. In addition to the ERK1/2 pathway, it has been previously shown that the p38 MAPK also plays a critical role in CVB3 infection through facilitating viral spread and propagation [338, 359, 360]. The significance of p38 pathway in viral infection is confirmed in the current study. Thus, the pro-viral function of GAB2 is likely executed by activating the p38 pathway, rather than the ERK1/2 pathway. We speculate that the inability of

GAB2 knockdown to block ERK1/2 phosphorylation is due to a compensatory function of GAB1 in ERK1/2 activation.

We have previously shown that the N-terminal cleavage fragment of GAB1 (GAB1-N1-174) further enhances ERK1/2 activation and facilitates viral growth[145]. However, this effect was not detected for GAB2-N1-237. Although GAB1-N1-174 and GAB2-N1-237 share a highly conserved PH domain (~67% identity), GAB2-N1-237 appears to contain distinct and additional docking sites, which likely result in a differential preference for harboring downstream signaling molecules.

In this study, we demonstrate that GAB2 is cleaved following CVB3 infection by viral protease $2A^{pro}$ and knockdown of GAB2 results in reduced viral replication, supporting a pro-viral activity for GAB2. Why does CVB3 induce the cleavage of a protein that promotes its infection? We postulate that GAB2 is an innocent bystander of protease $2A^{pro}$. It is conceivable that any proteins comprising a consensus cleavage recognition motif, such as GAB2, could possibly be targeted by viral proteases. Indeed, it has been previously shown that CVB3 infection results in the cleavage of inhibitor of κBα (IκBα) to generate a proteolytic fragment that subsequently limits viral replication, and thus cleavage of IκBα is regarded as a crucial step for the host to recognize and respond to the pathogens[144].

To establish a causal relationship between GAB2 cleavage and viral infection, one could test whether expression of a non-cleavable GAB2 can enhance viral replication. However, in this study, we showed that overexpression of a non-cleavable form of GAB2 ($GAB2^{G238E}$) failed to further increase viral replication compared to $GAB2^{WT}$-transfected cells. This is likely due to the fact that HeLa cells express high-levels of endogenous GAB2, obscuring the role of exogenous GAB2 in viral replication. In addition, as mentioned earlier, a large portion of the exogenously

transfected GAB2^{WT} remains uncleaved following infection, further attenuating the difference in viral replication between GAB2^{WT}- and GAB2^{G238E}-transfected cells.

In **Figure 22D**, we showed that the protein levels of GAB2-N and GAB2-C were markedly reduced following viral infection. There are two possible explanations for this observation. First, CVB3 infection results in the shutoff cap-dependent host protein translation as a result of viral protease-mediated cleavage of eIF4G and PABP. Thus, upon CVB3 infection, a decrease in protein synthesis of transfected GAB2-N and GAB2-C is expected. Second, at the late stage of viral infection, cellular proteases are activated, which further contributes to the reduced expression of GAB2 fragments.

In conclusion, our findings in this study that GAB2 is cleaved upon CVB3 infection through the proteolytic activity of virus-encoded protease 2A^{pro} represent a novel host anti-viral strategy against CVB3 infection. The balance between pro-viral GAB1-cleavage and anti-viral GAB2 cleavage represents a precise mechanism by which the host controls viral infection.

Chapter 6: Closing Remarks

6.1 Research Summary and Conclusion

The major findings in my dissertation are summarized below:

Chapter 3: Coxsackievirus Type B3 Is a Potent Oncolytic Virus against *KRAS*-Mutant Non-Small-Cell Lung Cancer

1. CVB3 selectively infects and lyses $KRAS^{mut}$ NSCLC cells.

2. $EGFR^{mut}$ NSCLC cells and normal lung epithelial cells are resistant to CVB3 infection.

3. Intratumoral injection of CVB3 leads to a significant regression of $KRAS^{mut}$ xenograft NSCLC and moderate cardiotoxicity in immunodeficient mice.

4. Enhanced expression of CAR in $KRAS^{mut}$ cells is associated with increased susceptibility of NSCLCs to CVB3.

5. Aberrant ERK1/2 activation is responsible for effective viral replication in $KRAS^{mut}$ NSCLCs.

Chapter 4: Enhanced Enteroviral Infectivity via Viral-Protease-Mediated Cleavage of GAB1

1. GAB1 is cleaved at Glycine 175 and Glycine 436 during CVB3 infection by virus-encoded protease $2A^{pro}$.

2. Knockdown of GAB1 attenuates CVB3 replication and viral particle release.

3. The resulting N-terminal cleavage fragment of GAB1 promotes CVB3 replication and viral particle release via enhancement of activation of ERK1/2 signaling

4. The resulting N-terminal cleavage fragment of GAB1 is constitutively localized on the plasma membrane.

Chapter 5: Cleavage of Grb2-Associated Binding Protein 2 by Viral Proteinase 2A during Coxsackievirus Infection

1. GAB2 is cleaved at Glycine 238 during CVB3 infection via viral-encoded protease $2A^{pro}$.

2. Knockdown of GAB2 attenuates CVB3 replication and viral particle release.

3. Cleavage of GAB2 does not further enhance CVB3 replication.

In conclusion, my study demonstrates that CVB3 is a potent oncolytic agent against $KRAS^{mut}$ NSCLCs that have an enhanced expression level of CAR and an aberrant ERK1/2 activation. *In vivo* studies demonstrate that intratumoral injection of CVB3 leads to a significant regression of $KRAS^{mut}$ xenograft NSCLC but moderate cardiotoxicity. On the other hand, my research demonstrates that CVB3 cleaves GAB1 to manipulate its downstream ERK1/2 signaling, in return, promoting CVB3 life cycle. This finding suggests a novel mechanism by which CVB3 manipulates the host machinery to gain advantage for its replication. Taken together, my study indicates that CVB3 is a promising OV against $KRAS^{mut}$ NSCLCs. Specific elimination of CVB3 replication in the heart may avoid CVB3-induced cardiotoxicity and enhance the safety of CVB3-based virotherapy.

6.2 Research Significance

Since *KRAS* mutations are commonly observed in NSCLCs, it has attracted a considerable attention as a drug target. Thus far, however, the quest for therapeutic inhibitors of *KRAS* has fallen short of expectations. No direct *KRAS* inhibitors have been proven to be clinically effective. Therefore, further efforts to develop therapies for $KRAS^{mut}$ NSCLCs are urgently needed.

As a key downstream effector of *KRAS*, ERK1/2 has been widely accepted as an alternatively attractive target for antitumor therapies because of its central role in controlling cell growth and survival[28, 37]. However, the results from existing clinical trials using ERK1/2 signaling inhibitors as anticancer agents do not demonstrate a positive outcome, which therefore limits its transition to the clinic[361]. Considering the highly lytic nature of CVB3 and its dependency on the activation of ERK1/2 for replication, I speculate that CVB3 is a promising candidate for the development of oncolytic virotherapy. My studies demonstrated that CVB3 is a potent OV specifically against $KRAS^{mut}$ NSCLCs *in vivo*. The current study is the first to provide evidence that CVB3-based virotherapy is effective towards $KRAS^{mut}$ NSCLCs, which are generally considered to be the most refractory type of NSCLC for first-line chemotherapy.

Further, my studies demonstrated that CAR gene expression is regulated by *KRAS* mutation. The induction of endogenous CAR expression appears to be an important mechanism of NSCLC susceptibility to CVB3 infection. *KRAS* mutations are widely observed in pancreatic cancer, colon cancer and NSCLC, indicating a potentially wide spectrum of cancer patients that can benefit from a CVB3-based oncolytic virotherapy[38, 362, 363]. Although the involved mechanism remains to be elucidated, the discovery of enhanced CAR expression in $KRAS^{mut}$ NSCLCs has provided a strong rationale to develop not only enterovirus-based virotheapy, but also treatment derived from adenovirus that shares CAR for virus entry.

To date, viral myocarditis and its sequelae DCM remain a severe infectious disease without effective therapies. Virus-mediated manipulation of the host signaling machinery has been widely discovered in a variety of viruses[364-366]. Hence, host signaling molecules have emerged as a potential target for new drug development. Elucidation of the mechanisms by which CVB3 hijacks the host signaling can provide solid evidence to understand the pathogenesis of these diseases. My results showed that CVB3 induces the cleavage of two functionally similar scaffolding adaptor proteins (GAB1/GAB2) via the activity of CVB3-encoded protease $2A^{pro}$. The resulting N-terminal fragment of GAB1 further activates ERK1/2 signaling, contributing to the enhancement of CVB3 replication. This finding indicates a novel mechanism by which CVB3 cleaves a pro-viral protein to obtain a gain-of-function to benefit its replication. Furthermore, my studies suggest that GAB1 has emerged as a predominant target for the virus to manipulate host signaling machinery, shedding light on a novel direction for drug development.

Cleavage of host proteins by viral protease $2A^{pro}$ occurs strictly on the basis of a core consensus sequences [L/I/M•X•T/S•X | G•X•X•X] (L, leucine; I, isoleucine; M, methionine; T, threonine; S, serine; G, glycine; X stands for any amino acid; |, stands for the scissile site). A list of host proteins targeted by viral protease $2A^{pro}$ was identified when using this formula. These findings provide valuable information for developing a specific viral protease $2A^{pro}$ inhibitor. My laboratory has proposed to design a small molecule that mimics the targets of viral protease $2A^{pro}$, preventing native substrates, such as GAB1 and GAB2, from cleavage during CVB3 infection. Using this potential molecule as an antiviral agent may provide an option for the neutralization of CVB3-induced cardiotoxicity.

6.3 Limitations and Future Directions

The patient-derived tumor cell xenograft model is one of the most widely used tools to examine response to therapy due to the following advantages: 1) it features the complexity of genetic and epigenetic abnormalities that exist in human tumor; 2) it can be used for screening for effective molecular therapeutics for individual patients; and 3) the results can be obtained in a short time[367, 368]. However, using subcutaneous xenografts to test treatment response is not always correlated with clinical responses in patients. This poor correlation is largely due to the fact that: 1) subcutaneous xenografts fail to mimic tumor microenvironment of patients; and 2) host immunity is at least partially abolished when using immunocompromised mice[369]. Thus, I speculate that impaired host immunity may promote virus spread within the xenograft, exaggerating the efficacy of CVB3-based virotherapy. On the other hand, as another major component of OV-mediated tumor eradication, OV-mediated antitumor immunity is thought to be beneficial for the enhanced efficacy of virotherapy due to 1) cross-primed $CD8^+$ cytotoxic T cells serve as an army to constitutively infiltrate and eliminate tumor cells; 2) cross-primed $CD8^+$ cytotoxic T cells exert systemic effects on metastatic lesions; and 3) activated $CD8^+$ cytotoxic T cells provide durable antitumor effects without the necessity for multiple rounds of dosing[370]. However, I cannot exclude the possibility that activated $CD8^+$ cytotoxic T cells can clear viral particles rapidly and excessively, leaving a relatively small window of opportunity for OV to replicate[371]. Thus, the balance between host antiviral immunity and host antitumor immunity emerges as a critical determinant for the success of an OV application.

To address this issue, future studies can use an orthotropic model to recapitulate the real tumor microenvironment. Furthermore, immunological deficits could potentially largely be overcome by grafting human tumor cells into NOD/SCID mice that also receive an injection of

immunocompetent peripheral blood or bone marrow cells. Genetically engineered mouse (GEM) models which have complete host immunity can also serve as a great tool to study drug response. For example, *KRAS*LSL-G12D transgenic mice that are highly predisposed to early onset NSCLCs can be utilized for testing the efficacy of CVB3-based virotherapy[372].

Due to the fact that CVB3 preferentially infects cardiomyocytes, I found that intratumoral injection of CVB3 is associated with an increased mortality rate in immunodeficient mice. This problem has become a major obstacle when developing CVB3 as an oncolytic agent. To address this issue, several methods can be utilized to reduce cardiotoxicity. For example, I propose to use microRNAs (miRNA)-based approaches to modify the CVB3 genome to further enhance its tumor-specificity and decrease its toxicity to normal tissues[373]. MiRNAs are a class of endogenous small non-coding RNAs that are evolutionarily conserved and act as key regulators in a wide range of fundamental cellular functions, including cell proliferation, differentiation, and apoptosis, by binding to the 3'UTR of the targeted mRNAs[374]. Subsequently, they promote either mRNA degradation or suppression of gene expression. Recent evidence suggests that miRNAs also play an important role in tumorigenesis and progression of cancers. MiRNAs are commonly observed to be downregulated in different types of cancer tissues compared to normal tissues. Taking advantage of pre-existing cellular miRNAs as a primed antiviral defense modulator, the tropism of an OV armed with complementary sequences of a specific miRNA can be selectively restricted to a certain type of host cells.

Although the cleavage events of GAB1 and GAB2 during CVB3 infection were confirmed in HeLa cells, I failed to verify the events in either HL-1 cells (a cell line derived from mouse cardiomyocytes) or CVB3-infected mouse hearts. The reason is probably due to a slight difference of amino acid sequences between *Homo sapiens* and *Mus musculus*. Using tissue extracts from

CVB3-infected mouse hearts, I still found that the expression levels of GAB1 and GAB2 were downregulated, suggesting that CVB3 still targets GAB1/GAB2 *in vivo.* But the mechanisms by which CVB3 downregulates GAB1/GAB2 expression in mouse hearts remain to be investigated. An alternative method to address this issue would be using human heart biopsies. This method allows for the detection of GAB1 and GAB2 cleavage by performing western blotting.

In my study, the cleavage sites were determined by site-directed mutagenesis techniques. Although this mutagenesis method has been widely accepted for cleavage studies, the possibility that conformational changes caused by point mutations sterically hinder potential proteolytic sites on the target proteins cannot be excluded. To address this issue, Edman degradation sequencing or mass spectrometry can be performed to verify the cleavage site. Briefly, the cleaved fragments of interest can first be harvested from either a Coomassie-stained SDS-PAGE gel or nitrocellulose membrane. The resulting products can then be processed for sequencing.

Another limitation in my studies involves forced expression by transient plasmid transfection. Overexpression by transient plasmid transfection has been widely used as a method to study the function of candidate protein *in vitro*. For example, in my study, the functional role of non-cleavable GAB1 for CVB3 infection was determined by forced overexpression of non-cleavable GAB1 in HeLa cells. However, due to the fact that HeLa cells also express endogenous WT-GAB1 that plays a positive role in supporting CVB3 replication, thus the inhibitory role of non-cleavable GAB1 in regulating CVB3 infection could be partially counteracted. $GAB1^{-/-}$ MEFs provide an instrumental model to solve this problem. By using $GAB1^{-/-}$ MEFs system, the difference between WT-GAB1 and non-cleavable GAB1 in CVB3 replication can be maximally distinguished.

Altogether, the discoveries described in this dissertation propose a promising approach to treat refractory *KRAS*^{mut} NSCLCs using CVB3-based virotherapy. Future studies will focus on the modification of CVB3 genome to enhance the safety of CVB3-based virotherapy.

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