

Curriculum Vitae

Name: Dominique R. Bollino

Contact information: drbollino@gmail.com

Degree and Date to be Conferred: Doctor of Philosophy, 2015

Collegiate Institutes Attended:

2009-2015 University of Maryland Baltimore; Baltimore, Maryland.
Doctor of Philosophy, Molecular Medicine, May 2015

2005-2008 Frostburg State University; Frostburg, Maryland.
Bachelor of Science, Biology with Biotechnology concentration, Dec. 2008

Publications:

Colunga, A., **Bollino, D.**, Schech, A. and Aurelian, L. "Calpain-dependent clearance of the autophagy protein p62/SQSTM1 is a contributor to Δ PK oncolytic activity in melanoma." *Gene Therap.* 2014; 21, 371-378.

Bollino D, Aurelian LA. HSPB8 (heat shock 22kDa protein 8); *Atlas Genet Cytogenet Oncol Haematol.* 2014; 18(12):907-918.

Bollino D, Balan I, Aurelian LA. "Valproic acid induces neuronal cell death through a novel calpain-dependent necroptosis pathway." *J Neurochem.* 2015;133(2):174-86

June HL, Liu J, Warnock KT, Bell KA, Balan I, **Bollino D**, Puche A, Aurelian L. "CRF-Amplified Neuronal TLR4/MCP-1 Signaling Regulates Alcohol Self-Administration." *Neuropsychopharmacology.* 2015; 40(6):1549-59

Bollino D, Colunga A, Li B, Aurelian, LA. "Multiple immunogenic cell death features define the oncolytic activity of the Δ PK virus." *Molecular Cancer.* In revision.

Oral Presentations:

April 2015 "Molecular Mechanisms of Oncolysis by the HSV-2 mutant Δ PK"
University of Maryland, Baltimore, MD. Graduate Program in Life Sciences, Student Seminar Series.

June 2014 "Co-treatment with valproic acid enhances the anti-tumor activity of the oncolytic virus Δ PK, in melanoma and breast cancer cells."
University of Maryland, Baltimore MD. Annual Cancer Biology Retreat.

Sept 2013 "ICP10PK-mediated neuroprotection in valproic acid induced cell death." University of Maryland, Baltimore, MD. Program in Molecular Medicine Student Seminar Series.

Abstracts:

Bollino D and Aurelian, L. “The HSV-2 gene ICP10PK protects neurons from valproic acid induced dysfunction and death associated with autism spectrum disorder.” American Society for Neurochemistry. Baltimore, MD. March 2012.

Bollino D and Aurelian, L. “Mechanisms of valproic acid induced neuronal cell death and protection by ICP10PK.” Greater Baltimore Chapter of the Society for Neurochemistry. Baltimore, MD. November 2012.

Bollino D and Aurelian, L. “Herpes Simplex Virus 2 Gene, ICP10PK, Protects Against Neuronal Cell Death Associated with Valproic Acid-Induced Autism Spectrum Disorder.” ASGCT. Salt Lake City, Utah. May 2013.

Abstract

Title of Dissertation: Oncolytic Mechanisms of the HSV-2 Mutant Δ PK and Enhancement by Valproic Acid

Name: Dominique Raphaelle Bollino, Doctor of Philosophy, 2015

Dissertation Directed by: Laure Aurelian, Ph.D., Professor
Department of Pharmacology

Carcinogenesis is the process by which normal cells become malignant. It typically occurs through the accumulation of mutations that dysregulate intracellular signaling pathways and lead to unchecked growth and proliferation. Oncolytic viruses (OV) are replication conditional virus mutants that take advantage of these overactivated growth pathways to selectively replicate in and lyse tumor cells. In addition to the direct infection and lysis of tumor cells, OVs also kill tumor cells through disruption of tumor vasculature and the induction of potent anti-tumor immune responses. Clinical efficacy of OVs remains relatively poor, attributed to therapeutic barriers such as poor tumor penetration, premature viral clearance, and the presence of highly resistant cancer stem cell (CSC) subpopulations. Efforts to enhance OV efficacy include the addition of transgenes to enhance anti-tumor immunity, as well as combination therapy with cytotoxic and/or immunosuppressive drugs to increase tumor cell death and reduce innate antiviral responses.

The growth compromised HSV-2 mutant Δ PK, has robust oncolytic activity in both melanoma cultures and xenografts associated with the induction of multiple pathways of programmed cell death. However, the impact of Δ PK on putative CSC populations, as well as its ability to harness immune responses that contribute to tumor cell death is still poorly understood. This thesis work sought to answer these questions as well as

investigate the potential benefits of combining Δ PK with valproic acid (VPA), a histone deacetylase inhibitor with demonstrated cytotoxic and immunosuppressive properties.

We report that: (i) Δ PK prevents anchorage-independent growth and lyses 3D cultures through calpain-dependent clearance of the autophagy protein p62/SQSTM1, (ii) Δ PK oncolysis includes several features of immunogenic cell death, such as the inhibition of Th2-based immunosuppressive conditions, promotion of a Th1-biased microenvironment, and the induction of anti-tumor immune surveillance mechanisms, (iii) VPA induces a novel calpain-dependent necroptotic form of cell death in neuronal cells, and (iv) the combination of VPA and Δ PK treatment in melanoma increases Δ PK -induced cell death through enhanced caspase activation. These findings suggest that Δ PK is a multi-mechanistic OV with particularly promising cancer therapeutic potential, and warrant further *in vivo* investigation into the oncolytic potential of the Δ PK and VPA combination.

Oncolytic Mechanisms of the HSV-2 Mutant Δ PK
and Enhancement by Valproic Acid

by
Dominique Raphaelle Bollino

Dissertation submitted to the faculty of the Graduate School of the
University Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2015

Table of Contents

Chapter 1: Introduction	1
A. Carcinogenesis.....	1
B. Intracellular Signaling	1
B1. Ras.....	2
B2: MAPK Signaling.....	3
B3: ERK	4
B4: JNK and p38MAPK.....	5
B5: PI3K/Akt.....	6
C. Melanoma	7
C1: Epidemiology and Development	7
C2: Common Genetic Alterations in Melanoma	8
C3: Cancer stem cells	11
C4: Tumor cell immune evasion	12
C5: Treatment	14
D. Programmed cell death.....	18
D1: Apoptotic cell death.....	19
D2: Caspase-independent cell death.....	21
D3: Necroptosis	24
D4: Pyroptosis	26
D5: Autophagic cell death	28
D6: Immunogenic cell death.....	29
E. Oncolytic virotherapy	30
E1: Immune system elements of virotherapy	31
E2: Herpes Simplex virus	34
F: Improving OV efficacy.....	39
F1: Apoptosis inducing genes and pro-drug converting enzymes.....	39
F2: Fusogenic peptides	40
F3: Enhancing anti-tumor immunity	41
F4: Drug combinations	42
Chapter 2: Experimental Overview	47

Chapter 3: Materials and Methods	50
A. Cell culture	50
B. Virus	51
C. Neuronal differentiation	51
D. Antibodies and reagents	51
E. Cell death	52
F. Cytoplasmic and nuclear separation and Immunoblotting.....	54
G. Immunofluorescent staining and Immunohistochemistry	54
H. Microarray analysis	55
I. Soft Agar Growth Assay	56
J. Spheroid Formation Assay	56
K. ELISA.....	57
L. <i>In vivo</i> studies	57
M. Statistical analysis.....	58
Chapter 4: Experimental Results	59
A. Calpain-dependent clearance of the autophagy protein p62/SQSTM1 is a contributor to Δ PK oncolytic activity in melanoma cancer stem cells	59
A1: Introduction	59
A2: Results.....	60
A3: Discussion.....	65
B. Melanoma cell lysis by the oncolytic Herpes simplex virus type 2 mutant Δ PK includes multiple features of immunogenic cell death.....	69
B1. Introduction.....	69
B2: Results.....	70
B3: Discussion.....	82
C. Valproic acid induces neuronal cell death through a novel calpain-dependent necroptosis pathway	90
C1. Introduction.....	90
C2 Results.....	91
C3: Discussion.....	105
D. The histone deacetylase inhibitor valproic acid enhances the oncolytic activity of Δ PK in melanoma.....	113

D1: Introduction	113
D2: Results	115
D3. Discussion of results	122
Chapter 5: General Discussion and Future Directions.....	127
Chapter 6: Literature Cited	138

List of Figures

Figure 1: Schematic of major MAPK intracellular signaling pathways	4
Figure 2: Intrinsic and extrinsic apoptosis pathways	22
Figure 3: Necroptosis	25
Figure 4 : Pyroptosis	27
Figure 5: ICP10PK pro-survival signaling and Δ PK construction	38
Figure 6: Elimination of anchorage independent growth by Δ PK is calpain and autophagy dependent	61
Figure 7: Δ PK induces LC3-II accumulation and calpain-dependent clearance of p62/SQSTM1	64
Figure 8: Δ PK upregulates pro-inflammatory and pro-apoptotic genes; alters cytokine secretion	71
Figure 9: Δ PK activates the JNK/c-Jun pathway in melanoma cells.....	73
Figure 11: Δ PK induces pro-inflammatory cytokine expression through autophagy-related activation of the TLR2/NFkB pathway.....	77
Figure 12: Δ PK-induced pyroptosis activates caspase-1 resulting in mature IL- β production.	78
Figure 13: Cytokine modulation contributes to Δ PK-induced melanoma cell death.....	80
Figure 14: Δ PK inhibits CTLA-4 expression.....	81
Figure 15: Δ PK inhibits tumor growth and upregulates MICA and TNF- α in melanoma xenografts.....	83
Figure 16: Schematic representation of the Δ PK-modulated ICD-associated functions ..	84
Figure 17: VPA induces neuronal cell death; inhibited by calpain, JNK and necroptosis inhibitors.	92
Figure 18: VPA induced cell death.	94
Figure 19: VPA does not activate caspases.	96
Figure 20: Calpain is activated in VPA-treated PC12, but not PC47 cells.....	98
Figure 21: VPA activates JNK1, leading to increased RIP-1 expression.	99
Figure 22 VPA induces AIF cleavage to a 57 kDa (tAIF) band and promotes its nuclear translocation.....	102
Figure 23: VPA induces tAIF mitochondrial release and increases levels of γ H2AX. ..	104
Figure 24:VPA increases Smac/DIABLO and decreases XIAP.....	106
Figure 25: Schematic representation of VPA-induced neuronal cell death.....	108
Figure 26: VPA-induced death is caspase dependent in melanoma	116
Figure 27: VPA enhances Δ PK-induced cell death through caspase activation.....	117
Figure 28: VPA enhances Δ PK-induced caspase activation in a cell type specific manner	119
Figure 29: VPA induces cell death in breast cancer cultures.....	121
Figure 30: Δ PK enhancement by VPA cell-type specific for breast cancer	122

List of Abbreviations

3-MA	3-Methyladenine
AAV	Adeno associated virus
ACT	Adoptive cell transfer
AIF	Apoptosis inducing factor
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD
ASD	Autism spectrum disorder
Atg	Autophagy related genes
ATP	Adenosine triphosphate
BAD	BCL2-associated agonist of cell death
BAX	BCL2-associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
BIK	BCL2-interacting killer
CARD	Caspase activation and recruitment domain
Caspase	Cysteine dependent aspartate directed proteases
Cdk	Cyclin-dependent kinase
cKIT	Tyrosine protein kinase Kit
c-Myc	Myelocytomatosis viral oncogene homolog
CNS	Central nervous system
CREB	cAMP responsive binding protein 1
CSC	Cancer stem cells
CTLA	Cytotoxic T-lymphocyte-associated antigen 4
Cyt C	Cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DAMP	danger associated molecular patterns
DC	dendritic cells
DD	Death domains
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E binding protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EtHD	Ethidium homodimer
Ets-1	Erythroblastosis virus E26 oncogene homolog 1
FBS	Fetal Bovine Serum
FADD	Fas associated death domain
FITC	Fluorescein isothiocyanate

GABA	γ -Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
γ H2AX	Gamma H2A histone family, member X
GM-CSF	granulocyte/macrophage stimulatory factor
GPCR	G protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HLA	Human leukocyte antigen
HMGB1	High-mobility group protein B1
HSP	Heat shock protein
HSV	Herpes simplex virus
hTERT	Human Telomerase reverse transcriptase
HVEM	Herpes virus entry mediator
IAP	Inhibitor of apoptosis
ICD	Immunogenic cell death
IE	Immediate early
IFN	Interferon
IKK	Inhibitor κ B kinase
IL 1	Interleukin
JNK	c-Jun N-terminal kinase
LC3	Light Chain 3
LAT	Latency-Associated Transcript
LT α	Lymphotoxin alpha
MAP-2	Microtubule associated protein 2
MAPK	Mitogen activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MDSC	Myeloid-derived suppressor cells
MEK	Mitogen activated protein kinase/ERK kinase $\frac{1}{2}$
MHC-1	Major histocompatibility complex class 1
MICA	MHC class I polypeptide-related sequence A
MITF	microphthalmia-associated transcription factor
MKK4	Mitogen activated protein kinase kinase 4
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target of Rapamycin
Nec-1	Necrostatin-1
NF- κ B	Nuclear factor kappa light chain enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural killer
NLR	Nod-like receptors
oHSV	Oncolytic herpes simplex virus
OV	Oncolytic viruses

PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PARP-1	Poly (ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cell line
PCD	Programmed cell death
PD-1	Programmed cell death-1
Pfu	Plaque forming unit
PH	Pleckstrin homology
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol 4,5 biphosphate
PIP3	Phosphatidylinositol 2,4,5 phosphate
PK	Protein kinase
PtdIns3K	class III phosphatidylinositol 3-kinase
PTEN	Phosphate and tensin homolog
PYD	Pyrin domain
RANTES	Regulated upon activation, normal T-cell expressed and secreted
Ras	rat sarcoma viral oncogene homolog
Rb	Retinoblastoma protein
RIP1	Receptor interacting protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Ribonucleotide reductase
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
Sh2	Src homology 2 domain
SMAC	Second mitochondria- derived activator of caspases
SOS	Son of sevenless
TAA	Tumor associated antigens
TAK1	Transforming growth factor β activated kinase 1
Th1	T helper type 1
Th2	T helper type 2
TK	Thymidine kinase
TLR	Toll-like receptor
TM	Transmembrane
TNFR	Tumor necrosis factor receptor
TNF α	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis inducing ligand
Treg	T regulatory cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Vps34	vacuolar protein sorting 34

VEGFR	Vascular endothelial growth factor receptor
VP5	Virion protein 5
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis protein
zVAD-fmk	z-V-A-D fluoromethylketone

Chapter 1: Introduction

A. Carcinogenesis

Carcinogenesis, the process by which normal cells are transformed into cancer cells, is caused by mutations in normal cells which upset the balance between proliferation and cell death. Two broad categories of genes are affected by these changes: oncogenes and tumor suppressor genes. Oncogenes may be normal genes that are expressed at inappropriately high levels, or genes that gain novel properties due to alterations. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Mutations in proto-oncogenes, which are the normally quiescent counterparts of oncogenes, can modify their expression and function, increasing the amount or activity of the product protein. When this occurs, proto-oncogenes become oncogenes, upsetting cell cycle regulation and making uncontrolled growth possible. Tumor suppressor genes inhibit cell division, survival, or other properties of cancer cells and are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell ¹ and these changes ultimately result in the dysregulation of intracellular signaling pathways involved in life/death decisions.

B. Intracellular Signaling

To respond to their environment, cells must be able to receive signals from their surroundings and translate them into changes in gene expression. Cells utilize surface receptors embedded in the plasma membrane to respond to external stimuli. These receptors consist of an extracellular domain that binds ligands, a transmembrane domain that anchors the receptor in the plasma membrane, and an intracellular cytosolic domain

that interacts with a diverse group of signaling proteins. A variety of cellular events such as growth, survival, differentiation, metabolism, and programmed cell death are mediated by molecular networks that cross-regulate one another. Stimuli or mutations that result in either dysregulated growth or premature cell death are the foundation of complex human diseases. In cancer cells, for example, intrinsic and/or acquired mutations push affected cells towards uncontrolled survival, proliferation and invasion, overpowering opposing signals and leading to tumor formation.

The modulation of these opposing pro-survival and pro-death signals is a major focus of the work in Dr. Aurelian's laboratory and the body of work contained in this thesis pertains to the manipulation of these pathways in cancer, namely melanoma, to induce cell death. An overview of several major intracellular signaling pathways that are important in carcinogenesis and cellular life/death decisions follows.

B1. Ras

One of the major classes of cell surface receptors is the receptor tyrosine kinases (RTK) which respond to a variety of ligands, such as growth factors and cytokines. Upon ligand binding, RTKs autophosphorylate at specific tyrosine residues in the cytosolic domain and the resulting phosphotyrosines serve as docking sites for other proteins involved in signal transduction. One family of responders to receptor signaling is the Ras family of G-proteins, whose highly conserved function is the activation of diverse intracellular pathways ultimately leading to gene expression. These small proteins are localized to the inner surface of the plasma membrane, and are activated by guanine nucleotide exchange factors, (GEFs) which convert GDP to GTP. Activation of Ras is highly coordinated, and requires a series of adaptor proteins. When ligand binds a RTK,

the SH2 (Src homology 2) domain of the adaptor protein binds the phosphorylated tyrosine residue, thereby bringing the Ras-GTP exchange factor, Sos (son of sevenless) that also binds Grb2 at another site in close proximity to Ras, resulting in Ras activation². Ras activation is negatively regulated by GTPase activating proteins (GAPs), which convert Ras-GTP back to Ras-GDP³, therefore inhibition of GAPs is one way to activate Ras. The ras oncogenes were among the first to be discovered in cancer research and activating mutations in the Ras family of proto-oncogenes (comprising H-Ras, N-Ras and K-Ras) are found in 20% to 30% of all human tumors⁴. Ras is an important upstream activator of several signaling pathways, including the MEK/ERK and PI3-K/Akt pathways, both of which are discussed below.

B2: MAPK Signaling

Downstream of Ras signaling, the mitogen activated protein kinases (MAPKs), mediate responses such as growth, survival, and apoptosis. The MAPK signaling cascade is separated into three signaling modules. In general, the pathway begins with the activation of a serine/threonine specific mitogen activated protein kinase kinase kinase (MAPKKK), such as Raf-1 or B-Raf, immediately downstream of Ras activation. The MAPKKK phosphorylates a MAP kinase kinase (MAPKK), followed by MAPKK phosphorylation of a downstream MAPK, which then regulates diverse cellular responses. The canonical MAPK signaling cascade is illustrated in Fig. 1. Three of the major MAPK classes are: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. ERK activation is generally associated with proliferation, cell cycle progression, and survival, while activation of JNK and p38 MAPK are associated with inflammation and stress stimuli responses.

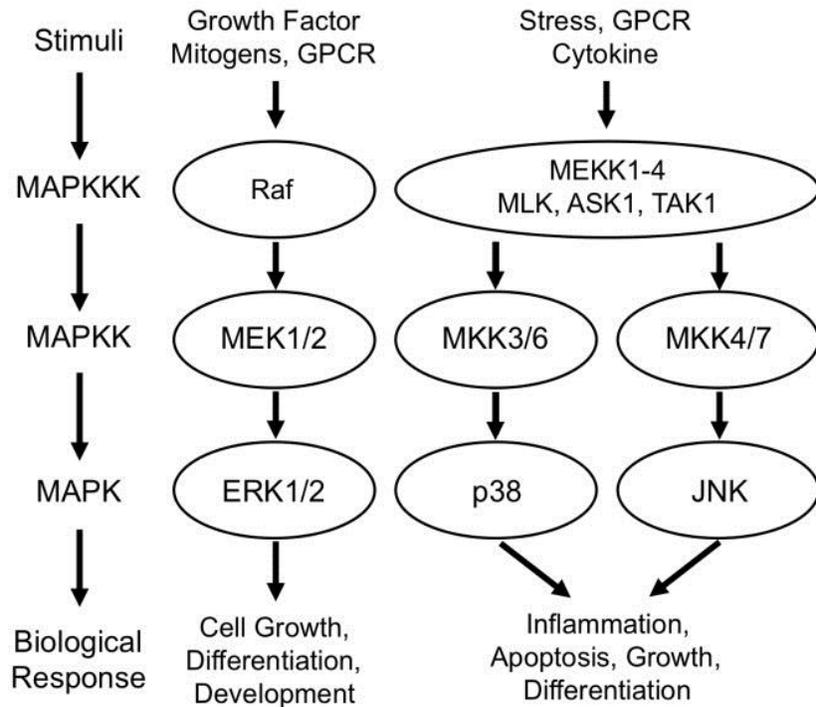


Figure 1: Schematic of major MAPK intracellular signaling pathways

Adapted from Cell Signaling Technologies. www.cellsignal.com

B3: ERK

ERK is a MAPK that functions as a major effector of Ras and its activation is typically associated with cell survival and growth. Upon activation, Ras activates Raf kinase through binding and phosphorylation. The Raf family of serine/threonine kinases consists of A-Raf, B-Raf, and C-Raf, each with its own tissue specific distribution and activity. Although primarily activated by Ras, Raf has multiple phosphorylation sites that can be modulated by alternate kinases and phosphatases⁵. MEK1/2 (Mitogen-activated

protein kinase/ERK kinase 1/2) is a dual specificity tyrosine and serine/threonine kinase which is canonically activated by Raf-mediated phosphorylation of serine residues in its catalytic domain. Activated MEK1/2 can then activate the serine/threonine kinases ERK1/2, which upon activation, can translocate to the nucleus and either directly or indirectly activate several transcription factors including CREB (cAMP responsive element binding protein 1), NFκB (nuclear factor kappa-light chain- enhancer of activated B cells), Ets-1 (erythroblastosis virus E26 oncogene homolog 1), c- Myc (myelocytomatosis viral oncogene homolog), c-Jun, and c-Fos ⁶. The Ras/Raf/MEK/ERK cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, migration, survival, differentiation, metabolism, and proliferation.

B4: JNK and p38 MAPK

In addition to ERK, there are two other well characterized MAPK family members, JNK and p38 MAPK which are activated by a variety of environmental stresses, inflammatory cytokines, growth factors, and G-protein coupled receptor (GPCR) agonists. The JNK family of protein kinases is made up of JNK1 and JNK2 which are ubiquitously expressed, and JNK3 which is expressed primarily in the brain. JNK family members are involved in diverse biological phenomena through the phosphorylation and regulation of many types of proteins including several transcription factors and members of the Bcl-2 family ⁷. Activated JNK can phosphorylate the transcription factor c-Jun, resulting in its nuclear translocation. c-Jun is part of the activating protein-1 (AP-1) transcriptional unit that is responsible for the induction of numerous stress response genes containing AP-1 response elements in their promoter regions⁸. The diversity of JNK

target genes has defined differential functions for JNK, and the analyses of pathways regulated by JNK have demonstrated that is indispensable for both cell proliferation and apoptosis⁹. JNK activity is induced by oncogenes in some tumor types^{10,11}, and growth inhibition in myeloma and breast cancer cells treated with JNK inhibitors has been reported¹², supporting a role of JNK activity in cell survival, proliferation, and carcinogenesis.

p38 MAPKs are members of the MAPK family that are also activated by a variety of environmental stresses and inflammatory cytokines. p38 MAPK is involved in regulation of the heat shock protein HSP27, and several transcription factors including ATF-2, Stat1, the Max/ Myc complex, MEF-2, Elk-1, and indirectly CREB¹³. Mounting evidence associates the activation of the p38 MAPK pathway with many aspects of carcinogenesis. For example, mas, an oncogene that encodes a novel G-protein coupled receptor, was shown to induce transformation through the induction of JNK and p38 MAPK signaling¹⁴. Furthermore, high levels of p38 MAPK kinase activity have been reported in several types of tumors, including non-small cell lung carcinomas and breast cancer^{15,16}, and activated p38 MAPK in prostatic intraepithelial neoplastic lesions was associated with proliferation rather than apoptosis¹⁷, suggesting that defects in p38 MAPK function may contribute to cell cycle defects and tumorigenesis.

B5: PI3K/Akt

The PI3K/Akt pathway is a cell survival pathway that is independent of the MEK/ERK signaling pathway. It can be activated by ligand binding to RTKs or by Ras (Cully et al. 2006). Once activated, PI3K phosphorylates phosphatidylinositol 4, 5 bisphosphate (PIP2), converting it to phosphatidylinositol 2, 4, 5 phosphate (PIP3) which acts as a

second messenger for numerous signaling cascades. When PIP2 is phosphorylated by PI3K, it can promote the assembly of signaling complexes near the inner side of the cell membrane and recruit signaling proteins with a pleckstrin homology (PH) domain. The serine/threonine-specific protein kinase Akt contains a PH domain and is preferentially recruited after PI3K activation. Activated Akt induces survival by a number of different mechanisms, including phosphorylation and inactivation of the pro-apoptotic Bcl-2 family member Bcl-2 associated death promoter (Bad), dissociation of the transcription factor NF- κ B from its inhibitor I κ B (allowing the transcription of inhibitor of apoptosis proteins [IAPs]), CREB activation, and phosphorylation of the initiator caspase procaspase-9, resulting in its aberrant proteolytic processing¹⁸. Akt-induced survival has also been associated with up-regulation of heat shock proteins, specifically Hsp70¹⁹. The PI3K signaling cascade is inhibited by the rapid dephosphorylation of PIP3 by Phosphatase and tensin homolog (PTEN). Because of its role in modulating the PI3K/Akt pathway, PTEN is a key tumor suppressor and is downregulated, mutated, or lost in numerous cancers²⁰. In prostate cancer, JNK signaling has been identified as a target of PTEN, also implicating JNK in PI3K-driven cancers²¹.

C. Melanoma

C1: Epidemiology and Development

Malignant melanoma is a type of cancer that initiates in melanin-producing cells called melanocytes. While not as commonly diagnosed as other types of skin cancers, such as basal cell carcinoma and squamous cell carcinoma, melanoma is far more dangerous. Melanoma accounts for 75% of all deaths associated with skin cancer²² and is commonly associated with aggressive local growth and metastases. According to the

most recent report from the CDC, 70,853 people in the United States were diagnosed with melanomas of the skin in 2011 with 12,212 resulting deaths (U.S. Cancer Statistics Working Group). Primary melanoma is the result of the malignant transformation of melanocytes of the basal layer of the epidermis. Melanocytes are derived from neural crest progenitors, and their development is modulated by the receptor tyrosine kinase c-KIT and microphthalmia-associated transcription factor (MITF)²³. The process by which transformation of melanocytes occurs is not yet fully understood, but it develops in sequential transformation stages. Melanomagenesis occurs when activating mutations induce hyperproliferation and subsequent formation of benign nevi that can acquire more mutations, such as those that inactivate tumor suppressors, which promote further tumor growth and invasiveness. Some melanomas, however, arise in the absence of a pre-existing nevus. After melanocytes proliferate into noninvasive radial growth phase melanoma, which then multiply in a horizontal direction within the epidermis, they penetrate the dermis during the vertical growth phase and become invasive. Although melanoma tends to spread to non-visceral sites such as skin or lymph nodes, the most common visceral sites involved in metastatic melanomas are lung, liver, brain, bone, and small intestine²⁴.

C2: Common Genetic Alterations in Melanoma

C2.1: MAPK signaling dysregulation

The MAPK signal transduction pathway is the subject of intense study in oncology. As this pathway normally regulates cell growth, survival, and invasion, it has unsurprisingly been implicated in the development of a broad spectrum of cancers. Melanomas arise as the result of numerous, often disparate, but advantageous mutations.

Genetic alterations that result in the loss or mutation of one or more tumor suppressor genes and/or constitutive activation of the Ras/Raf/MEK/ERK pathway are common in melanoma, with upwards of 90% of patient samples exhibiting mutations in either N-Ras or B-Raf²⁵. Mutations that inhibit Ras GTPase activity or its binding partners lead to constitutive activation of Ras, the primary node of activation for both Raf signaling and PI3K/Akt signaling which plays a role in proliferation, survival and invasion. Tumors with activating mutations in Ras, therefore, are difficult to target through single pathway agents alone because of the activation of multiple prosurvival pathways²⁶. More than 80% of BRAF mutations in melanoma are V600E mutations, which constitutively activate the BRAF enzyme and result in 10.7-fold kinase activity versus wild-type BRAF²⁷. The second most common mutation is V600K (10%-20%)²⁸. Mutant BRAF is constitutively active, causing hyper-activation of MEK and ERK, and providing pro-growth and pro-survival signals.

Also downstream of Ras activation, the PI3K/Akt pathway plays diverse roles in cellular proliferation, apoptosis, and cytoskeletal rearrangement and is one of the most commonly altered pathways in human tumors. Activated Akt is a major mediator of PI3K signaling and phosphorylates numerous downstream substrates including murine double minute 2 (MDM2), NFκB, mammalian target of rapamycin (mTOR), Bad, human telomerase reverse transcriptase (hTERT), and the cyclin dependent kinase (cdk) inhibitor p27, which promote cell survival, proliferation, and invasion²⁹. Phosphorylation of MDM2 induces p53 degradation, and phosphorylation of p27 inhibits its activity, allowing for cell cycle progression. Akt is a major activator of melanoma progression. Aberrant expression of Akt is seen in 60% of malignant melanomas³⁰. PTEN, a negative

regulator of the PI3K/Akt pathway, has been shown to be inactivated or deleted in up to one-third of melanomas and ectopic expression of PTEN in PTEN-deficient melanoma cells suppresses cell growth, inhibits colony formation, and reduces tumorigenicity and metastasis in mice³¹.

C2.2: Tumor suppressor inactivation

In addition to mutations that influence the MAPK pro-survival/growth signaling pathways, melanoma is also characterized by the inactivation of several tumor suppressors that function in cell cycle control. Loss of function of tumor suppressor genes is a critical step in carcinogenesis for many tumor systems.

C2.2a HspB8 (H11)

HspB8 is a heat shock protein that is expressed in normal melanocytes, where it causes growth arrest through β -catenin phosphorylation at the transcriptional activity site Ser (552) and inhibition of the Cyclin E/Cdk2 complex. Like the established tumor suppressors, it is silenced by aberrant DNA methylation in most melanoma tissues and in other cancers (e.g. prostate cancer, Ewing's sarcoma, and hematologic malignancies), and its restored expression induces cell death^{32,33}. Tumor cell death is through the activation of death pathways that lead to apoptosis as well as the activation of additional tumor suppressor functions, including upregulation of the haploinsufficient tumor suppressor Beclin-1³⁴. The role of HspB8 as a tumor suppressor is further supported by the finding of a pro-tumorigenic mutation associated with increased autokinase activity (W51C). This mutation indicates that the autokinase activity is required for the HspB8 proliferative, but not anti-proliferative (pro-apoptotic) activity^{32,35,36}.

C2.2b: CDKN2A

A major gene associated with melanoma is cyclin-dependent kinase inhibitor 2A (CDKN2A), an upstream regulator of the retinoblastoma gene pathway, acting through the cyclin D1/cyclin-dependent kinase 4 complex. CDKN2A, also called p16, controls the passage of cells through the cell cycle and provides a mechanism for holding damaged cells at the G1/S checkpoint to permit repair of DNA damage before cellular replication. CDKN2A encodes two proteins, p16 and p14, both inhibitors of cellular senescence. When the alternate reading frame for exon 1 is transcribed instead of the standard reading frame, p14 is produced and it exerts its effects through the tumor suppressor p53 pathway and mediates cell cycle arrest at the G1 and G2/M checkpoints, facilitating repair of DNA damage³⁷. Mutations in CDKN2A resulting in loss of function, account for 35% to 40% of familial melanomas³⁸.

C2.2c: MEN1

Multiple endocrine neoplasia 1 (MEN1) is a nuclear protein that has been reported to play a role in a variety of cellular processes, including transcription, cell cycle regulation, and proliferation. MEN1 interacts directly with several transcription factors, including NFκB, and while expressed in melanocytes and melanocytic nevi, its expression is either significantly decreased or lost in a high percentage of melanoma cell lines and cultures. MEN1 has growth-suppressive activity in melanoma, as evidenced by human melanoma cell line proliferation upon ectopic MEN1 expression³⁹.

C3: Cancer stem cells

Cancer stem cells (CSC) have been identified in hematological malignancies and several solid cancers. Like normal adult tissue stem cells, CSC are a minority of the

whole tumor and are the only cells that are able to maintain tumor growth indefinitely. Although the majority of cells differentiate and die, the driving force of the tumor is the self-renewal properties of cancer stem cells. Conventional anti-cancer treatments might eradicate most malignant cells in a tumor, but they are potentially ineffective against CSC, which may ultimately be responsible for recurrence and progression⁴⁰. Functional assays of cancer stem cells include the ability to produce colonies when plated in soft agar, grow into spheroids under anchorage-independent conditions, and induce tumor formation when injected at low concentrations⁴¹. In recent years, several reports have demonstrated a CSC subpopulation in melanoma⁴¹⁻⁴³. It has been shown that stem cell associated markers such as CD133, CD166, nerve growth factor receptor (CD271), and nestin are expressed by human melanomas⁴⁴. For effective therapy, it is critical that tumor-forming CSC be targeted and eliminated.

C4: Tumor cell immune evasion

In addition to the presence of tumor-forming CSCs that need to be eliminated, another obstacle for melanoma therapeutics is the immunosuppressive tumor microenvironment (TME), which promotes tumor cell escape from immune surveillance and can contribute to tumor progression. Several molecular mechanisms have been described by which tumors can evade immune responses. These include loss of expression of histocompatibility antigens on the tumor cell surface, the production of immunosuppressive cytokines, the activation of regulatory T cells, and the expression of inhibitors of the immune response⁴⁵. In melanoma, the absence of co-stimulatory molecules⁴⁶, downregulation of tumor-associated antigens⁴⁷, MHC class I molecules⁴⁸ and natural killer cell receptor ligands⁴⁹, have all been described. The melanoma TME is

also characterized by intensive secretion of immunosuppressive factors such as VEGF, TGF- β , IL-10, and nitric oxide⁵⁰, which induce the recruitment and activation of various immunosuppressive leukocytes, including myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), tumor-associated M2 macrophages regulatory dendritic cells (regDC), and Th2 lymphocytes.

One of the NK cell ligands downregulated in melanoma is the MHC class I polypeptide-related sequence A (MICA), which acts as a ligand for the activating receptor NKG2D expressed on NK, $\gamma\delta$ T, cytotoxic $\alpha\beta$ CD8⁺ T, and NKT cells. Its expression on tumor cells can engage NKG2D, resulting in the cytotoxic killing of the target cells and the release of danger associated molecular patterns (DAMPs) and tumor associated antigens (TAAs) that can further stimulate immune cells⁵¹. In melanoma, IL-10 decreases MICA expression, thereby reducing NKG2D-mediated cell cytotoxicity and facilitating tumor cell escape from immune surveillance⁵².

Expression of immune checkpoint inhibitors is commonly associated with tumor immune evasion. CTLA-4 is a glycoprotein of the immunoglobulin superfamily that functions as an inhibitory receptor of T cell activation and effector functions and is implicated in the maintenance of immune tolerance (immune checkpoint)⁵³. Recent studies have shown that CTLA-4 is constitutively expressed in several solid tumors including melanoma^{54,55}, and CTLA-4 blockade with specific monoclonal antibodies resulted in impressive tumor regression⁵⁶. Additional information about immunotherapy using CTLA-4 blockade can be found in Section C5.2.

C5: Treatment

While there are predominant trends such as the high prevalence of N-Ras/B-Raf and CDK2NA mutations, melanomas are highly heterogeneous. There are cell-to-cell disparities in surface marker expression and proliferation rates, and disease prognosis is poor due to resistance to conventional radio and chemotherapies. Although melanoma can be cured by surgical excision if an early diagnosis is made, less than 15% of patients with metastatic melanoma survive past 3 years⁵⁷. Several mechanisms of drug resistance have been suggested, including deregulation of tumor apoptotic pathways, impaired cell cycle checkpoints, and enhanced DNA repair⁵⁸. Until recently, the only approved treatment option available for patients were dacarbazine, a DNA alkylating agent, and high dose interleukin-2 (HD IL-2), an immunotherapy that stimulates T cell proliferation and activity⁵⁹. Dacarbazine has long been used as the first choice of chemotherapeutic drug in patients with unresectable or metastatic melanoma, although the response rate is marginal (10–20 %) and patient survival benefit has never been reported⁶⁰, underscoring the need for more efficient therapies. In the following sections, the development of promising targeted therapies and immunotherapies for the treatment of melanoma is reviewed.

C5.1: Targeted therapies

The first targeted therapy to demonstrate substantial efficacy against melanoma was vemurafenib, an adenosine triphosphate–competitive BRAF inhibitor⁶¹. In the phase III clinical trial, vemurafenib conferred a survival advantage compared to dacarbazine chemotherapy in patients with BRAF^{V600E}-mutant melanomas, with 48% of patients having a detectable decrease in tumor size, compared to 5% in the dacarbazine treatment

group⁶². Another BRAF inhibitor, dabrafenib, provides similar clinical benefit⁶³. Vemurafenib and dabrafenib were approved by the U.S. Food and Drug Administration (FDA) for treatment of advanced BRAF-mutant melanoma in 2011 and 2013, respectively. Although these BRAF inhibitors are impressive examples of bench-to-bedside translation in melanoma and produce rapid initial disease stabilization, their efficacy is restricted to the subset of patients with BRAF^{V600E}-mutant melanomas. The inhibition of targets downstream of B-Raf may also hold some promise. Although MEK inhibitors initially exhibited profound off target effects and poor bioavailability, newer compounds are demonstrating some efficacy in the clinic. Unfortunately, tumor regressions in patients on BRAF and MEK inhibitors are almost inevitably followed by the emergence of drug resistance and disease progression, with median progression-free survival limited to 5 to 7 months⁶⁴. As a result, BRAF and MEK inhibitor combination therapies have been explored, and while they resulted in some delay in disease progression, most patients still relapsed within a year⁶⁵.

C5.2: Immunotherapies

Harnessing the body's own immune system to fight cancer has long been considered the ultimate treatment for cancer because of its potential to specifically target transformed cells while limiting normal tissue damage. Because of the innate ability of melanomas to evade the immune response, immunotherapy has been an active area of research, although major successes have been elusive until recently. Commonly used strategies to boost immune responses against tumors include cytokines, adoptive T cell transfer, and immune checkpoint regulators.

IL-2, a T cell growth factor first identified in 1976 has long been evaluated in cancer treatment because of its ability to stimulate the proliferation and activity of T cells and cause tumor regression⁶⁶. High-dose IL-2 became the first non-chemotherapeutic FDA-approved therapy for advanced melanoma in 1998. Although its use is limited by high toxicity and low response rates, IL-2 can produce long-term remissions in a small subset of patients⁶⁷. Apart from IL-2, interferons (IFNs), cytokines with antiviral and immunomodulatory effects, have also been tested in melanoma for their ability to activate immune cells such as macrophages and up regulate antigen presentation to T cells. Pegylated interferon α -2b (p-IFN α -2b) is IFN α -2b conjugated to polyethylene glycol in order to reduce the rate of absorption and clearance and increase immunogenicity. It was approved by the FDA in 2011 as an adjuvant therapy for surgically treated melanoma patients⁶⁸.

Another treatment that can yield durable responses is adoptive cell transfer (ACT) with autologous T cells, in which lymphocytes are harvested from patient tumors, expanded and activated *ex vivo*, then reinfused⁶⁹. The interest in ACT increased after the recent finding that BRAF inhibition led to an increase in expression of melanoma differentiation antigens, improved recognition by antigen-specific lymphocytes, and increased infiltration of the tumor with CD4+ and CD8+ lymphocytes, which correlated with better tumor regression⁷⁰. ACT is usually preceded and/or accompanied by lymphodepletion using cyclophosphamide or fludarabine, as it was found that the effectiveness of adoptive T cell transfer and the chances of tumor regression were increased when the number of circulating lymphocytes were decreased prior to the

transfer due to the elimination of suppressor T cells and/or the decreased competition by endogenous lymphocytes for homeostatic regulatory cytokines⁷¹.

The most successful immunotherapy approach to date has been immune checkpoint inhibition. T cell activation requires T cell receptor recognition of an antigenic peptide/major histocompatibility complex on the surface of an antigen-presenting cell (APC) as well as a co-stimulatory interaction between the T cell and APC. The second costimulation step can be mediated by either a stimulatory or inhibitory pair of receptor-ligands known as “immune checkpoints”⁷². Two of the best-studied checkpoints involve CTLA-4 and programmed cell death-1 (PD-1), two co-inhibitory T cell receptors that mediate immune tolerance. Ipilimumab, a humanized CTLA-4 blocking monoclonal antibody, was the first treatment to prolong survival in advanced melanoma and was approved by the FDA in 2011. Although ipilimumab responses were limited to 11% of patients in the phase III trial, many of these responses were durable⁷³. Activated T cells can also induce up-regulation of PD-1 ligands PD-L1 and PD-L2 in many peripheral tissues and APCs. One important example of tumor-mediated immune evasion is PD-L1 up-regulation on cells in inflammatory tumor microenvironments⁷⁴. Monoclonal antibodies that antagonize PD-1 or PD-L1 have been even more impressive than CTLA-4 blocking antibodies in clinical studies, with higher response rates and less autoimmune toxicity⁷⁵. Under investigation is the co-inhibition of CTLA-4 and PD-1, since the two checkpoints play non-redundant roles in the regulation of immune responses⁷⁶.

Another compelling strategy is combination of immune checkpoint inhibitors with targeted therapies such as BRAF or MEK inhibitors. Although the phase I trial of ipilimumab plus vemurafenib was closed due to severe hepatotoxicity⁷⁷, several trials

with other combinations of targeted therapies, checkpoint inhibitors, and cytokines are ongoing. Unfortunately, an issue when developing novel drug combinations is that of increases in adverse events because of either overlapping toxicities or unpredictable drug-drug interactions. Despite advances in our understanding of melanoma biology and pathogenesis and success in developing targeted therapies and immunotherapies for melanoma⁷⁸, there remains a significant need for better therapies with improved long-term efficacy and decreased toxicity.

D. Programmed cell death

Cell death is a crucial process during development, homeostasis, and immune regulation of multicellular organisms, and its dysregulation is associated with numerous pathologies. Cell death was originally characterized based on morphology as either necrotic or apoptotic. Apoptosis describes the tightly regulated, energy-dependent process mediated by the cysteine proteases known as caspases. Cell death via apoptosis results in specific morphological changes including cell shrinkage, DNA-laddering, and packaging of small cell fragments into “apoptotic” bodies for disposal by macrophages. Necrosis, on the other hand, describes an unorganized, passive process whereby cells swell and rupture, releasing their contents into the extracellular space, which can trigger an immune response. It has become increasingly evident that cell death can occur through pathways outside of apoptosis and necrosis. The term programmed cell death (PCD), emerged to describe any ordered cellular process that is initiated by the cell and results in cell death. The present cell death paradigm is that caspase-dependent apoptosis is the major cell death pathway, but (i) caspase-independent mechanisms can cooperate with (or substitute for) caspases in the execution of lethal signaling pathways, (ii) the major

programmed necrotic cell death pathway(necroptosis) is mediated through the serine/threonine kinase receptor interacting protein 1 (RIP1), (iii) signaling involved in caspase-1-mediated inflammatory cell death (termed pyroptosis) differs from classical caspase dependent apoptosis, and (iv) autophagic cell death is a type of cell death occurring together with (but not necessarily by) autophagic vacuolization. These major forms of programmed cell death are described below.

D1: Apoptotic cell death

Apoptosis is the proto-typical and most extensively studied PCD pathway. Two major protein families are involved in apoptosis, namely the Bcl-2 family of proteins, which regulate mitochondrial integrity⁷⁹, and the cysteinyl aspartate-specific proteases (caspases), which are responsible for apoptosis execution. In humans, twelve caspases involved in apoptotic and inflammatory pathways have been identified and broadly classified as initiators (caspase-2, -8, -9, -10), executioners (caspase-3, -6, -7) or inflammatory (caspases-1,-4,-5)^{80,81}. All caspases are expressed as inactive proenzymes consisting of an N-terminal prodomain of variable length, followed by a large subunit (p20) and a small C-terminal subunit (p10). Initiator caspases contain protein interaction domains, such as caspase activation and recruitment domains (CARDs) and death effector domains (DEDs), which enable caspases to interact with other molecules that regulate their activation. Initiator caspases, present in the cell as inactive monomers, are recruited to platform molecules via these protein-protein interaction domains and are subsequently activated by oligomerization and proximity-induced autoproteolysis⁸². In addition to aggregation and auto-activation, caspases can often activate other procaspases, allowing initiation of a proteolytic cascade, which amplifies the apoptotic

signaling pathway and leads to rapid cell death. Depending on how the pathway is initiated, apoptosis is categorized as either intrinsic or extrinsic.

Intrinsic apoptosis can be initiated by various stimuli, such as cytotoxic insults and DNA damage, and acts through the mitochondria, which are controlled by the Bcl-2 family of proteins⁸³. Under normal conditions, the anti-apoptotic Bcl-2 family members maintain mitochondrial integrity by preventing the pro-apoptotic Bcl-2 family members Bax and Bak from causing mitochondrial damage. During cellular stress, Bcl-2-homology 3 (BH3)-only proteins are activated and antagonize the anti-apoptotic Bcl-2 family members. Consequently, the inhibition of Bax and/or Bak is relieved, resulting in their oligomerization and formation of pores in the mitochondrial membrane leading to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c (cyt c) into the cytosol. Cyt c can then associate with Apaf-1 and ATP, forming a platform for recruitment and activation of procaspase-9, also known as the apoptosome⁸⁴. Active caspase-9 cleaves and activates the downstream executioner caspases-3, -6 and -7, which are crucial for the execution of apoptotic cell death. In addition to cyt c, other pro-apoptotic proteins are released from the mitochondria and contribute to cell death. For example, second mitochondria-derived activator of caspases (Smac/DIABLO) antagonizes the action of IAPs such as XIAP, cIAP1, and cIAP2. XIAP is an endogenous inhibitor of programmed cell death that can bind to and inhibit activation of caspases and binding of Smac/DIABLO to XIAP relieves its inhibitory interaction with caspase-9, -3 and -7. cIAP1 and cIAP2 do not directly inhibit caspases and instead interact with tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), which leads to their

recruitment to TNFR1. There, they mediate the ubiquitination of RIP1, resulting in promotion of TNF-induced NF- κ B activation⁸⁵.

The extrinsic pathway of apoptosis is initiated by ligand binding to TNF family death receptors located in the plasma membrane. For example, Fas and TRAIL ligand binding to their respective receptors leads to recruitment of the adaptor protein Fas-associated death domain (FADD), which contains a “death effector domain”(DED) that is also found in the procaspase 8 and 10. FADD and procaspases-8 and/or -10 then associate via their DEDs, forming the death-inducing signaling complex (DISC), and promoting the self-cleavage and activation of caspases -8 and -10 and activation of downstream executioner caspases. Aggregation of TNFR1 following ligand binding leads to the sequential formation of two complexes⁸⁶. Complex I consists of TNFR1, TNFR-associated death domain (TRADD), TRAF2, RIP1, cIAP1 and cIAP2 and is formed at the plasma membrane. These proteins are important mediators of TNF- induced activation of NF- κ B and MAPKs. Endocytosis of TNFR1 is followed by the formation of complex II, which is analogous to the DISC induced by Fas and TRAIL, resulting in caspase 8 and/or 10 activation. In addition to activating executioner caspases, activated caspase-8 can also cleave the BH3-only protein Bid, activating the mitochondrial pathway of apoptosis and amplifying death receptor induced apoptosis. The extrinsic and intrinsic pathways of apoptosis are schematically represented in Fig 2.

D2: Caspase-independent cell death

In the absence of caspase activity, pro-apoptosis stimuli that trigger mitochondrial outer membrane permeabilization (MOMP) can also induce cell death, so called caspase-independent cell death⁸⁷. Caspase-independent cell death, although similar to apoptosis

has distinct morphological and biochemical properties. The permeabilization of the mitochondria is often viewed as the ‘point-of-no-return’ and in the absence of caspase activation is most notably mediated by apoptosis inducing factor (AIF) and the calpain protease family. Under normal conditions, AIF, expressed as a 62 kDa precursor, is anchored to the inner mitochondrial membrane, where its oxidoreductase activity

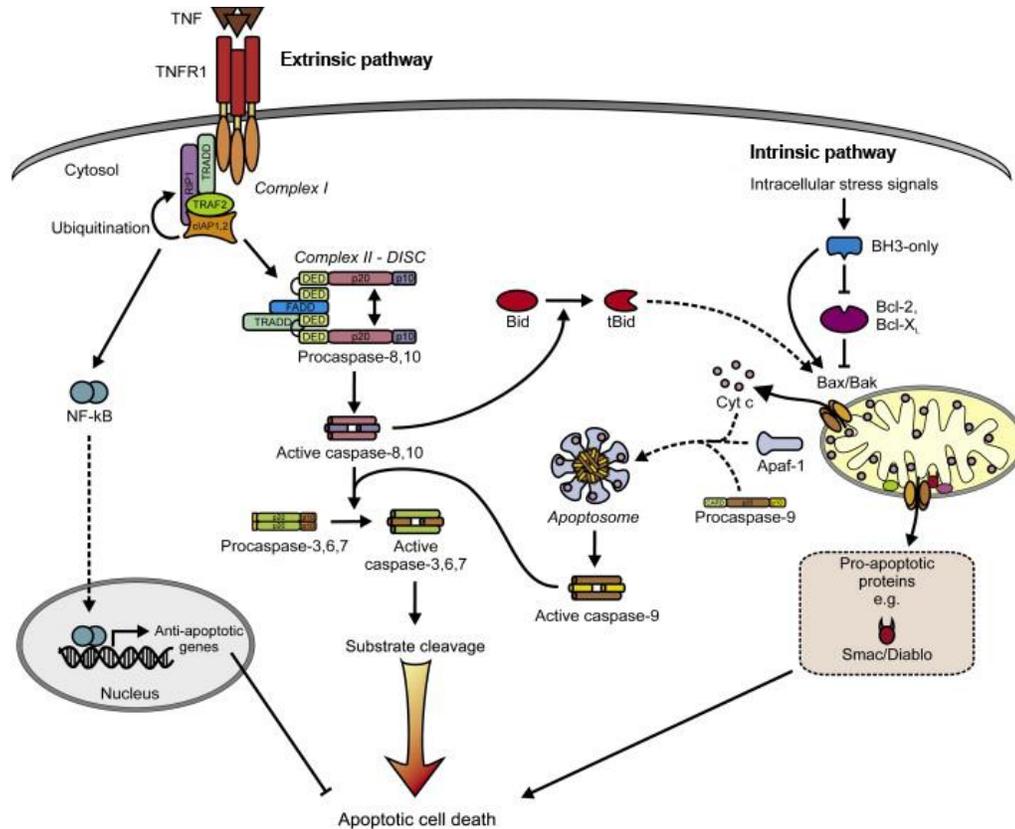


Figure 2: Intrinsic and extrinsic apoptosis pathways

Apoptosis can be triggered by ligand binding to death receptors (via extrinsic pathway) leading to formation of the DISC complex and activation of the initiator caspase-8. Damage to the mitochondria can also trigger apoptosis via the intrinsic pathway. Cytochrome c is released from damaged mitochondria where it forms a complex with Apaf-1 and caspase 9 known as the apoptosome. The apoptosome can cleave and activate the executioner caspase-3. Activated caspase-8 can also truncate Bid which translocates to the mitochondria and forms pores in the mitochondria which allows for cytochrome c release. Both pathways converge on activation of executioner caspases. Adapted from ⁸⁸

plays a vital role in the maintenance and maturation of the mitochondrial respiratory chain complex 1⁸⁹. When the mitochondrial membrane is permeabilized following a cell death insult, AIF is processed into a soluble, truncated 57kDa form (tAIF). This cleavage is specifically achieved through the activation of two families of cysteine proteases that are different from caspases: calpains and cathepsins. Calpains regulate AIF cleavage in a Ca²⁺-dependent context, whereas cathepsins B, L, or S activation of AIF is Ca²⁺-independent^{90,91}. Once in the cytoplasm, tAIF translocates to the nucleus where it interacts with DNA to cause chromatin condensation and large-scale DNA fragmentation⁹². AIF does not display intrinsic endonuclease properties, therefore its DNA-degrading activity depends on the recruitment of downstream nucleases, including Endo G and cyclophilin A^{93,94}.

The calpains are similar to the caspases in that they are cysteine proteases, but their activity is regulated by cytosolic Ca⁺² concentrations. The most important and mostly studied calpains are the conventional μ -calpain (calpain I) and m-calpain (calpain II). The terms μ - and m-calpain were first used to refer to the micromolar or millimolar Ca²⁺ concentration needed to activate μ - and m-calpain, respectively⁹⁵. Both μ - and m-calpain are heterodimeric enzymes (80 kDa) that share a common small regulatory subunit protein of 28 kDa that is critical for calpain function. Calpains are activated by binding of Ca²⁺ followed by an autolytic cleavage at the N-terminal moiety of the protein. In addition to cleaving AIF and promoting caspase independent cell death, calpains have also been suggested to be involved in the regulation of caspase activity during apoptosis. The cleavage of upstream caspases-9 and -8, as well as executioner caspases-3 and -7 by calpains has been described^{96,97}. ER stress-induced apoptosis mediated via murine

caspase-12 has also been shown to require calpain activation⁹⁸. Conversely, several reports support the role of calpains as negative regulators of caspase activity. For example, calpain-generated fragments of caspases-7, -8 and -9 were inactive and/or unable to activate downstream executioner caspases, and calpain potently inhibited the ability of cyt c to activate executioner caspases⁹⁹. A recent study also demonstrated calpain-dependent cleavage of the cytochrome c binding protein Apaf-1¹⁰⁰ and proapoptotic members from the Bcl-2 family, such as Bax and Bid, are processed and activated by calpains¹⁰¹.

D3: Necroptosis

Various stimuli can engage a non-apoptotic form of programmed necrosis called necroptosis that morphologically resembles necrosis¹⁰². The best-characterized inducers of necroptosis are death receptor ligands, TNF in particular, and DNA alkylating agents. TNFR stimulation induces formation of a complex at the membrane that includes TRADD, TRAF2/5, RIP1 and cIAP1/2¹⁰³. When the conditions do not favor apoptosis, this complex dissociates and RIP1 is released into the cytosol where it can interact with receptor interacting protein-3 (RIP3) to form the necrosome, as schematically represented in Figure 3¹⁰⁴. As demonstrated by the use of Necrostatin-1 (Nec-1), a small molecule identified in a chemical library screen as a potent inhibitor of the RIP1 kinase activity, the formation of this complex requires the phosphorylating properties of RIP1¹⁰⁵. Necrosome formation is also highly regulated by ubiquitylation. The ubiquitin ligases cIAP-1 and cIAP-2 negatively affect its formation by ubiquitylating RIP1, whereas the deubiquitylase CYLD counteracts this and promotes necrosome formation^{106,107} and blockade of cIAP

alkylating agents, such as Methylnitrosoguanidine (MNNG), have also been shown to induce necroptosis through the induction of DNA damage that results in disproportionate activation of poly ADP-ribose polymerase-1 (PARP-1) and the phosphorylation of histone H2AX at Ser139 (γ H2AX)^{111,112}. PARP-1 is a nuclear enzyme activated by DNA strand breaks, and plays a key role in repairing DNA damage. Over-activation of PARP-1 potentially leads to NAD⁺ reduction, ATP depletion, cellular energy failure, and release of AIF from the mitochondria. Once in the cytosol, AIF translocates to the nucleus, where, in cooperation with γ H2AX and cyclophilin A, it provokes DNA degradation and cell death¹¹¹.

D4: Pyroptosis

Pyroptosis is a more recently recognized form of programmed cell death that is morphologically and biochemically distinct from necrosis and apoptosis. As the name suggests, pyroptosis is an inflammatory type of cell death and unlike apoptosis, it depends on the activation of caspase-1¹¹³. During pyroptosis, recognition of bacterial, viral or host danger signals induces the formation of inflammasomes and/or pyroptosomes, caspase-1 activating complexes analogous to the apoptosome seen for intrinsic apoptosis. Several inflammasomes have been identified and are formed by the oligomerization of Nod-like receptors (NLR), such as NLRP3 and AIM2, with adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which recruits procaspase 1. Caspase 1 is then activated by autocatalytic cleavage. As shown in Fig 4, ASC and caspase 1 can also oligomerize alone without NLRs, forming the pyroptosome. Active caspase-1 is the central executor of pyroptotic cell death and initiates an

inflammatory response by the cleavage of the proinflammatory cytokines pro-IL-1 β and pro-IL-18, which are released by the cell upon their activation ¹¹⁴.

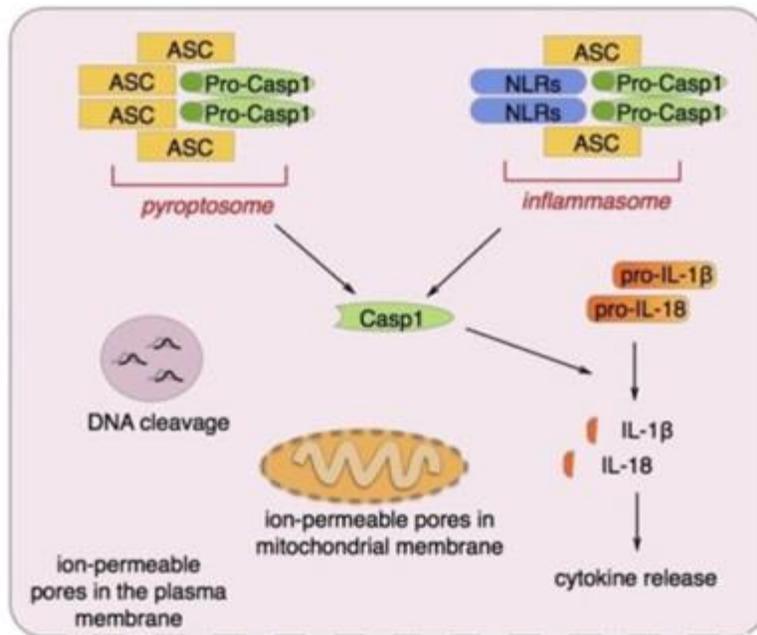


Figure 4 : Pyroptosis

Pyroptosis is a caspase-1 dependent programmed cell death. Caspase-1 can be activated by pyroptosomes and inflammasomes. The pyroptosome is composed of oligomerized ASC dimers while inflammasomes are composed of NLRs and ASC. Both activate caspase-1 and active caspase-1 induces maturation of pro-IL-1 β and pro-IL-18, DNA cleavage, and the formation of ion-permeable pores in the plasma and mitochondrial membrane. From ¹¹⁵.

However, this inflammatory response is not required for the execution of cell death.

Caspase 1 also induces the formation of discretely sized ion-permeable pores in the plasma membrane, leading to cell swelling and cell lysis ¹¹⁶. This untimely release of cytoplasmic content also functions in the elicitation of an immune response, and

pyroptosis is therefore considered a form of immunogenic cell death, further described in Section D6.

D5: Autophagic cell death

Autophagy is an evolutionarily conserved catabolic pathway that allows eukaryotes to degrade and recycle cellular components. Although basal levels of autophagy are required for maintenance of intracellular homeostasis and a pro-survival role for autophagy is well established, autophagy has also long been implicated in cell death¹¹⁷ and inhibiting autophagy has been shown to protect cells from death in some systems¹¹⁸. However, in most instances autophagy appears to be associated with, rather than actually causing, such cell death. Autophagy is a lysosome- dependent process that degrades various cargoes varying from molecules to whole organelles¹¹⁹. Proteins and organelles are sequestered in specialized double-membrane vesicles, called autophagosomes and mature autophagosomes fuse with lysosomes leading to the breakdown of engulfed material by lysosomal hydrolases. Autophagy is a complex process carried out by dedicated proteins, called autophagy related genes (Atg). The autophagy process can be divided into distinct steps, including induction, cargo recognition and selection, vesicle formation, autophagosome-vacuole fusion, and breakdown of the cargo. Different sets of Atg proteins are involved in these steps and consist of the core autophagic machinery. The synthesis of autophagic vacuoles requires the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which is composed of the PtdIns3K Vps34 (vacuolar protein sorting 34), a myristoylated serine/threonine kinase Vps15 (p150 in mammalian cells), Atg14, and Beclin 1^{120,121}. The function of Beclin 1 in autophagy is regulated by Bcl-2, which can bind and sequester Beclin-1, thereby inhibiting autophagy¹²². Activation of the PI3KC3 (Vps34)-complex results in the generation of PI3P and the recruitment of other Atg proteins that facilitate vesicle elongation. Two conjugation systems are implicated in

this process, the first being the formation of an Atg12-Atg5-Atg16 multimer complex involved in vesicle curvature¹²³. During the second conjugation step, LC3 is lipidated by binding to phosphatidylethanolamine (PE). In contrast to the cytoplasmic localization of LC3, LC3-II, which is formed when phosphatidylethanolamine conjugates to LC3, specifically binds to the autophagic membranes, and for that reason it is generally used as an autophagic marker¹²⁴. Targeted components such as protein aggregates, mitochondria, and invading pathogens are recruited to the autophagosome by adaptor proteins such as p62. The autophagosome fuses with a lysosome, resulting in the degradation of the autophagic contents as well as p62 and LC3-II by hydrolases.

D6: Immunogenic cell death

In recent years, the concept of immunogenic cell death (ICD), or cell death that stimulates an immune response against dead cell antigens, has emerged. The original model was first proposed in the context of anticancer chemotherapy, and only included “immunogenic apoptosis”, a form of apoptosis which involved the exposure of “danger signals” at the cell surface that were capable of activating dendritic cells (DC) and mounting an anti-tumor immune response¹²⁵. However, in recent years the definition of ICD has been expanded to include necroptosis, necrosis, autophagic cell death, and pyroptosis. The signals that mediate the immunogenicity of tumor cells involve elements of the DNA damage response, elements of the endoplasmic reticulum stress response, as well as elements of the apoptotic response. For pre-apoptosis cells, common features of ICD include the exposure of calreticulin, heat-shock proteins (HSPs), and other endoplasmic reticulum proteins on the cell surface, the secretion of ATP during the blebbing phase of apoptosis, or the secretion/release of immunostimulatory factors, such

as cytokines, ATP, and the non-histone chromatin protein high-mobility group box 1 (HMGB1), to stimulate innate immune effectors. Pyroptosis and necrosis/necroptosis involve the release of cytoplasmic contents, including danger associated molecular patterns (DAMPs), such as ATP, HMGB1, and uric acid, into the extracellular space, which are highly immunogenic¹²⁶. Emerging clinical and experimental data indicate that clinical responses to cytotoxic cancer therapy can be improved if immunogenic cell death pathways are also concurrently activated¹²⁷.

E. Oncolytic virotherapy

Oncolytic virotherapy is the use of growth restricted virus mutants to reduce tumor burden through tumor-selective growth. While there are some naturally occurring virus mutants with tumor-selective growth, the majority of OVs achieve tumor selectivity through mutation and/or deletion of viral genes important for replication, impairing the ability of the virus to grow in normal cells. Highly proliferative tumor cells, however, can support replication of the mutants, because the replication cycle of many viruses exploits the same cellular pathways that are altered in cancer cells¹²⁸. Therefore, unlike traditional chemotherapeutic treatments, oncolytic viruses (OVs) can target and kill tumor cells, leaving normal tissue intact. OVs are advantageous because they are known to destroy tumors by several distinct mechanisms, which typically do not overlap with the mechanisms induced by traditional therapies. While direct viral oncolysis and tumor spread have previously been considered the primary determinants of OV therapeutic efficacy, recent studies have highlighted the importance of post-oncolytic anti-tumor activity^{129,130}. In addition to directly destroying infected tumor cells as a result of infection, infection with many oncolytic viruses can result in the development of a potent

adaptive anti-tumor immune response. This immune response can overcome localized immune suppression, and may even create an *in situ* vaccination effect through cross-presentation of tumor associated antigens to the host immune response. A key factor in the development of adaptive anti-tumor immunity in response to virotherapy is the mode of cancer cell death induced by the virus. ICD, including necrosis and pyroptosis, results in the release of DAMPs and tumor associated antigens(TAAs), potentiating anti-tumor immunity¹³¹. Another advantage of OV is that they are not subject to the typical mechanisms of drug resistance such as drug efflux pumps and defective apoptotic signaling¹³², making them potential candidates for the targeting of CSC. Direct studies of CSC-lysis by OV, however, are relatively scant. In addition to death induced through direct cell lysis and the anti-tumor immune response, OV also induce necrotic/apoptotic death of uninfected cells induced by anti-angiogenesis and vasculature targeting^{133,134}.

OVs have been developed from virtually all of the virus families, with each having its own advantages and disadvantages. Overall, oncolytic viruses have excellent clinical tolerability, but their efficacy is limited by a number of factors, including relatively poor level of intratumoral replication/spread, premature viral clearance due to host innate anti-viral immunity, and the inability to eliminate tumor-forming CSC. Strategies to improve OV efficacy are discussed in Section F of this introduction.

E1: Immune system elements of virotherapy

The immune system is a double edged sword in the context of virotherapy. In order for successful viral propagation as well as possibility of retreatment, the immune response to the virus must be minimized, while at the same time, an immune response against the tumor must be elicited. This response depends on modulation of both the

innate and adaptive immune responses as they relate to the virus and the tumor. Innate and adaptive immune responses in the context of virotherapy are discussed below.

E1.1: Innate immune response

A key property of the innate immune system is the ability to recognize viruses as ‘foreign’. Viral proteins and nucleic acids are distinguished from cellular counterparts by cellular proteins called pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), which can be present in the cytoplasm or on the cell surface. Engagement of PRRs leads to the synthesis of pro-inflammatory cytokines, including interferon- α and - β (IFN- α , IFN- β), TNF- α , interleukin-6 (IL-6), IL-12, and IFN- γ which function locally by binding receptors on other cells, resulting in the production of antiviral genes as well as immune cell recruitment. Key immune cells that participate in innate responses are macrophages, neutrophils, natural killer (NK) and DCs. While these cells are immediately available to combat a wide range of pathogens they are constrained by a limited and invariant repertoire of PRRs. These receptors recognize PAMPs such as peptidoglycans, lipopolysaccharides, and unmethylated CpG DNA, and engagement of these receptors on immune cells leads to pathogen engulfment and degradation. DC activation through engagement of their pattern recognition receptors is an important step in the development of an adaptive response, as discussed below. Innate immunity is present in all individuals at all times, but is not specific for any individual pathogen; therefore it does not lead to lasting immunity and does not increase with repeated exposure to a pathogen. Cells of the innate immune system can recognize the presence of a growing tumor which has undergone stromal remodeling, causing local tissue damage. This is followed by the induction of inflammatory signals, such as the production of IFN-

γ , which is essential for recruiting cells of the innate immune system to the tumor site¹³⁵.

The host innate immune responses are an obstacle for virotherapy since they are involved in the recognition and elimination of viral vectors; therefore OV_s with natural or engineered immune evasion mechanisms are desirable. Co-treatment with anti-inflammatory drug agents that dampen the innate immune responses, such as histone deacetylase inhibitors, are also being used to improve OV efficacy, as discussed in Section F4.1.

E1.2: Adaptive immune response

The adaptive immune response refers to the response of antigen-specific lymphocytes to antigen. The induction of an adaptive immune response begins when DCs ingest a pathogen then migrate to peripheral lymphoid tissues where they mature into antigen presenting cells (APC) that express both pathogen antigen and the co-stimulatory molecules necessary to activate naïve T lymphocytes. Lymphocytes activated by antigen then give rise to clones of antigen-specific effector cells that mediate adaptive immunity. Unlike innate immunity, the adaptive immune response is capable of generating immunological memory, meaning that after exposure to a pathogen, any subsequent exposure will produce an immediate and stronger response¹³⁵. This memory response is of particular concern for oncolytic virotherapy, since the development of an adaptive immune response against the virus would prohibit retreatment, a necessary component of therapy. The elicitation of an anti-tumor immune response, another critical aspect of oncolytic virotherapy, is also dependent on adaptive immune responses. OV_s that induce ICD, such that there is a release of DAMPS and TAA into the extracellular space, can

promote the formation of an antigen-specific adaptive immune response against tumor antigens.

E2: Herpes Simplex virus

Because this thesis work focuses on the molecular mechanisms of an oncolytic Herpes simplex virus (HSV) generated in the Aurelian lab, HSV and oncolytic HSVs are discussed in more detail in the following section.

E2.1: General Overview and Pathogenesis

HSVs belong to the alpha herpesviridae family of double stranded DNA viruses and are further classified into two serotypes, HSV type 1 (HSV-1) and HSV type 2 (HSV-2), which respectively affect upwards of 90% and 25% of the world population. The viral particle consists of a double-stranded DNA core surrounded by an icosahedral capsid that is surrounded by protein unstructured matrix called tegument, which, in turn, is surrounded by a lipid bilayer envelope with embedded branched glycoproteins¹³⁶. The viral genome is quite large as compared to other viruses used as vectors, encoding upwards of 84 unique proteins¹³⁷. The genome is packaged as a linear molecule but rapidly circularizes upon reaching the host cell nucleus. HSV virions bind to three known receptors, nectins-1 and -2, the lymphotoxin alpha receptor also known as the herpes virus entry mediator (HVEM/ Lymphotoxin- α receptor), and heparin sulfate giving HSV broad cellular tropism. The major viral glycoprotein responsible for cell binding and entry is gD, however gI, gL, gH are involved in the subsequent fusion of viral and cellular membranes.

During virus entry, the viral glycoproteins and cell membrane fuse, releasing tegument proteins and capsids into the cytosol. The viral capsid is rapidly translocated to

the nuclear membrane surface where it docks and releases the viral genome into the nucleus. Virus replication is carefully orchestrated through three successive waves of gene expression, named Immediate Early (IE or α), Early (E or β) and Late (L or γ). IE genes are regulatory and function to induce the expression of E genes, which are responsible for viral DNA replication. Late genes encode viral structural proteins involved in virion assembly. The late gene VP5 encodes the major capsid component, and serves as an indicator of productive virus replication. HSV has the unique ability to latently infect neurons of the sensory ganglia nearest to the primary infection site ¹³⁶. During latency, the viral genome is maintained as an extrachromosomal episome and all viral protein expression ceases. Cellular stress such as UV irradiation, neuronal damage, and hormonal fluctuations are known to trigger latency reactivation. The mechanism of reactivation is also still poorly understood but believed to be related to the Latency-Associated Transcript (LAT), which is expressed and processed into several smaller RNAs which possess microRNA gene silencing characteristics that target the neurovirulence gene ICP34.5 and ICP0, which is required for activation of gene expression ¹³⁸. Reactivation of HSV-2 has been associated to stress induced activation of the AP-1 transcription factor complex (c-Jun/cFos) which bind to the promoter region of the gene encoding the large subunit of ribonucleotide reductase domain, also known as ICP10 in HSV-2 ¹³⁹.

E2.2: Oncolytic HSV

Most oncolytic HSV (oHSV) were developed from HSV-1 and their generation involves the deletion or mutation of one or more genes required for efficient virus replication. The most commonly targeted genes in oHSVs encode ICP34.5, a

neurovirulence gene, R1, the large subunit of the viral ribonucleotide reductase, and ICP47, which is responsible for immune evasion. ICP34.5 binds to and activates protein phosphatase-1 α , which dephosphorylates the transcription factor eIF2 α allowing protein synthesis to continue despite other antiviral responses typically mediated by protein kinase R (PKR)¹⁴⁰. ICP47 binds to the human transporter associated with antigen presentation (TAP) to inhibit peptide transport into the endoplasmic reticulum for loading onto MHC class I proteins. ICP47 therefore presumably limits the development of an adaptive immune response against HSV by preventing CD8⁺ T cell recognition of HSV infected cells, since the CD8⁺ T cell receptor can only recognize peptides bound to MHC class I. However, the contribution of ICP47 to pathogenesis is unclear. Because tumor cells have high levels of ribonucleotide reductase, deletion of R1 (also called ICP6 in HSV-1 and ICP10 in HSV-2), confers tumor-selective virus growth. The HSV-2 R1, ICP10, has two independent functions. A kinase function that is not conserved by the HSV-1 homologue ICP6 and the large subunit of ribonucleotide reductase function that is conserved in both virus isotypes. ICP10 is made up of an N-terminal extracellular domain, a transmembrane (TM) domain, a unique protein kinase (PK) domain and it is followed by the ribonucleotide reductase domain¹⁴¹. Subsequent thorough characterization of ICP10 demonstrated that the PK domain functions as a constitutively activated growth factor receptor. As illustrated in Fig 5A, the signaling cascades that are activated by ICP10PK include Ras/Raf/MEK/ERK, PI3K/AKT, and Adenylate cyclase/PKA illustrating the multiple cellular processes that HSV-2 uses for efficient virus replication and latency reactivation. ICP10PK signaling, like cellular RTK's requires localization at the plasma membrane where it binds to the GEF, SOS which

activates Ras through exchange of GDP to GTP. ICP10PK also binds and phosphorylates RasGap inhibiting its ability to downregulate Ras activation¹⁴². This signaling is critically involved in the ability of HSV-2 to induce immediate early gene expression and replicate¹⁴³. Of note, most oncolytic HSV-1 possess R1 (ICP6) deletions whereas in oncolytic HSV-2 viruses, only a portion of the R1 (ICP10) subunit has been deleted, leaving the ribonucleotide reductase domain intact and presumably enhancing their replication.

The majority of oHSVs are based on HSV-1 and while oHSV constructs were tolerated well in early clinical trials, efficacy was limited by a relatively poor level of intratumoral replication due to deletion of ICP34.5, and /or R1¹⁴⁰. Indeed, in glioblastoma, an oHSV with an intact PKR-inhibiting domain of ICP34.5 had superior oncolytic activity than a virus in which the entire ICP34.5 was deleted¹⁴⁴, and an oHSV missing both copies of ICP34.5 and no other mutations was unable to replicate in a Phase I study of oral squamous cell carcinoma¹⁴⁵. The most successful oHSVs in clinical trials have been genetically modified in various ways to increase efficacy. These alterations are discussed in Section F.

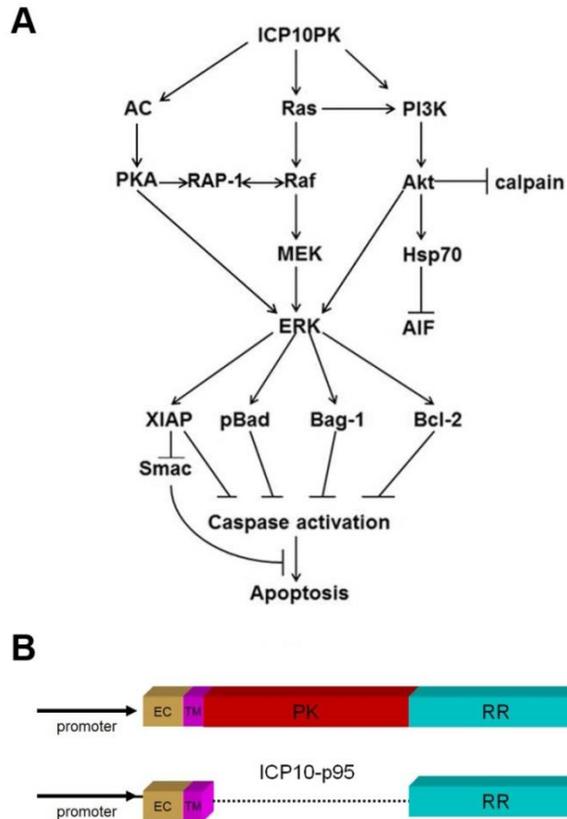


Figure 5: ICP10PK pro-survival signaling and Δ PK construction

Adapted from¹⁴⁶

E2.3: Δ PK

Δ PK is an HSV-2 mutant that is deleted only in the R1 protein kinase domain (known as ICP10PK), which is not conserved in HSV-1 (Fig 5B). The loss of ICP10PK imparts tumor selectivity while leaving R1 and ICP34.5 intact for improved replication, thereby presumably increasing the clinical efficacy of Δ PK as an oncolytic agent¹⁴⁷. Work completed in the Aurelian lab has previously demonstrated that Δ PK has strong oncolytic activity in melanoma cultures and xenografts¹⁴⁸. Cell death, however, is largely independent of virus replication and is caused by the induction of PCD pathways

requiring sequential activation of calpain and caspases-7 and -3, and is also associated with autophagy and pyroptosis proteins. Δ PK has strong *in vivo* activity, demonstrated by the long term elimination of melanoma xenografts with animals remaining tumor-free for at least a year. Although this implies that CSCs are not resistant to Δ PK, their lysis has not been documented and the role of virus-induced PCD pathways and tumor cell type, if any, are still poorly understood. Δ PK is particularly promising because it stimulates a Th1 biased immune response¹⁴⁹ and was tolerated well in Phase I/II clinical trials¹⁵⁰. Δ PK treatment of tumor xenografts enhanced macrophage infiltration and increased expression of activated caspase 1 and proinflammatory cytokine TNF α ¹⁴⁸, suggesting that increased inflammation and immune surveillance is a mechanism that contributes to the ability of Δ PK to induce lasting tumor elimination.

F: Improving OV efficacy

Arming oncolytic viruses that are inefficient in pre-clinical models or clinical trials with transgenes designed to enhance tumor selectivity, improve intratumoral replication, modify immune responses, or express antitumorigenic genes, can increase their potency. Some of the modifications made to OVs to increase efficacy include the expression of cytotoxic genes and pro-drug converting enzymes, fusogenic alterations to increase spread, expression of immunostimulatory genes, and use of combinatorial therapy.

F1: Apoptosis inducing genes and pro-drug converting enzymes

Arming oncolytic viruses with transgenes that are capable of inducing apoptosis is an attractive strategy for improving anti-cancer activity. Adenoviruses that are engineered to express TNF-related apoptosis-inducing ligand (TRAIL) were found to be more oncolytic

than the parental viruses in cancer cell lines and in xenograft models¹⁵¹, and an adenovirus armed with apoptin, a small apoptosis-inducing protein derived from chicken anemia virus, inhibited primary transplanted tumors and significantly extended survival times of animals¹⁵². Another adenovirus armed with the tumor suppressor gene, p53, killed human cancer cells of various tissue origins and p53 status more effectively than did its parental control virus without the insert¹⁵³.

Pro-drug therapies seek to reduce dose-limiting toxicities of chemotherapeutics and increase the efficacy of OVs by selectively generating the chemotherapeutic agent at the target tumor site. Such pro-drug-based cancer therapies have two basic components: an inactive, nontoxic pro-drug and a pro-drug-activating enzyme. Thymidine kinase (TK) and cytosine deaminase (CD) and their respective pro-drugs [ganciclovir (GCV) and 5-fluorocytosine (5-FC), respectively] are the most advanced of the pro-drug-based therapies¹⁵⁴. An oHSV-1 expressing cytosine deaminase displayed increased cytotoxicity¹⁵⁵. An HSV-1 based oncolytic virus engineered to express cytochrome P450 which converts cyclophosphamide into freely diffusible alkylating metabolites, exhibited an enhanced antitumoral effect when the prodrug was administered with the virus¹⁵⁶. Another approach used an oncolytic adenovirus armed with carboxypeptidase G2 driven by a telomerase promoter was used to convert the alkylating mustard prodrug ZD2767P into a cytotoxic compound that enhanced its efficacy over that of an oncolytic adenovirus lacking the transgene¹⁵⁷.

F2: Fusogenic peptides

Two limiting factors to virus spread are recognition by the host immune system and slow diffusion of virions to neighboring cells. To improve upon these fronts, mutant

viruses which possess fusogenic activity have been employed. Fusogenic glycoproteins cause infected cells to fuse with neighboring cells and the resulting syncytium allow for faster dissemination of virus. Fusogenic proteins are generally controlled under tumor-specific promoters and exhibit higher pre-clinical efficacy over their non-fusogenic counterparts¹⁵⁸. An adenovirus encoding a fusogenic membrane glycoprotein driven by the human endothelial receptor tyrosine-kinase promoter triggered in vivo fusion between endothelial cells and epithelial cells, thereby facilitating penetration of the virus¹⁵⁹. FusOn-H2, an HSV-2 oncolytic that differs from Δ PK in that both the ICP10PK catalytic and transmembrane domains were replaced with EGFP and the resulting protein was placed under the direction of the promiscuous CMV promoter has demonstrated fusogenic activity imparted by an unrelated genetic alteration¹⁶⁰.

F3: Enhancing anti-tumor immunity

Many OV's expressing cytokines (such as IL-2, IL-12, IL-18); chemokines (such as CCL5), or co-stimulatory molecules (such as B7.1 and CD40L) have been studied and some exciting antitumor immunity and therapeutic results have been documented in animal models and in human cancer patients. One of the most clinically successful approaches so far has been the expression of cytokine granulocyte macrophage colony stimulating factor (GM-CSF), in both vaccinia virus and oHSV. T-VEC, an oHSV armed with the GM-CSF, recently passed Phase III clinical trials in stage III and IV melanoma¹⁶¹. When administered intratumorally to patients with hepatocellular carcinoma, an oncolytic vaccinia virus also engineered to express GM-CSF lead to objective responses in 3 of the 10 evaluable patients¹⁶². IFN α is another proinflammatory cytokine with proven antitumoral efficacy; however, when delivered systemically it frequently elicits

severe toxicities. Intratumoral injection of an oncolytic vaccinia virus that expresses IFN α has exhibited enhanced benefits compared to the parent virus¹⁶³. An adenovirus armed with TNF- α was shown to have potent tumor cell killing both *in vitro* and *in vivo*, associated with apoptotic and necrotic tumor cell death and antitumor immune responses¹⁶⁴.

F4: Drug combinations

Despite the development of novel OV's with improved efficacy and tumor selectivity, their efficacy is still limited, in part, by their inability to evade host innate immunity and induce efficient and sustained viral propagation in tumor cells. Compounds that specifically dampen tumor cell antiviral responses can be used to regulate the extent of virus replication and tumor killing. Patients given low dose cyclophosphamide in conjunction with an oncolytic adenovirus exhibited increase cytotoxic T cells, induced Th1 type immunity, and survival and overall survival versus virus alone. An oncolytic adenovirus used in conjunction with the chemotherapeutic mixture of mitomycin-C, doxorubicin, and cisplatin was well tolerated, however antitumor activity was only observed in one out of six patients in a Phase I/II study¹⁶⁵. Sunitinib, a drug already approved for renal cancer treatment, has been shown to augment the growth of oncolytic vesicular stomatitis virus (VSV) in mouse models¹⁶⁶, to have anti-angiogenic properties, and to enhance anti-tumor immunity¹⁶⁷. Taken together, these studies demonstrate the promising potential of chemotherapeutics to augment the efficacy of oncolytic virotherapy.

Of particular relevance to this thesis work is the use of histone deacetylase inhibitors (HDACis) to augment OV efficacy. HDACis have been shown to increase the killing of virus-resistant tumor cells and have the added benefit of promoting tumor specific immune responses in preclinical models¹⁶⁸. Since epigenetic processes, such as histone deacetylation and DNA methylation, are known to contribute to the cancerous transformation of cells by silencing critical genes, it has been hypothesized that HDACis act through the stimulation of silenced tumor suppressor genes¹⁶⁹. More information about the mechanism and utility of HDACis is contained in the remaining sections.

F4.1: Histone deacetylase inhibitors

Chromatin is formed of DNA packaged in nucleosome structures, constituted by 146 base-pair DNA sequence winding around an octamere of histones (two copies of each histone: H2A, H2B, H3, and H4) held in place by histone H1. The condensed form of chromatin (heterochromatin) is inactive in terms of transcription whereas the decondensed form (euchromatin) corresponds to an active form. The transition between euchromatin and heterochromatin is dependent upon two families of proteins: histone acetyl transferases (HATs), and histone deacetylases (HDACs). It has been established that histone acetylation leads to relaxation of the nucleosome structure, releasing the DNA and allowing transcription. Inhibition of HDAC promotes decondensed chromatin formation, thereby promoting the expression of genes. HDACs have become natural targets for cancer therapies because they have been shown to regulate the expression and activity of numerous proteins that are involved in both cancer initiation and progression¹⁷⁰. In addition, the balance between histone transacetylases and deacetylases is often damaged in cancer, leading to altered expressions of tumor suppressor genes

and/or proto-oncogenes^{171,172}. Several HDACis have been shown to arrest the growth and/or induce apoptosis of cancer cells^{170,173} and are being investigated in clinical trials in mono- and combination therapy. Moreover, HDACis have been shown to upregulate the transcriptional activity of virally delivered transgenes both *in vitro* and *in vivo*^{174,175} and to prevent the transcriptional activation of IFN-stimulated genes in response to viral infection^{176,177}, making them attractive as potential pharmacologic agents to augment oncolytic virotherapy.

F3.1.1: Valproic acid

Valproic acid (VPA) is an HDACi, the primary indication of which is for the treatment of epilepsy and mood disorders^{178,179}. In the human brain, VPA alters the activity of the neurotransmitter Gamma Aminobutyric acid (GABA) by potentiating the inhibitory activity of GABA through several mechanisms, including inhibition of GABA degradation, inhibition of GABA Transaminobutyrate, increased GABA synthesis, and decreased turnover¹⁸⁰. Moreover, VPA attenuates N-Methyl-D-Aspartate-mediated excitation¹⁸¹ and blocks Na⁺ channels, Ca²⁺ channels, and voltage-gated K⁺ channels¹⁸². While VPA was reported to have neuroprotective activity in some models of CNS injury and neurodegenerative disorders¹⁸³⁻¹⁸⁵, a growing body of evidence indicates that it causes HDAC-related and -unrelated neuronal cell death^{186,187}. Indeed, VPA was shown to exacerbate the death of cerebellar granule neurons¹⁸⁸, reduce the proliferation of hippocampal neurons resulting in cognitive impairments¹⁸⁹, and induce apoptosis in various neuronal cell populations^{186,190}. In humans, VPA has established teratogenic activity. It causes neural tube defects and increases the incidence of children with autism spectrum disorder (ASD), when taken during pregnancy^{191,192}. Developmental

neurotoxicity was attributed to the generation of free radicals, oxidative stress¹⁹³ and caspase-dependent apoptosis^{194,195}, and the morphological changes seen in the brains of autistic children confirmed that VPA induces PCD^{194,196}. Rats and mice exposed to VPA *in utero* or shortly after birth present with behavioral and structural abnormalities similar to those observed in humans with ASD, making them a useful disease model^{197,198}. VPA-induced neurodegeneration was seen in both cultured neuronal cells and experimental animals^{188,189,195,198,199}. VPA reduced the proliferation of hippocampal neurons and caused cognitive impairment in intraperitoneally injected rats¹⁸⁹ and neonatal mice and rats given clinically relevant doses of anticonvulsant VPA therapy exhibited widespread apoptotic neurodegeneration in several brain regions^{198,200}. The pathways involved in the VPA-induced neurotoxicity are still poorly understood²⁰¹ and while caspase-independent PCD was reported²⁰², its exact mechanism is still unknown.

In addition to its controversial roles in neuroprotection/neurodegeneration, VPA has more recently seen increased interest in cancer therapy due to its HDACi activity. VPA has demonstrated potent antitumor effects in a wide variety of cancers in preclinical studies, *in vitro* and *in vivo*, through modulation of multiple pathways including cell cycle arrest, angiogenesis, apoptosis, differentiation, and senescence. These effects appear to be cell type-specific and could be dependent upon the degree of cell differentiation and, in the case of cancer, dependent upon the degree of genetic alteration²⁰³. VPA is also a particularly promising candidate for augmenting the efficacy of OV. It has been shown to increase viral propagation and reduce the host IFN response to an oHSV in a model of glioma¹⁷⁷. When used in combination with equine herpes virus

type 1(EHV-1), VPA treatment resulted in a significant increase in virus entry, replication, cell to cell spread and cell lysis in human glioma cells²⁰⁴.

Chapter 2: Experimental Overview

Carcinogenesis occurs through the accumulation of multiple aberrant genetic mutations leading to uncontrolled cellular survival and proliferation. These initiating mutations lead to genome wide dysregulation of gene expression and intracellular signaling, resulting in a heterogeneous mass of interrelated but diverse cells. Tumor cell heterogeneity promotes the natural selection of tumor progeny with greater proliferative capacity and invasive potential^{129,205} and this heterogeneity gives tumors inherent evolutionary advantages when challenged by anticancer therapies. As a result, treatment methods that address a singular therapeutic strategy may be insufficient to completely eliminate tumor growth.

Oncolytic virotherapy is an innovative treatment modality which uses virus to selectively replicate in, and damage cancerous tissue while leaving normal tissue intact²⁰⁶. The anticancer activities of OV's are derived from multimodal cancer killing mechanisms. In addition to direct lysis of tumor cells, OV's also induce anti-angiogenesis resulting in the death of surrounding uninfected cells^{133,207}, and activate innate and tumor-specific immune cells to elicit an immune response against remaining tumor cells²⁰⁸. For the best therapeutic outcome, OV's should induce tumor cell death through both viral replication and the induction of PCD, eliminate CSC, and elicit a potent anti-tumor immune response. Recent efforts have focused on engineering OV to improve efficacy, however, as a stand-alone therapeutic they have not been shown to induce complete, long-term regression of established tumors *in vivo*^{206,209}, therefore chemotherapeutic agents are increasingly sought for combination therapy.

This thesis work was based upon the central hypothesis that in addition to PCD pathways already established by our lab, Δ PK oncolytic activity also involves the elimination of CSC and immune modulation that favors the development of anti-tumor immunity, and oncolytic activity could be enhanced by combination therapy with the HDACi, valproic acid.

In part A of my experimental results, I demonstrate that indeed Δ PK infection prevents anchorage-independent growth, a defining characteristic of CSC, in melanoma cultures, and growth is restored by treatment with autophagy and calpain inhibitors. Δ PK also effectively lyses 3D melanoma spheroid cultures and this activity involves calpain-dependent clearance of the autophagy associated protein p62/SQSTM1, but not caspase activation, revealing a significant difference between adherent cultures and their stem-like counterparts in terms of the mechanisms that mediate their lysis.

In part B, I show that Δ PK oncolytic activity involves the induction of multiple features of immunogenic cell death in melanoma cultures and xenografts, including (i) JNK/c-Jun dependent inhibition of the immunosuppressive cytokine IL-10 and the resulting upregulation of MICA, the ligand for the activating receptor NKG2D expressed on NK and cytotoxic T cells, (ii) upregulation of pro-inflammatory cytokines TNF- α , GM-CSF, and IL-1 β through activation of the TLR2/NF κ B pathway and pyroptosis, and (iii) inhibition of the negative immune checkpoint regulator CTLA-4.

In part C, I examine the mechanism of action of VPA in neuronal cells and demonstrate that VPA activates a previously unrecognized calpain-dependent necroptosis cascade that initiates with the activation of JNK1/RIP-1 signaling and is followed by AIF cleavage/nuclear translocation and H2AX phosphorylation as well as an altered

Smac/DIABLO to XIAP balance. This mode of cell death is unique from the caspase-dependent form of cell death induced by Δ PK in melanoma (which are of neuronal lineage), supporting the hypothesis that VPA could complement and enhance Δ PK oncolytic activity.

In part D, I demonstrate that rather than inducing calpain-dependent necroptosis in melanoma, VPA –induced cell death in melanoma is caspase-dependent. Nonetheless, combining VPA with Δ PK treatment enhances Δ PK-induced cell death associated with cell-type specific enhanced caspase activation.

Collectively, the data in this thesis underscore the therapeutic potential of Δ PK stemming from its multi-mechanistic induction of PCD pathways that include multiple features of ICD and the lysis of tumor-forming CSC, and highlight the promise of combinatorial therapy to further enhance Δ PK efficacy.

Chapter 3: Materials and Methods

A. Cell culture

PC12 cells were grown in DMEM/F12 medium (Media Tech, Herndon, VA, USA) with 10% FBS, 0.36% D-glucose (Sigma-Aldrich, St. Louis, MO, USA), and 0.009% gentamicin (Invitrogen, Waltham, MA USA). The establishment and properties of stably transfected PC12 cells (PC47 and PC70) that have constitutively activated survival pathways were previously described²¹⁰. Primary rat cortical neurons were purchased from Life Technologies (Grand Island, NY). They were cultured on poly-D-lysine coated polystyrene plates and grown in NeuroBasal medium with 0.5mM GlutaMAX supplement and 2% B-27 supplement (Life Technologies), as per manufacturer's instructions. SK-N-SH human neuroblastoma cells (American Type Culture Collection, Manassas, VA) were grown in MEM with 2 mM L-glutamine and Earle's BSS containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, and 10% FBS.

Melanoma cell lines A2058 and A375 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium with 10% FBS (Gemini Bioproducts, Calabasos, CA, USA). The medium was supplemented with 4.5 g/L glucose, 1500 mg/mL sodium bicarbonate and 4 mM glutamine. LM cells were established from a histologically confirmed metastatic melanoma and passaged only 6-8 times prior to study. Growth was in RPMI 1640 medium with 10% FBS, as previously described¹⁴⁸.

B. Virus

The generation and properties of the HSV-2 mutant Δ PK were previously described¹⁴³. Δ PK is deleted in the sequences that encode the kinase function of ICP10 (also known as HSV-2 R1). The ICP10 kinase activity functions independently of the R1 activity and is required for virus growth. Δ PK expresses the kinase negative (ICP10PK deleted) protein p95 under the direction of the authentic immediate early (IE) ICP10 promoter. Δ PK was grown in Vero cells. Cell lysates were cleared of cell debris by centrifugation at 3000 g for 30 min at 4°C.

C. Neuronal differentiation

For neuronal differentiation, PC12 and PC47 cells were cultured on rat tail collagen-coated cover glass or polystyrene flasks and grown in NeuroBasal medium with 2 mmol/L L-glutamine, B-27 supplement, 0.009% gentamicin (Invitrogen) and 100 ng/mL nerve growth factor (NGF,2.5S; Millipore, Billerica, MA, USA). Fresh NGF was supplied every other day. Differentiation was confirmed by the formation of neurites and staining with antibody to the neuronal differentiation marker MAP-2²¹¹, as previously described²¹⁰. We confirmed neuronal differentiation and its failure to alter transgene expression and similar results were obtained when differentiation was for 4, 7 or 12 days.

D. Antibodies and reagents

Antibodies to XIAP, RIP-1, caspases- 1, -9, -8, phosphorylated and total JNK, phosphorylated c-Jun, phosphorylated and total Akt, TLR2, LC3, and phosphorylated H2AX were purchased from Cell Signaling Technology (Danvers, MA, USA).

Antibodies to actin, GAPDH, AIF, Smac/DIABLO, MAP-2, ASC, NF κ B, MyD88,

MICA, p62, CTLA-4, caspases -3, -7, -4, TNF α , and calpain were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to phosphorylated (activated) ERK 1/2 (pERK1/2) was purchased from Promega (Madison, WI). The calpain inhibitor PD150606 and caspase 1 inhibitor YVAD-fmk were purchased from Calbiochem (La Jolla, CA, USA), the JNK inhibitor SP600125, the autophagy inhibitor 3-methyl adenine (3-MA), the pancaspase inhibitor z-VAD-fmk and the necroptosis inhibitor necrostatin-1 (Nec-1) from Sigma-Aldrich (St. Louis, MO, USA). The Ambion Silencer Select RIP-1 siRNA was purchased from Life Technologies and was transfected into neuronally differentiated PC12 cells using Lipofectamine RNAiMAX Reagent (Life Technologies) as per manufacturer's instructions. Alexafluor 488-conjugated anti-goat secondary antibody was purchased from Invitrogen (Carlsbad, CA, USA) and horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and the mammalian derived recombinant human IL-10 (hIL-10) were purchased from Cell Signaling Technologies.

E. Cell death

Cell death was determined by trypan blue, ethidium homodimer, propidium iodide, and/or TUNEL staining. For trypan blue staining, an aliquot of the cell suspension was incubated with an equal volume of 0.4% trypan blue and the percentage of dead cells (identified by blue staining) was calculated relative to the total cell numbers in 4 independent fields using a hemacytometer. For ethidium homodimer staining, cells were incubated with ethidium homodimer (Life Technologies) for 30 min at room temperature according to the manufacturer's instructions, mounted in Vectashield with DAPI (Vector, Burlingame, CA, USA) and visualized with an Olympus BX50 fluorescent microscope (Center Valley, PA, USA). Stained cells were counted in four randomly selected fields,

for a total of at least 250 cells. For propidium iodide staining, propidium iodide solution (1mg/mL) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells grown on glass coverslips were incubated with propidium iodide solution (50µg/mL) for 30 min at room temperature in the dark. Slides were rinsed with phosphate buffered saline (PBS), mounted in Vectashield with DAPI (Vector Burlingame,CA) and visualized with an Olympus (BX50 fluorescent microscope Center Valley, PA, USA). Stained cells were counted in four randomly selected fields, for a total of at least 250 cells.

For TUNEL, the In situ Cell Death Detection kit, Fluorescein (Roche San Francisco,CA) was used according to the manufacturers' instructions. Briefly, cells grown on glass slides were fixed in 4% paraformaldehyde in PBS, pH 7.4 (1 h at 24°C) followed by permeabilization in 0.1% Triton-X (in 0.1% sodium citrate) for 2 min on ice. DNA breaks were labeled by incubation (60 min at 37°C) with terminal deoxynucleotidyl transferase and nucleotide mixture containing fluorescein isothiocyanate (FITC)-conjugated dUTP [terminal deoxynucleotidyl transferase FITC-dUTP nick end labeling (TUNEL reagent), followed by anti-FITC antibody conjugated to alkaline phosphatase (30 min at 37°C). Chromogenic reaction was carried out by adding alkaline phosphatase substrate solution containing 0.4 mg of nitroblue tetrazolium chloride per mL and 0.2 mg of 5-bromo-4-chloro-3-indolylphosphate toluidine salt (NBT-BCIP, Roche) per mL in 0.1 mol/L Tris-HCl (pH 9.5)- 0.05 mol/L MgCl₂-0.1 mol/L NaCl-1 mmol/L levamisole (10 min at 24°C). Slides were mounted in Vectashield with DAPI (Vector) and visualized with an Olympus (Center Valley, PA, USA) BX50 fluorescent microscope. Stained cells were counted in four randomly selected fields, for a total of at least 250 cells.

F. Cytoplasmic and nuclear separation and Immunoblotting

Cytoplasmic and nuclear fractions were separated as previously described²¹⁰. Briefly, cell pellets were suspended in 50 mM Tris-HCl buffer (pH 8.0) with 50 mM NaCl, 1% NP-40, 1 mM dithiothreitol and protease inhibitors on ice. The cells were centrifuged (7000 g; 1 min) and the resulting supernatant (cytoplasmic fraction) was removed. The nuclear pellet was resuspended in the same buffer but with 450 mM NaCl and sonicated until in solution. For whole cell extraction, cell pellets were lysed with radioimmunoprecipitation buffer [20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Nonidet P-40 (Sigma), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate] supplemented with protease and phosphatase inhibitor cocktails (Sigma). Protein concentrations were determined using the bicinchoninic assay (Pierce, Rockford, IL, USA) and 50µg of extracts were resolved using SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 1 hour in TNT buffer [0.01 M Tris-HCl (pH 7.4), 0.15M NaCl, 0.05% Tween 20] containing 5% milk or bovine serum albumin (Sigma-Aldrich). They were incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Quantitation was performed by densitometric scanning with the Bio-Rad GS-700 imaging densitometer (Bio-Rad, Hercules, CA, USA). The results of three independent experiments are expressed as the mean actin or GAPDH-adjusted densitometric units ± SE.

G. Immunofluorescent staining and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde (30 min; room temperature (RT)) and permeabilized (2 min; 4°C) with 0.1% Triton X-100 in 0.1% sodium citrate buffer. They

were incubated with primary antibody diluted in 5% bovine serum albumin and 5% normal goat serum in PBS overnight at 4°C, washed in phosphate-buffered saline (PBS) with 0.1% Tween 20 and exposed to fluorochrome-labeled secondary antibodies (1 h; 37°C). Slides were mounted in Vectashield with DAPI (Vector) and visualized with an Olympus BX50 fluorescent microscope (Center Valley, PA, USA). Staining cells were counted in four randomly selected fields, for a total of at least 250 cells. When used, Mitotracker Red 580 was added to cells (30 min, 37°C) as per manufacturer's instructions prior to fixation with paraformaldehyde. For immunohistochemical staining, tumor sections were post-fixed (30 min) in 4% paraformaldehyde in PBS (w/v), treated (10 min) with 0.3% H₂O₂ to remove endogenous peroxidases, permeabilized and blocked in blocking solution (10% goat serum, 1% BSA and 0.3% Triton-X 100 in PBS) for 1 h. Sections (20 µm-thick) were exposed overnight (4 °C) to the primary antibody diluted in blocking solution followed by horseradish peroxidase-conjugated secondary antibody diluted in 5% goat serum and 5% BSA (1 h). The reaction was developed with ImmPACT DAB substrate (Vector Laboratories) and sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich) followed by dehydration and mounting in Permount (Sigma-Aldrich). Sections were visualized under brightfield conditions with an Olympus BX50 microscope. The stained cells were counted in representative 50 µm² fields and the percentage of positive cells was calculated relative to the total cells per field.

H. Microarray analysis

Total RNA was isolated and purified from A2058 and LM cultures, mock-infected with PBS or infected with ΔPK (moi= 0.5 pfu/cell; 24hrs) using the RNeasy kit (Qiagen,

Valencia, CA). Biotin-labeled cRNA target was prepared with the TrueLabeling-AMP 2.0 kit (SABiosciences, Frederick, MD) then hybridized onto inflammatory and cell death targeted oligoarrays (Oligo GeArrays, SABiosciences) according to the manufacturer's instructions. Microarray data analysis was done using GEArray Expression Analysis Suite 2.0 software (SABiosciences).

I. Soft Agar Growth Assay

Soft agar colonies were grown as previously described²¹². Briefly, top layers of 500 cells (Mock) or 5000 cells per well (24-well plate) were suspended in 0.3% low melting temperature agarose in 1xDMEM and were overlaid onto 0.6% agarose in 1x DMEM. The solidified agarose cell mixture was overlaid with 1x DMEM. Plates were incubated at 37°C for 14 days, and colonies (defined as a minimum of 50µm in diameter) were counted. The number of colonies per 10⁴ cells was calculated according to the appropriate dilution used.

J. Spheroid Formation Assay

Triplicate dilutions of 1x10⁴ cells per well were suspended in serum free DMEM supplemented with 20ng/ml basic fibroblast growth factor (bFGF, R&D Systems) and 20ng/ml epidermal growth factor (EGF, R&D Systems) and plated onto ultra-low attachment plates obtained from Corning (Corning, NY). Spheroid cultures were grown at 37°C for 7 days. Spheres were defined as at least 500µm in diameter. The assay was performed in triplicate for both mock- and ΔPK-infected (moi=1.0, 48 h p.i.) cells.

K. ELISA

Conditioned media from mock or Δ PK infected melanoma cultures in the absence or presence of specific inhibitors were assayed for TNF- α , GM-CSF, IL-1 β , and IL-10, using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

L. *In vivo* studies

The Animal Care and Use Committee of the University of Maryland School Of Medicine approved all the described studies. Male nude mice (6 to 8-week-old) (Balb/c nu/nu) were obtained from Charles River Laboratories (Wilmington, MA, USA). Melanoma xenografts were established by subcutaneous injection of A375 cells (10^7 in 100 μ l) into both the left and right hind flanks. When the tumors became palpable (≈ 200 mm³ in volume; day 7), animals were randomly assigned to treatment groups. Treatments consisted of intratumoral injections of partially purified Δ PK (10^6 or 10^7 pfu) in a total volume of 100 μ l of cell culture medium or 100 μ l of virus-free culture medium (control). The treatment protocol consisted of four injections given at weekly intervals (one injection per week). Every other day, minimum and maximum perpendicular tumor axes were measured and tumor volume was calculated as previously described¹⁴⁸. Animals were maintained in pathogen-free conditions and were euthanized when their tumors reached 1.5 cm in any one direction. Tumors were collected after euthanasia and processed for immunoblotting and immunohistochemistry.

M. Statistical analysis

Two-tailed student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA were used as appropriate, p value less than 0.05 was considered significant. All analyses were carried out using SigmaPlot 11.0 for Windows. ANOVA was followed by pairwise comparison with the Holm-Sidak test.

Chapter 4: Experimental Results

A. Calpain-dependent clearance of the autophagy protein p62/SQSTM1 is a contributor to Δ PK oncolytic activity in melanoma cancer stem cells

A1: Introduction

Oncolytic virotherapy is a promising therapeutic strategy for the reduction of tumor burden through selective viral replication and cell lysis in rapidly replicating tumor cells. Clinical efficacy is modest, however, attributed in part to the survival of slowly replicating cancer stem cells (CSC) that retain many of the properties of normal stem cells, including the ability to differentiate into multiple cell types, proliferate and maintain neoplastic clonality²¹³. CSC are characterized by their ability to grow in anchorage-independent conditions and initiate tumor formation, and are consequently believed to be responsible for tumor recurrence²¹⁴, making their elimination highly desirable. Elimination of CSC remains a clinical challenge due to their resistance to traditional drug and radiation therapy attributed to their increased expression of drug export machinery and enhanced DNA repair pathways. Despite their significant impact on therapeutic efficacy, direct CSC-lysis studies are relatively scant and the ability of oncolytic viruses to lyse these slowly replicating cells is unclear.

We have previously demonstrated that the HSV-2 mutant Δ PK has strong oncolytic potential in melanoma cell lines and xenografts. Δ PK is deleted in the protein kinase (PK) domain of the HSV-2 large subunit of ribonucleotide reductase (R1) (known as ICP10), which activates the Ras signaling pathway that is required for virus growth in normal cells, but it retains the R1 domain of ICP10 that is required for viral DNA replication¹⁴³. Deletion of ICP10PK imparts tumor selective replication and favors virus induction of

multiple programmed cell death (PCD) pathways¹⁴⁸. We have previously shown that Δ PK has strong *in vivo* activity, demonstrated by the long term elimination of melanoma xenografts in several animals that remained tumor-free for at least a year. Although this implies that CSCs are not resistant to Δ PK, their lysis has not been documented and the role of virus-induced PCD pathways, if any, is still poorly understood. We report that Δ PK eradicates melanoma cancer cells with CSC growth characteristics through an activation of autophagic cell death and calpain-dependent clearance of the autophagy associated protein p62/SQSTM1, underscoring its strong therapeutic potential.

A2. Results

A2.1: Δ PK abrogates anchorage-independent growth.

To examine the ability of Δ PK to lyse cells with CSC-like properties, we asked whether it can abrogate anchorage-independent growth potential, an established *in vitro* model of tumorigenicity⁴¹. To answer this question, 2D adherent melanoma cultures were infected with Δ PK and subsequently plated in anchorage independent conditions. A2058 and A375 cells were infected with Δ PK (moi=1) or mock infected with PBS for 48 hours then plated in either spheroid growth conditions or in soft agar. Spheroid and colony formation was assessed 7 and 14 days after plating, respectively. The results, as shown for A2058 cultures assayed in spheroid growth conditions, are expressed as spheroids /10⁴ cells \pm s.d. and indicate that Δ PK -infected cells do not grow under these conditions (Figure 6A). Similar results were obtained for A375 and in soft agar, confirming that Δ PK abrogates 3D, CSC-like growth potential in melanoma cultures.

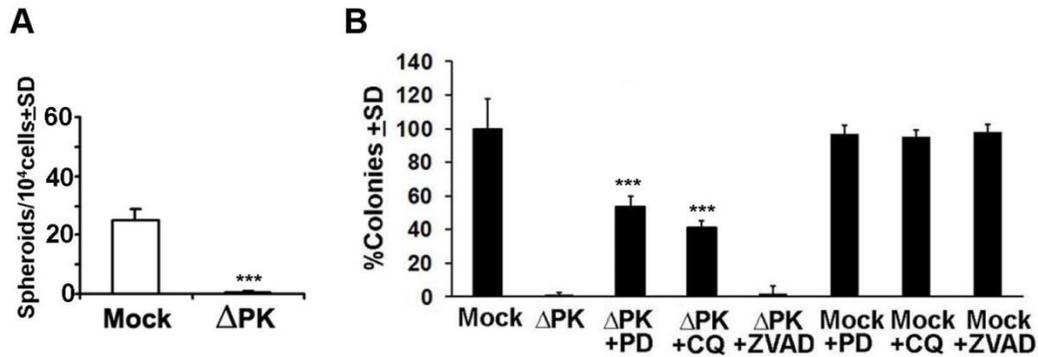


Figure 6: Elimination of anchorage independent growth by Δ PK is calpain and autophagy dependent

(A) A2058 cells mock-infected or infected with Δ PK (moi =1; 48 h) were assayed for growth in spheroid culture and the results are expressed as number of spheroids/ 1×10^4 cells \pm s.d. Similar results were obtained for A375 cells and for colony forming potential in soft agar. (B) A2058 2D cultures were mock infected or infected with Δ PK (moi=1; 48 h) in the absence or presence of PD150606 (100 μ M), chloroquine (CQ, 10 μ M) or zVAD-fmk (20 μ M) and plated under soft agar as described in Materials and Methods. Colonies were counted and the results are expressed as % colonies \pm s.d. calculated relative to the mock-infected cultures (100%). Similar results were obtained for growth in spheroid cultures. (***)P<0.001, n=3)

A2.2: Autophagy and calpain activation contribute to Δ PK -mediated eradication of cells with anchorage independent growth potential.

Having previously shown that calpain and caspase-mediated PCD pathways are involved in the Δ PK mediated killing of 2D melanoma cultures¹⁴⁸, and that Δ PK induces the expression of the autophagy protein beclin-1 in melanoma xenografts, we wanted to better understand the contribution of these death pathways to the Δ PK-induced elimination of anchorage independent growth. A2058 and A375 cells were mock infected with PBS or infected with Δ PK (moi=1; 48 h) in the absence or presence of either the calpain-specific inhibitor PD150606 (100 μ M), the pancaspase inhibitor z-VAD-fmk (20 μ M), or the autophagy inhibitor chloroquine (10 μ M), and assayed for

growth in soft agar or spheroid culture. Results are expressed as % colonies \pm s.d. calculated relative to the mock infected cells (100%) and are shown in Figure 6B for A2058 colony formation. Δ PK -infected cells failed to grow in 3D culture. The calpain-specific inhibitor PD150606 and autophagy inhibitor chloroquine restored 3D growth, but growth was not restored by treatment with the pancaspase inhibitor z-VAD-fmk, and the inhibitors alone did not affect the 3D growth of the mock-infected cultures. Similar results were obtained for spheroid growth and A375 cultures. The data indicate that calpain and autophagy, but not caspase activation, contribute to the ability of Δ PK to inhibit 3D growth in melanoma cultures.

A2.3: Δ PK infection induces LC3-II accumulation and p62 clearance in spheroids

To further investigate the mechanism of Δ PK to lyse cells with CSC-properties, we used A2058 and A375 cells grown as 3D multicellular tumor spheroids. These cultures include a gradient of proliferating cells similar to those found in tumor avascular microregions, and are often used as *in vitro* surrogates of tumorigenesis²¹⁵. The 3D spheroids were infected with Δ PK (moi= 1) or mock-infected with PBS and examined for cell death by regular microscopy and staining with propidium iodide (PI) followed by flow cytometry (FCM). The Δ PK, but not mock-infected, spheroids were largely reduced to debris and the majority of cells stained with PI, confirming the ability of Δ PK to lyse CSC-enriched cultures [experiment performed by Aric Colunga²¹⁶]. To examine the relationship between Δ PK-induced CSC lysis and autophagy, we asked whether infection induces autophagy markers. We focused on the membrane-bound phosphatidylethanolamine-conjugated form of microtubule associated protein 1 light chain 3 (LC3II), which binds autophagosome membranes, and p62/SQSTM1, a stress-

inducible protein that functions as an assembly factor for ubiquitinated proteins and organelles, both of which are widely used autophagy markers^{124,217}. In the first series of experiments, protein extracts from A2058 and A375 spheroids mock-infected with PBS or infected with Δ PK (moi=1) were immunoblotted with LC3 antibody and examined for the conversion of LC3I to LC3II. The stripped blots were probed with antibody to actin (loading control) and the results were quantified by densitometric scanning. Δ PK caused a significant increase in the LC3- II/LC3-I ratio relative to that seen in the mock-infected cultures in both melanoma cultures. LC3-II increase was seen as early as 1 h pi and it was still present at 24 h p.i. (Figure 7A).

In the second series of experiments, A2058 and A375 spheroids were mock-infected or infected with Δ PK in the absence or presence of CQ (10 μ M) and immunoblotted with antibody to p62/SQSTM1. The blots were stripped and reblotted with antibody to GAPDH and the levels of p62/SQSTM1 determined by densitometric scanning and analyzed relative to GAPDH. As shown in Figure 7B for A2058 cells, p62/SQSTM1 was expressed in the mock, but not Δ PK -infected, cultures, indicating that Δ PK induces p62/SQSTM1 clearance. Significantly, however, expression was not restored completely by treatment of the infected cells with the autophagy inhibitor CQ (Figures 7B-C), suggesting that the Δ PK -mediated clearance of p62/SQSTM1 is through a mechanism other than autophagy.

A2.4: Δ PK -mediated p62/SQSTM1 clearance is calpain-dependent

To better understand the mechanism responsible for the clearance of p62/SQSTM1, duplicate spheroid cultures were infected with Δ PK (moi=1; 24 h) in the absence or

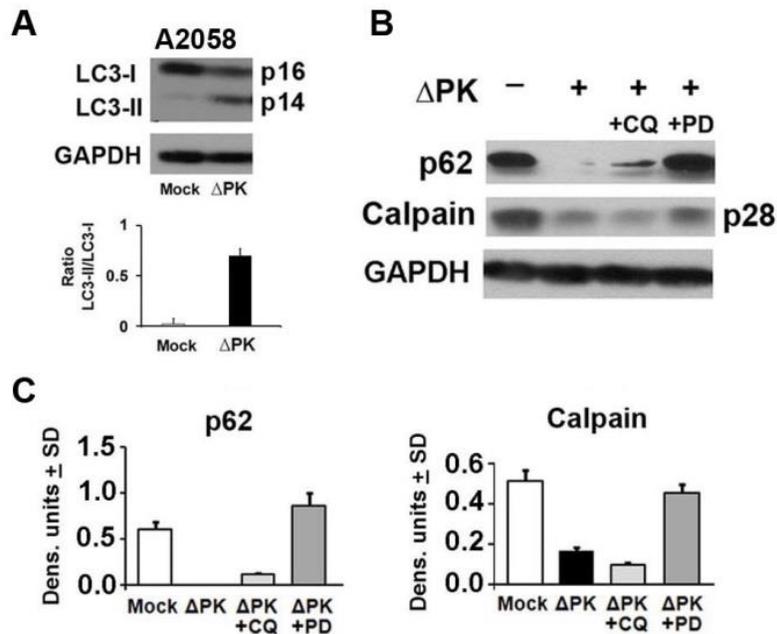


Figure 7: ΔPK induces LC3-II accumulation and calpain-dependent clearance of p62/SQSTM1

(A) A2058 and A375 spheroid cultures were dissociated into single cell suspensions and mock- or ΔPK -infected (moi=1) for 1, 4 or 24 h and protein extracts were immunoblotted with antibodies to LC3 followed by actin (loading control). Data were quantified by densitometric scanning and the results obtained for three replicate experiments are expressed as LC3-II/LC3-I ratio. (B) A2058 spheroid cultures dissociated into single cell suspensions were mock- or ΔPK -infected (moi=1; 24 h) in the absence or presence of PD150606 (100 μM) or CQ (10 μM) and protein extracts were immunoblotted with antibodies to p62/SQSTM1, the calpain p28 regulatory subunit, or GAPDH (loading control). The blots were stripped between antibodies and representatives of three replicate experiments are shown. Similar results were obtained in A375 spheroids. (C) Data were quantified by densitometric scanning and the results obtained for three replicate experiments are expressed as densitometric units±s.d. for p62/SQSTM1 and p28 (calpain).

presence of PD150606 (100 μM), and protein extracts were immunoblotted with antibody to p62/SQSTM1 followed by immunoblotting with antibody to the calpain regulatory subunit p28, the loss of which documents calpain activation⁹⁵. p62/SQSTM1 expression

was fully restored in the PD150606-treated Δ PK -infected spheroid cultures, indicating that its clearance is calpain dependent (Figures 7B-C). Indeed, Δ PK induced calpain activation as evidenced by (i) loss of the p28 regulatory subunit in Δ PK -infected cells and (ii) restored expression in infected cells treated with the calpain inhibitor PD150606 (Figure 7B-C). Collectively, the data indicate that calpain-mediated p62/SQSTM1 degradation is a key contributor to the Δ PK oncolytic activity in CSC-enriched 3D melanoma cultures.

A3: Discussion

Human malignant melanoma is a highly aggressive and drug-resistant cancer. Although conventional anti-cancer treatments might eradicate most malignant cells in a tumor, they are potentially ineffective against chemoresistant CSC, which are capable of self-renewal and may ultimately be responsible for tumor recurrence and progression. Elimination of CSC therefore remains a major, yet unfulfilled goal of cancer therapeutics. We report that Δ PK has strong oncolytic activity in CSC-enriched melanoma 3D cultures that involves autophagic cell death and the calpain-dependent clearance of autophagy associated protein p62/SQSTM1. The following comments seem pertinent to these findings.

Oncolytic virotherapy is a promising novel therapeutic strategy based upon tumor cell specific virus replication and cell lysis. Virtually all the virus families have been used to develop oncolytic viruses, each with its own advantages and disadvantages²⁰⁹. Clinical efficacy, however, remains modest, attributed in part to quiescent CSC subpopulations that are resistant to virus infection/replication. The majority of oHSV are based on HSV-1 and deleted in UL39 and/or ICP34.5 genes. UL39 encodes the large subunit of

ribonucleotide reductase (R1, also known as ICP6 in HSV-1 and ICP10 in HSV-2) that is required for viral DNA replication in normal and quiescent cells. Viruses that lack R1 are able to replicate in rapidly dividing tumor cells that express high levels of cellular ribonucleotide reductase, but mutant viruses are severely inhibited in their ability to replicate in CSC, which are quiescent cells. Indeed, an oHSV with an intact ICP34.5 was shown to lyse glioblastoma CSC more efficiently than an oncolytic virus in which ICP34.5 was entirely deleted¹⁴⁴. While direct CSC lysis studies are relatively scant, oHSV with genetic modifications to increase tumor penetration and virus spread have been shown to kill glioblastoma, neuroblastoma and rhabdomyosarcoma cells with CSC-like properties²¹⁸⁻²²¹. Δ PK differs from most oHSV in that it is based on HSV-2 and is deleted in ICP10PK, which is not conserved in HSV-1, but it retains the replication-associated ICP34.5 and ribonucleotide reductase activities¹⁴³. ICP10PK functions at the level of Ras activation and its deletion enables tumor selective virus growth and the activation of multiple PCD pathways^{148,222}. Δ PK was well tolerated in phase I/II human clinical trials¹⁵⁰ and has strong *in vivo* oncolytic activity in melanoma, with xenograft-bearing animals remaining tumor-free for at least 1 year after treatment¹⁴⁸. Although this implies that CSCs are not resistant to Δ PK, their lysis remains to be documented.

We found that Δ PK inhibited the 3D growth potential of melanoma cultures when measured by both spheroid and soft agar colony formation and 3D growth was restored by treatment with the calpain specific inhibitor, PD150606. Δ PK also lysed 3D spheroid cultures and lysis was associated with the loss of the p28 calpain regulatory subunit, restored by PD150606, indicating that calpain activation contributes to the oncolytic activity of Δ PK in 3D melanoma cultures. Interestingly, although both calpain and

caspase activation have been implicated in Δ PK oncolytic activity in adherent melanoma cultures, caspase inhibition with z-VAD-fmk was unable to restore 3D growth. While we cannot exclude the potential contribution of caspases that are not inhibited by zVAD-fmk^{223,224}, the levels of the anti-apoptotic Bcl-2 and survivin proteins are increased in anchorage-independent as compared to adherent cultures, making them resistant to caspase-mediated PCD²²⁵.

Although autophagy is primarily considered a protective process by which cells can remove and recycle protein aggregates and damaged organelles under starvation conditions, prolonged exposure to stress can induce autophagic cell death¹¹⁸. This dual role is most apparent in cancer cells where autophagy was shown contribute to²²⁶ and inhibit tumorigenesis²²⁷. We found that treatment with the autophagy inhibitor CQ restored the 3D growth potential of Δ PK infected melanoma and infected melanoma spheroids had increased levels of LC3-II accompanied by p62/SQSTM1 clearance, established signatures of autophagy²¹⁷. However, the expression of p62/SQSTM1 was not restored by CQ but rather by the calpain inhibitor PD150606. A possible interpretation of our findings is that the observed LC3-II accumulation reflects a residual effort by the cells to survive Δ PK-induced cell death, although implicit in this interpretation is the conclusion that the ability of CQ to restore 3D growth is through a pro-survival function other than autophagy.

Although p62/SQSTM1 is a substrate adaptor that plays critical roles in autophagy, studies have indicated that it is a multi-domain protein that interacts with other molecules and has a profound impact on signal regulation within the NF κ B, mTOR and Wnt signaling pathways^{228,229}. p62/SQSTM1 has been implicated in Ras-induced

tumorigenesis and its loss inhibited Ras-controlled transformation, apparently through ROS induced cell death²³⁰ and has been shown to enhance the motility of squamous cell carcinoma cells²³¹. High levels of p62/SQSTM1 were associated with worse prognosis and tumor progression in breast and non-small-cell lung cancer²³²⁻²³⁴, making its clearance by Δ PK potentially significant from a therapeutic standpoint. Although further studies are needed in order to test this interpretation for Δ PK and to better elucidate the contribution of autophagy, if any, to its oncolytic activity, our data highlight the versatility of Δ PK oncolytic activity.

B. Melanoma cell lysis by the oncolytic Herpes simplex virus type 2 mutant Δ PK includes multiple features of immunogenic cell death

B1. Introduction

Oncolytic viruses (OVs) were originally developed to lyse tumor cells through selective replication, but depending on the virus platform, their anti-tumor activity also includes induction of distinct programmed cell death (PCD) pathways¹³¹. Recent interest has focused on the ability of the OVs to activate/redirect cytotoxic immune responses through induction of immunogenic cell death (ICD), a process triggered by the induction of pro-inflammatory cytokines¹²⁶ and the release of danger-associated molecular pattern molecules (DAMPs), pathogen-associated molecular pattern molecules (PAMPs), and tumor-associated antigens (TAAs), which stimulate immune effector cells^{131,235-237}. However, clinical efficacy remains modest, likely related to the immunosuppressive tumor milieu, and much remains to be learned about the mechanisms by which OVs induce tumor cell death, the contribution of the specific virus platform, and how these can be exploited to enhance immunogenicity.

OVs developed from Herpes simplex virus (oHSVs) are primarily based on HSV type 1 (HSV-1) and are typically deleted in the neurovirulence gene ICP34.5 and/or the large subunit of the ribonucleotide reductase (R1). Some are also deleted in ICP47, which functions in virus immune evasion, but its role in OV-based immunotherapy is as of yet unclear²⁰⁸. T-Vec, an oHSV armed with granulocyte macrophage colony-stimulating factor (GM-CSF) has recently passed Phase III clinical trials in stage III and IV melanoma¹⁶¹. HSV-2 differs from HSV-1 in that many of its replicative aspects depend on the protein kinase function of R1 (also known as ICP10PK), which is poorly conserved and non-functional in HSV-1. ICP10PK activates Ras signaling pathways and

is required for virus growth in slowly replicating normal cells, which differ from tumor cells in that they have low levels of Ras activity^{142,143}. We have shown that an HSV-2 mutant deleted in ICP10PK (Δ PK) has strong oncolytic activity in melanoma xenografts, causing a profound and long-lasting reduction in tumor burden. This activity is associated with calpain and caspases-3 and -7 activation and includes heat shock protein (Hsp) upregulation/ release, autophagy induction, cancer stem cells (CSC) lysis, and intratumoral influx of CD11b+ antigen-presenting cells^{148,216}. Δ PK is a particularly promising OV platform because it was well tolerated in human phase I/II clinical trials¹⁵⁰. However, its ability to harness immune responses that contribute to tumor cell death is still poorly understood. We report that Δ PK-induced melanoma cell death has multiple ICD features, both in culture and xenografts. These include: (i) JNK/c-Jun dependent inhibition of the immunosuppressive cytokine IL-10 and the resulting upregulation of MICA, the ligand for the activating receptor NKG2D expressed on NK and cytotoxic T cells, (ii) upregulation of pro-inflammatory cytokines TNF- α , GM-CSF, and IL-1 β through activation of the TLR2/NF κ B pathway and pyroptosis, and (iii) inhibition of the negative immune checkpoint regulator CTLA-4.

B2: Results

B2.1: Δ PK increases transcription of pro-inflammatory and death-associated genes.

To examine whether Δ PK modulates the expression of cytokines and death-associated functions in melanoma cells, A2058 and LM cultures were mock-infected with PBS or infected with Δ PK [multiplicity of infection (moi) = 0.5 plaque forming units (pfu)/cell] and RNA isolated at 24 hrs post infection (pi) was analyzed with inflammatory and cell death targeted oligoarrays, as described in Materials and Methods. The data summarized

in Fig. 8A for A2058 cells, indicate that Δ PK caused a marked upregulation of several genes implicated in inflammation and cell death. They include inflammatory caspases-1, -4, and -5, the cytokines IL-1 α , IL-1 β , IL-6, IL-8, IL-12, TNF- α , LTA, and GM-CSF, and mitogen activated protein kinases (MAPK) involved in both PCD and inflammatory processes. Among these are MAP3K7IP1 (also known as TAB1), which is involved in TAK1-regulated signaling pathways induced by IL-1, ²³⁸, MAP4K4, the upstream activator of JNK ²³⁹, JNK and its downstream target c-Jun, MAP2K6, the upstream activator of the pro-apoptotic p38MAPK ²⁴⁰, and MAP3K14 (also known as NIK) that stimulates NF κ B signaling cascades common to the TNF- α and IL-1 receptors ²⁴¹. Similar results were obtained in LM cells.

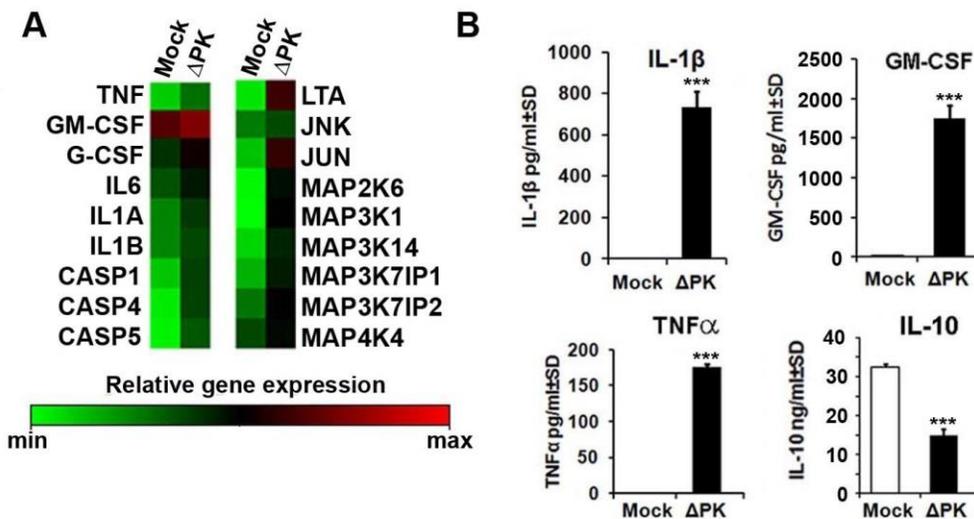


Figure 8: Δ PK upregulates pro-inflammatory and pro-apoptotic genes; alters cytokine secretion

(A) Total RNA was isolated from mock- or Δ PK-infected (moi=0.5; 24hrs) A2058 cells assayed with inflammatory and apoptosis targeted microarrays (SABiosciences, Oligo GeArrays) and analyzed using GEArray Expression Analysis Suite 2.0. (B) Conditioned media from A375 cells mock- or Δ PK infected (moi=1; 24hrs) were assayed for TNF- α , IL-10, GM-CSF, and IL-1 β by ELISA and results are expressed as ng or pg/ml \pm SD. (***)p<0.001 vs mock, by student's t-test, n=3)

B2.2: Δ PK alters the balance of secreted cytokines from anti- to pro-inflammatory.

An excess of secreted anti-inflammatory cytokines characterize the tumor immunosuppressive interstitial milieu. To examine whether Δ PK can alter this milieu by favoring the secretion of pro-inflammatory cytokines, we focused on IL-1 β , TNF- α and GM-CSF, that are involved in ICD^{242,243} and IL-10, which is an immunosuppressive factor that plays an important role in immune tolerance and is also secreted by regulatory T cells (Treg)^{244,245}. A2058 and A375 melanoma cells were mock-infected with PBS or infected with Δ PK (moi = 1) and the conditioned media collected at 24hrs pi were assayed for cytokine expression by ELISA. The mock-infected cultures secreted IL-10, but not TNF- α , IL-1 β or GM-CSF. By contrast, when infected with Δ PK the balance of the secreted anti- to pro-inflammatory cytokines was reversed, with a significant increase in the secretion of TNF- α , IL-1 β , and GM-CSF and a simultaneous decrease in the secretion of IL-10. This is summarized in Fig. 8B for A375 cells and similar results were obtained in A2058 cells.

B2.3: Δ PK inhibits IL-10 secretion through JNK/c-Jun activation.

Having seen that Δ PK induces the transcription of MAP4K4, JNK and c-Jun (Fig. 8A), we wanted to verify that this reflects its ability to activate the JNK/c-Jun pathway implicated in the regulation of inflammatory responses²⁴⁶. A375 cells were mock infected with PBS or infected with Δ PK (moi = 1) and protein extracts obtained at 1, 4, and 24 hours pi were immunoblotted with antibody that recognizes the phosphorylated JNK isoforms (pJNK1 and pJNK2/3). The blot was stripped and re-probed with antibody against total JNK used as loading control and expression levels were determined by densitometric scanning, as described in Materials and Methods. The results are expressed

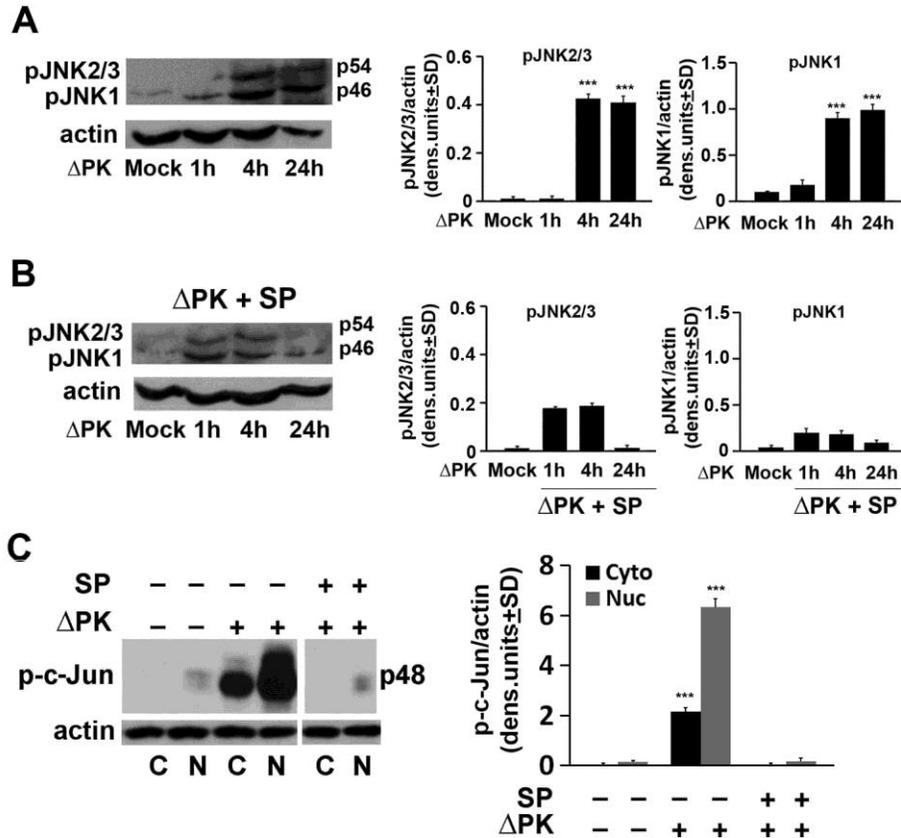


Figure 9: ΔPK activates the JNK/c-Jun pathway in melanoma cells.

(A) A375 cells were mock- or ΔPK-infected (moi=1) and protein extracts obtained at 1,4, and 24 hours pi were immunoblotted with antibody to phosphorylated JNK isoforms (pJNK1 and pJNK2/3). The blots were stripped and re-probed with antibody to total JNK and the results quantified by densitometric scanning are expressed as densitometric units ± SD. (B) Extracts from parallel cultures infected in the presence of the JNK inhibitor SP600125 (SP) were collected at 1,4, and 24 hours pi and immunoblotted with pJNK antibody. The blots were stripped and re-probed with antibody to β-actin used as gel loading control and the results are expressed as densitometric units ± SD. (C) A2058 cultures mock- or ΔPK-infected (moi = 1; 24hrs) in the absence or presence of SP600125 (100μM) were separated into nuclear and cytoplasmic fractions as described in Materials and Methods and immunoblotted with antibodies to phosphorylated c-Jun (p-c-Jun) followed by β-actin. The results are expressed as densitometric units ± SD. (***)P<0.001 vs mock, by two-way ANOVA, n=3).

as densitometric units ± SE. The pJNK antibody identified two bands respectively corresponding to pJNK1 (46 kDa) and pJNK2/3 (54kDa), the levels of which were significantly higher in the ΔPK- than mock-infected cultures (Fig. 9A) and reduced by

treatment with the JNK activation inhibitor SP600125 (100 μ M) (Fig. 9B). To confirm that c-Jun is also activated by Δ PK infection, protein extracts from duplicate A375 cultures mock-infected or infected with Δ PK (moi = 1; 24hrs) in the absence or presence of SP600125 were separated into nuclear and cytoplasmic fractions as described in Materials and Methods and immunoblotted with antibodies against phosphorylated c-Jun (p-c-Jun). Blots were stripped and re-probed with actin as a gel loading control. The levels of p-c-Jun were significantly increased in the Δ PK as compared to the mock-infected cultures, primarily in the nuclear fraction, and upregulation was inhibited by SP600125 (Fig. 9C). This is consistent with the established nuclear translocation of the activated c-Jun protein²⁴⁷, and it indicates that Δ PK activates the JNK/c-Jun pathway. Significantly, ELISA of the culture supernatants indicated that SP600125 restored the levels of IL-10 in the Δ PK-infected cultures (moi = 1; 24hrs) to those seen in the mock-infected controls, as shown in Fig. 10A, B for A2058 and A375 cells, respectively. SP600125 had no effect on GM-CSF, TNF- α and IL-1 β production (Fig. 10A,B), indicating that Δ PK inhibits IL-10 production through JNK/c-Jun activation, but this pathway is not involved in the production/secretion of the inflammatory cytokines.

B2.4: IL-10 inhibition induces expression of the MHC class I chain-related protein A (MICA).

MICA is a ligand for the activating receptor NKG2D expressed on NK, $\gamma\delta$ T, cytotoxic $\alpha\beta$ CD8⁺ T, and NKT cells. Its expression on tumor cells can engage NKG2D, resulting in the cytotoxic killing of the target cells and the release of DAMPS, PAMPS and TAAs that further stimulate immune cells⁵¹. IL-10 facilitates the ability of melanoma

cells to escape immune surveillance by decreasing MICA expression, thereby reducing NKG2D-mediated cell cytotoxicity⁵². Having seen that Δ PK induces JNK-dependent inhibition of IL-10 expression (Fig. 9), we wanted to know whether this results in increased MICA expression. A2058 and A375 cells were mock-infected or infected with Δ PK (moi = 1; 24 hrs) in the presence or absence of the JNK inhibitor SP600125 (100 μ M) or recombinant IL-10 (1.4 ng/ml) and protein extracts were immunoblotted with MICA antibody using antibody to β -actin as gel loading control. The results were quantitated by densitometric scanning and the data are summarized in Fig. 10C for A2058 cells. MICA was minimally expressed in mock-infected melanoma cells, and its expression was significantly increased by Δ PK infection. Both SP600125 and IL-10 restored the MICA levels to those seen in the mock-infected cells, indicating that Δ PK increases MICA expression through JNK-dependent IL-10 inhibition. Similar results were obtained in A375 cells.

B2.5: Pro-inflammatory cytokines are upregulated through autophagy-related activation of TLR2 signaling.

TLRs initiate signaling events that lead to NF κ B activation and the transcription of inflammatory cytokines²⁴⁸. We focused on TLR2 that mediates inflammatory cytokine production in response to HSV infection^{249,250} and autophagy that is linked to cytokine secretion^{251,252} and enhanced T cell presentation of TAAs^{253,254}. A2058 and A375 cells were mock-infected with PBS or infected with Δ PK (moi = 1; 4hrs) in the absence or presence of the autophagy inhibitor 3-methyladenine (3-MA) (5mM)²⁵⁵ and protein extracts were immunoblotted with antibody to TLR2, the TLR adaptor protein MyD88, or the mature NF κ B subunit (p50). A 95-110kDa doublet consistent with the mature TLR2

²⁵⁶, was seen in the Δ PK- but not mock-infected cells and its induction was inhibited by 3MA. MyD88 was also seen in the Δ PK- but not mock-infected cultures, as shown in Fig. 11A for A375 cells, and this was accompanied by increased expression of the mature NF κ B p50, the latter partially inhibited by 3MA(Fig 11B). Double immunofluorescent staining of the mock- and Δ PK-infected cells with antibodies to NF κ B p50 and TNF- α confirmed that Δ PK favored NF κ B nuclear localization (relative to mock-infected cells), which is indicative of pathway activation, and it was accompanied by increased TNF- α expression (Fig. 11C). We conclude that activation of the TLR2/ NF κ B pathway is involved in cytokine production, because ELISA of the supernatants from cells mock or Δ PK-infected cultures in the absence or presence of 3MA indicated that the levels of all 3 inflammatory cytokines were significantly increased by infection with Δ PK, but a similar increase was not observed in the presence of 3-MA (Fig. 11D). Similar results were obtained in A375 cells and 3MA had no effect on Δ PK-induced IL-10 inhibition. Collectively, the data indicate that Δ PK induces pro-inflammatory cytokine expression through autophagy-related activation of the TLR2/ NF κ B pathway.

B2.6: Δ PK -induced pyroptosis contributes to IL-1 β production.

Pyroptosis is a caspase 1-dependent form of inflammatory cell death, which is activated after an initial NF κ B -dependent priming step²⁵⁷. It is characterized by the oligomerization of apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1 (pyroptosome formation) leading to the cleavage (activation) of caspase 1 and the resulting activation of pro-inflammatory cytokines, such as IL-1 β ²⁵⁸.

Having seen that the levels of IL-1 β are increased in Δ PK-infected cells, we wanted to know whether this involves pyroptosis-

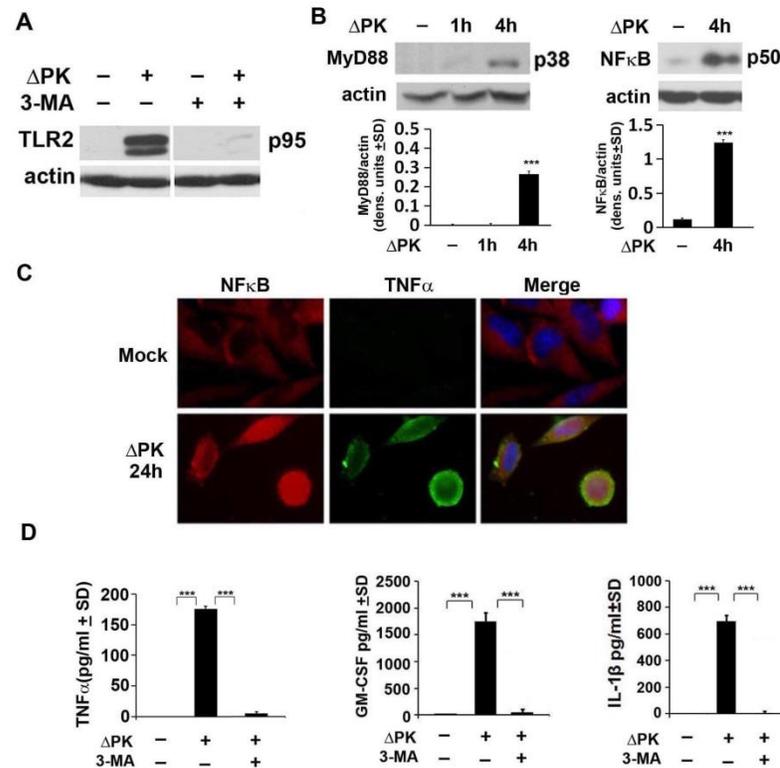


Figure 10: Δ PK induces pro-inflammatory cytokine expression through autophagy-related activation of the TLR2/NF κ B pathway.

(A) Protein extracts from A375 cells mock or Δ PK-infected (moi = 1) in the absence and presence of the autophagy inhibitor 3-methyladenine (3-MA, 5 mM) were collected at 1 and/or 4 hours pi and immunoblotted with antibody to TLR2 (top panel) and MyD88 (bottom panel). Blots were stripped and re-probed with antibody to β -actin used as gel loading control. (B) Extracts from A375 cells mock- or Δ PK-infected as in (A) (moi=1) were collected at 4 hours pi and immunoblotted with antibody to NF κ B p50. Blots were stripped and re-probed with antibody to β -actin used as gel loading control. The results were quantitated by densitometric scanning and expressed as densitometric units \pm SE (** p <0.001 and * p <0.05, by one way ANOVA). (C) A2058 cells were mock- or Δ PK-infected (moi=1; 24hrs) and stained in double immunofluorescence with Alexafluor 594-labeled NF κ B p50 (red) and Alexafluor 488-labeled TNF α (green) antibodies and counterstained with DAPI to visualize nuclei. (D) Supernatants from A2058 cells mock or Δ PK-infected as in (A) were collected at 24hrs pi and assayed for TNF α , GM-CSF, and IL-1 β by ELISA. (** p <0.001 by two-way ANOVA, n=3).

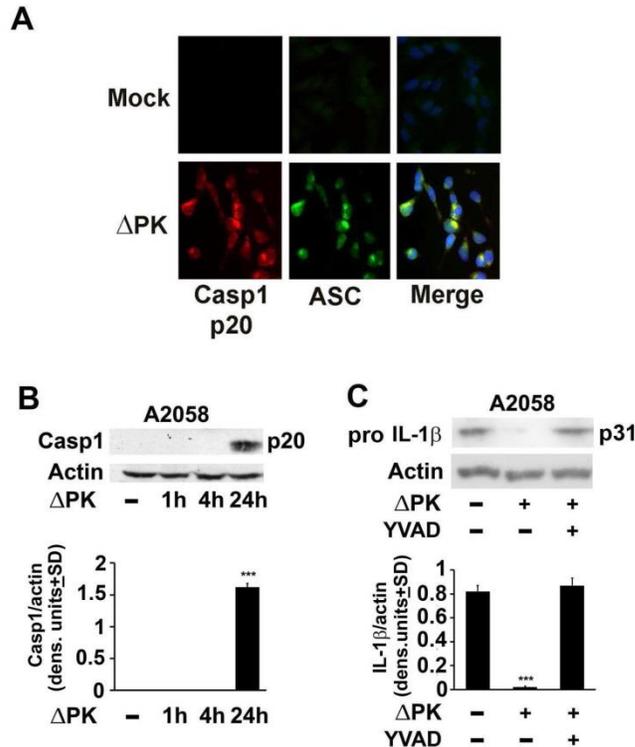


Figure 11: ΔPK-induced pyroptosis activates caspase-1 resulting in mature IL-β production.

(A) Mock or ΔPK-infected A375 cells grown on coverslips were fixed 24hours pi and stained in double immunofluorescence with Alexafluor 488-labeled ASC (green) and Alexafluor 594 labeled caspase 1 p20 (red) antibodies, as described in Materials and Methods. Coverslips were mounted in Vectashield with DAPI to visualize nuclei. (B) Extracts from A2058 cultures either mock or ΔPK-infected (moi=1) were collected at 1, 4, and 24 hours pi and immunoblotted with antibody to activated caspase 1 (p20) Blots were stripped and re-probed with antibody to β-actin used as gel loading control. Data were quantified by densitometric scanning and the results are expressed as densitometric units ± SD. (C) Extracts from A2058 cultures mock or ΔPK-infected (moi=1; 24hrs) in the presence or absence of the caspase 1 specific inhibitor z-YVAD-fmk (YVAD) (100uM) were immunoblotted with antibody to pro- IL-1β. Blots were stripped and re-probed with antibody to β-actin used as gel loading control. Data were quantified by densitometric scanning and are expressed as densitometric units ± SD (**p<0.001 vs mock, by two-way ANOVA, n=3).

related caspase-1 activation. A375 and A2058 cells were mock- or ΔPK-infected (moi = 1; 24 hrs) and stained in double immunofluorescence with antibodies to ASC and

activated caspase 1 (p20). Staining was virtually absent from mock-infected cells, whereas ASC and activated caspase 1 co-localized in the Δ PK-infected cells, as shown in Fig. 12A for A375 cells. Consistent with the oligoarray data (Fig. 8A), immunoblotting with antibody to activated caspase-1 confirmed that activation only occurred in the Δ PK-infected cells (Fig 12B). Caspase-1 activation was accompanied by IL-1 β production as evidenced by the loss of pro-IL-1 β and its restored expression in cells infected with Δ PK in the presence of the caspase 1 specific inhibitor z-YVAD-fmk (YVAD) (Fig 12C). Collectively, the data indicate that Δ PK-induced IL-1 β expression involves pyroptosome-dependent caspase-1 activation and pro-IL-1 β cleavage.

B2.7: The altered cytokine balance contributes to Δ PK-induced melanoma cell death.

To examine the contribution of the altered cytokine balance to Δ PK-induced melanoma cell death, we first determined the levels of cell death induced by Δ PK alone at 24 and 48h by examining mock- or Δ PK-infected A2058 and A375 cultures for cell death by trypan blue staining. As shown in Fig 13A, Δ PK induced $30.8 \pm 1.1\%$ at 24hr which increased to $54.7 \pm 0.8\%$ at 48 hrs for A2058 cultures, and $22.3 \pm 0.7\%$ death at 24hrs which increased to $59.2 \pm 1.2\%$ at 48hrs for A375 cultures. Because of the higher levels of cell death seen at 48 hours, duplicate mock- or Δ PK-infected A2058 and A375 cultures grown in the presence or absence of SP600125 (100uM) that regulates JNK-dependent IL-10 expression, or 3-MA (5mM) or z-YVAD-fmk (100uM), which regulate TLR2 and pyroptosis-dependent expression of the pro-inflammatory cytokines, were assayed for cell death by trypan blue exclusion only at 48hours pi. Δ PK-induced cell death was significantly inhibited by the addition of SP600125 ($p < 0.001$), 3-MA ($p < 0.001$) or z-

YVAD-fmk ($p=0.002$) in A2058 cells (Fig 13B) and similar results were obtained in A375 cells (Fig 13C), indicating that immunogenic modulation through the respective pathways is involved in the Δ PK-induced cell death.

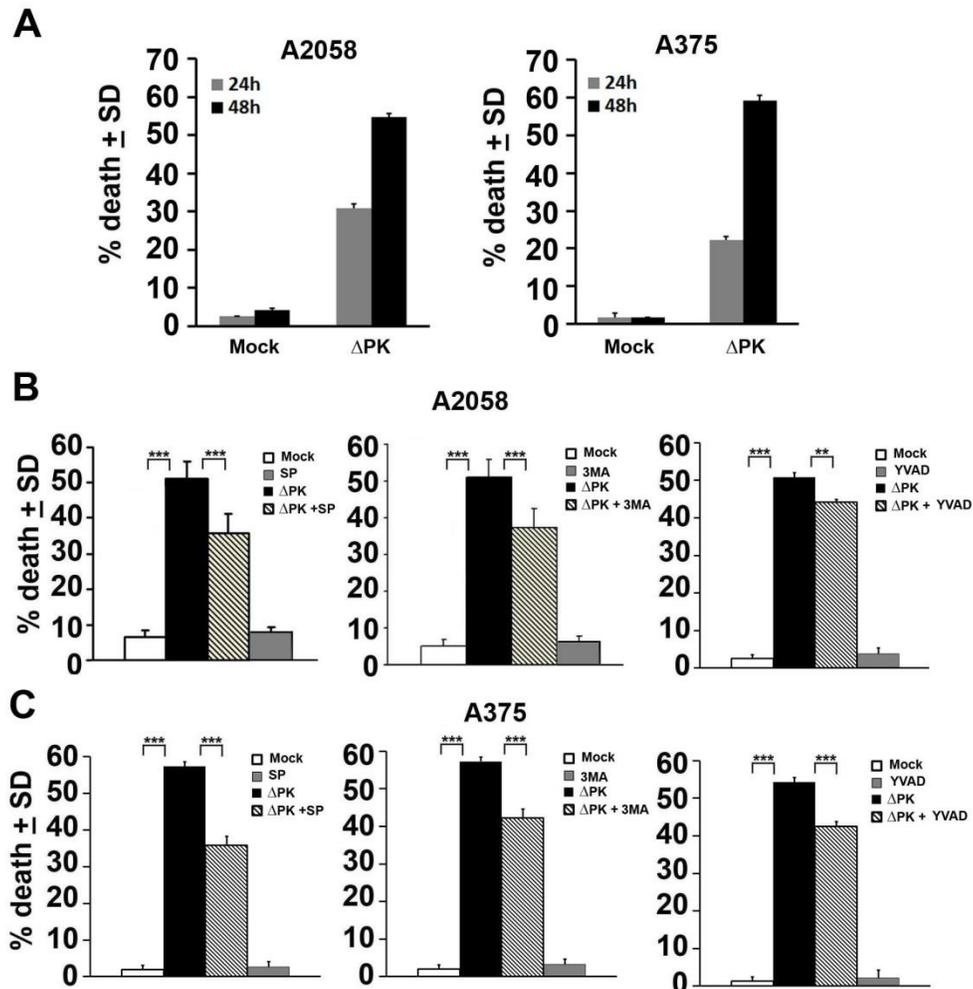


Figure 12: Cytokine modulation contributes to Δ PK-induced melanoma cell death.

(A) A2058 and A375 cultures were mock- or Δ PK-infected ($moi=1$) and examined for cell death by trypan blue exclusion at 24h and 48h pi. Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death \pm SD. (B) A2058 and (C) A375 cultures were mock- or Δ PK-infected ($moi=1$) in the presence or absence of SP600125 (100uM, left) 3-MA (5mM, middle), or z-YVAD-fmk (100uM, right) and examined for cell death by trypan blue exclusion at 48 hours pi as in (A). The results are expressed as % death \pm SD. (***) $p<0.001$, (**) $p<0.01$, by two-way ANOVA, $n=3$).

B2.8: Δ PK inhibits expression of the negative immune checkpoint regulator CTLA-4.

CTLA-4 is a glycoprotein of the immunoglobulin superfamily that functions as an inhibitory receptor of T cell activation and effector functions and is implicated in the maintenance of immune tolerance (immune checkpoint) ⁵³. Recent studies have shown that CTLA-4 is constitutively expressed in several solid tumors including melanoma ^{54,55}, and CTLA-4 blockade with specific monoclonal antibodies resulted in impressive tumor regression ⁵⁶. To examine the effect of Δ PK on CTLA-4 expression, A2058 and A375 cells were mock-infected or infected with Δ PK (moi = 1) and protein extracts obtained at 4 and 24 hrs p.i were immunoblotted with CTLA-4 antibody. Two bands (p30 and p43) that respectively represent the cytosolic and glycosylated (membrane-associated) proteins were seen in mock-infected cultures. Their expression was inhibited by Δ PK, as seen for p30 at 4hrs pi and for p43 at 24hrs pi (Fig. 14), but the mechanism of inhibition is still unclear.

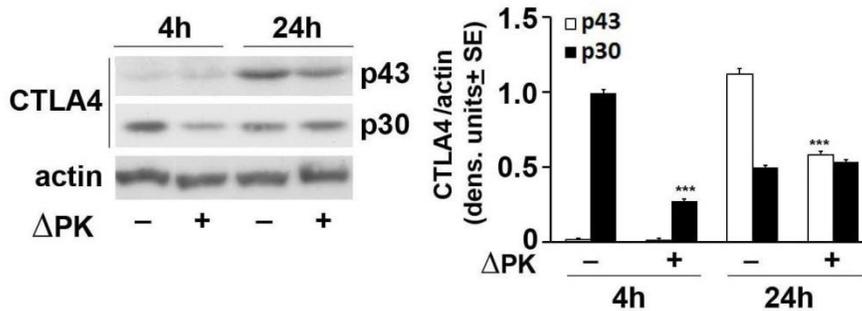


Figure 13: Δ PK inhibits CTLA-4 expression

Cell extracts from mock and Δ PK -infected A2058 cells (moi = 1) were collected at 4 and 24 hours pi and immunoblotted with antibody to CTLA-4 [recognizes the glycosylated (p43) and cytosolic (p30) protein forms]. Blots were stripped and re-probed with antibody to β -actin used as gel loading control. Data were quantified by densitometric scanning and the results are expressed as densitometric units \pm SE. (***)P<0.001 vs mock, by two-way ANOVA, n=3).

B2.9: Δ PK inhibits tumor cell growth associated with increased expression of MICA and TNF- α .

We have previously shown that Δ PK inhibits the growth of melanoma xenografts, associated with apoptosis, caspase-1 activation, CSC lysis, increased autophagy and Hsp expression and characterized by tumor infiltration with CD11b+ antigen presenting cells¹⁴⁸. To examine whether altered expression of pro-inflammatory cytokines and MICA is also associated with tumor growth inhibition, A375 cells were implanted into Balb/c nude mice by subcutaneous (s.c.) injection into both flanks and when the tumors became palpable (day 7; approximately 200mm³), the animals were given intratumoral injections (100 μ l) of partially purified Δ PK (10⁶ per injection) or culture medium control and tumor volume was calculated as described in Materials and Methods. Confirming our previous findings (Colunga et al., 2010), mock-infected animals evidenced time-dependent tumor volume increases that reached maximal levels on day 37, but Δ PK caused a significant (p<0.001) decrease in tumor growth (Fig 15A). This was associated with increased TNF- α and MICA expression, as respectively evidenced by immunohistochemistry and immunoblotting of xenografts collected from three mock and three Δ PK- treated animals at 7 days after the last Δ PK injection (Fig. 15B). Collectively, the data indicate that Δ PK induces melanoma cell death in culture and xenograft tissues through the simultaneous modulation of multiple ICD-associated functions, as schematically represented in Fig. 16.

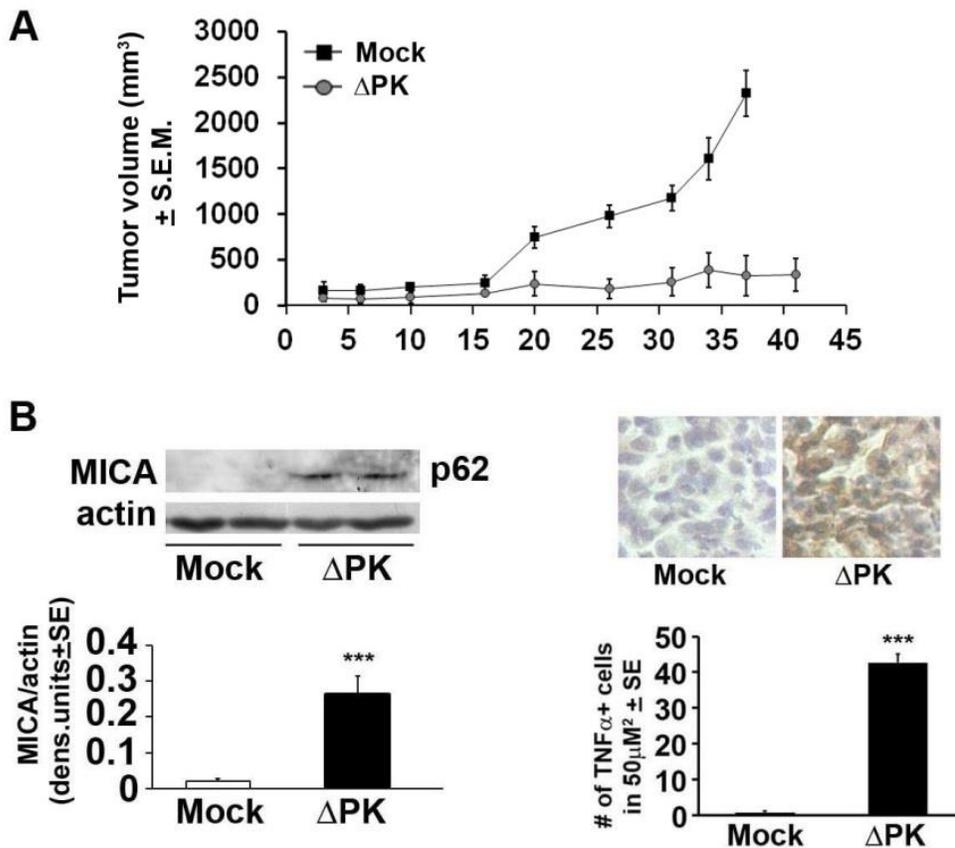


Figure 14: Δ PK inhibits tumor growth and upregulates MICA and TNF- α in melanoma xenografts.

(A) A375 xenografts were established as described in Materials and Methods and were given four i.t. injections of Δ PK (n=6; 10^6 pfu) or growth medium (n=3) at weekly intervals beginning on day 7, when the tumors were palpable. The difference between mock and Δ PK treatment became statistically significant at day 20 and remained significant by the end of the study ($P < 0.001$ by two-way ANOVA). (B) A375 xenograft tissues mock-treated or treated with Δ PK as in (A) were collected 7 days after the last Δ PK injection and extracts were immunoblotted with antibody to MICA (left panel), stripped and re-blotted with antibody to β -actin used as gel loading control. Each lane represents a tumor from a different animal. Data were quantified by densitometric scanning for all tumors and results are expressed as densitometric units \pm SE. Duplicates of the A375 xenografts were stained with TNF- α antibody by immunohistochemistry and counterstained with Mayer's hematoxylin (right panel). Staining cells were counted in three randomly selected fields ($50 \mu\text{m}^2$) and the mean number of positive cells per area was calculated (***) $P < 0.001$ vs mock, by one way ANOVA).

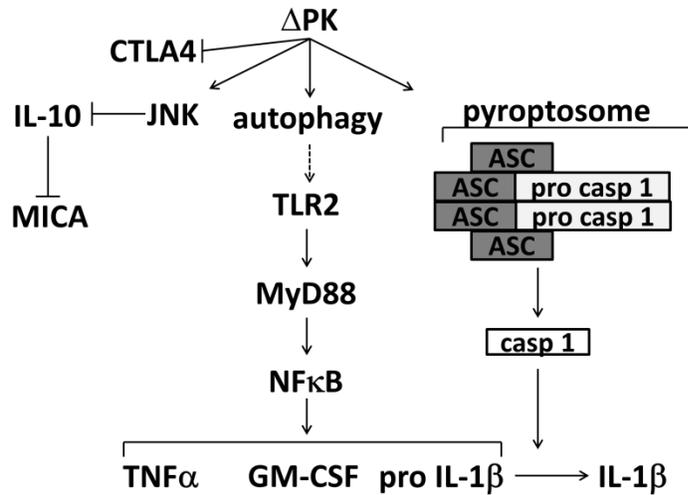


Figure 15: Schematic representation of the Δ PK-modulated ICD-associated functions

Δ PK induces melanoma multiple ICD-associated signals associated with its ability to cause tumor cell death. They include: (i) JNK/c-Jun dependent inhibition of the anti-inflammatory cytokine IL-10 and the resulting upregulation of MICA, the ligand for the NK and cytotoxic T cells activating NKG2D receptor, (ii) upregulation of pro-inflammatory cytokines TNF- α , GM-CSF and IL-1 β through autophagy-related activation of the TLR 2/NF κ B pathway and pyroptosis, and (iii) inhibition of the negative immune checkpoint regulator CTLA-4.

B3: Discussion

The salient feature of the data presented in this report is the finding that Δ PK-induces melanoma cell death through multiple ICD-associated signals that include: (i) JNK/c-Jun dependent inhibition of the anti-inflammatory cytokine IL-10 and the resulting upregulation of MICA, the ligand for the activating receptor NKG2D expressed on NK and cytotoxic T cells, (ii) upregulation of pro-inflammatory cytokines TNF- α , GM-CSF and IL-1 β through activation of the TLR 2/ NF κ B pathway and pyroptosis, and (iii) inhibition of the negative immune checkpoint regulator CTLA-4. The following comments seem pertinent with respect to these findings.

Oncolytic virotherapy is a unique mode of cancer therapeutics that encompasses

direct tumor cell lysis through virus replication, the induction of PCD programs, and immunotherapy resulting from enhanced innate immunity and the elicitation of tumor immunogenicity^{129,131,237}. Virtually all virus families were pursued in OV development, each with its specific mechanism of tumor cell lysis and unique advantages and disadvantages. However, overall clinical efficacy is limited, associated at least in part, with the failure to lyse CSC and tumor immune evasion resulting from the low TAA immunogenicity and the highly immunosuppressive interstitial milieu. Indeed, tumor-associated immune tolerance is a major obstacle in cancer immunotherapy and is characterized by a large number of regulatory T cells (Tregs)²⁵⁹ and an anti-inflammatory Th2-biased programming, both of which decrease TAA presentation by MHC I^{260,261}.

Recent efforts to improve immunotherapeutic potential have focused on arming the OVs with pro-inflammatory cytokines that can break immune tolerance by skewing the Th balance in favor of an anti-tumor Th1-biased programming^{262,263}. An adenovirus-based OV armed with TNF- α was shown to have increased cancer-eradicating potency^{164,264} as were HSV or vaccinia-based OVs armed with GM-CSF^{265,266}. Combinatorial therapy using chemotherapeutic drugs together with OVs has also received particular attention, as shown with a parvovirus-based OV that stimulates IL-1 β secretion²⁶⁷. Alternatively, OVs have been combined with immune checkpoint inhibitors in order to break immune tolerance, as recently reported for an oncolytic Newcastle disease virus and systemic CTLA-4 blockade²⁶⁸. However, individual cytokines deliver immunostimulatory signals in a relatively non-specific manner and the most potent OV-potentiating ICD mode is still unclear. Recent studies suggest that cytokines may even

contribute to the establishment of Treg-mediated tolerance, as shown for exogenously delivered GM-CSF²⁶⁹. Indeed, it is becoming increasingly evident that multi-mechanistic OV's are particularly desirable because they could mimic the effects of combinatorial therapy by inducing various death pathways, lysing CSC, and eliciting multiple diverse and potent anti-tumor immune responses at the relatively lower toxicity of a single agent therapy.

We have previously shown that the HSV-2 mutant Δ PK causes a profound and long-lasting reduction of tumor burden in melanoma xenografts through, in addition to virus replication, the induction of multiple PCD pathways that include apoptosis and autophagy, Hsp release, and CSC lysis^{148,216}. We reasoned that Δ PK is a particularly promising OV because it was well tolerated in phase I/II clinical studies¹⁵⁰ and it induces multiple and complementary death pathways, at least in melanoma. We found that Δ PK also induces a range of anti-tumor immune responses that include an altered balance of Th1 vs Th2-biased programming, TLR2/NF κ B activation and pyroptosis and inhibition of the negative immune checkpoint CTLA-4. Inhibition of the immunosuppressive cytokine IL-10 that is expressed and secreted by melanoma cells, is particularly relevant from a therapeutic standpoint, because IL-10 plays a crucial role in immune tolerance by inhibiting DC maturation and their antigen presenting function²⁷⁰, and the expression of MHC class II and co-stimulatory molecules²⁷¹. While a Reovirus-based OV was also shown to inhibit IL-10 in melanoma cells²⁷², the inhibitory mechanism and its contribution to cytotoxic cell engagement is unclear. We show that Δ PK inhibits IL-10 through JNK/c-Jun activation and this results in increased expression of MICA, the MHC I-related ligand that stimulates the engagement of the NKG2D receptor expressed by NK

and cytotoxic T cells^{51,273}. Indeed, Δ PK increased the levels of phosphorylated (activated) JNK and c-Jun and p-c-Jun nuclear translocation, both inhibited by the JNK-specific inhibitor SP600125, and SP600125 also inhibited Δ PK-induced melanoma cell death. Significantly, both SP600125 and recombinant IL-10 blocked the ability of Δ PK to induce MICA expression, indicating that Δ PK stimulates the cytotoxic cell engagement characteristic of ICD through JNK/c-Jun mediated IL-10 inhibition^{51,273}. The ability of the activated JNK/c-Jun pathway to inhibit IL-10 secretion is in contrast to previous findings for monocytes/macrophages,²⁷⁴⁻²⁷⁶, but MICA upregulation was also associated with the ability of Δ PK to inhibit the growth of melanoma xenografts, consistent with previous findings that its expression on tumor cells enhances NK-mediated tumor cell lysis NKG2D engagement^{277,278}.

TLRs are a family of pattern recognition receptors that recognize PAMPs and DAMPs, and trigger the activation and maturation of DCs. They play a crucial role in antigen presentation, tumor clearance, and pyroptosis, which features rapid release of proinflammatory intracellular contents that are recognized by APCs and promote the formation of T-cell-mediated adaptive immunity^{114,279}. Consistent with previous findings that Δ PK can alter a natural Th2- to a Th1-biased response¹⁴⁹, we found that IL-10 inhibition was accompanied by the simultaneous upregulation/release of the inflammatory cytokines TNF- α , GM-CSF and IL-1 β in cultured melanoma cells and TNF- α was also increased in Δ PK infected xenografts. Upregulation was through activation of the TLR2/NF κ B pathway and pyroptosis, a caspase-1 dependent inflammatory cell death modality¹³¹. Indeed, Δ PK upregulated the expression of TLR2 and its adaptor protein MyD88 and caused NF κ B activation, as evidenced by the

expression and nuclear translocation of the p50 active subunit. The autophagy inhibitor 3MA blocked both TLR2 and NFκB p50 expression and it abolished the ΔPK-induced expression of the inflammatory cytokines, associating the activation of the TLR2/NFκB pathway with the ability of ΔPK to induce autophagy²¹⁶. Previous studies have shown that: (i) HSV antigens are recognized by TLR2, resulting in the production of several pro-inflammatory cytokines^{249,280} and NK cells activation²⁵⁰ and (ii) autophagy is involved in innate immunity stimulation and cytokine secretion^{251,252}. We do not exclude the possibility that the ability of 3MA to inhibit the ΔPK-induced activation of the TLR2/NFκB pathway and pro-inflammatory cytokine production is an off-target effect unrelated to autophagy. However, 3MA inhibited the oncolytic activity of ΔPK, confirming that TLR2/NFκB pathway activation and inflammatory cytokine production contribute to ΔPK-induced melanoma cell death.

Significantly, TLR/ NFκB activation is a priming signal that acts to induce the expression of pro-IL-1β²⁸¹, which, in turn is cleaved by caspase-1 activated through pyroptosis²⁵⁸. Consistent with this interpretation, double immunofluorescence and immunoblotting studies confirmed that ΔPK induced pyroptosome formation through ASC and pro-caspase-1 oligomerization resulting in caspase-1 activation and pro-IL-1β cleavage. Cleavage was inhibited by the caspase-1 specific inhibitor z-YVAD-fmk that also inhibited the ability of ΔPK to induce melanoma cell death, confirming the contribution of pyroptosis to ΔPK-induced cell death. Pyroptosis induction is particularly critical from the standpoint of ICD, because it induces the release of DAMPs and TAAs that are recognized by immune and non-immune cells and induce tumor cell death^{131,235,236}. Finally, ΔPK inhibited the expression of CTLA-4, a glycosylated cell surface

receptor that is constitutively expressed in several solid tumor-derived cells, including melanoma^{54,55}, and behaves as a negative regulator of T cell function²⁸². While the exact contribution of the melanoma-expressed CTLA-4 is still unclear, it is important to point out that its blockade was previously shown to induce tumor regression⁵⁶ and it is clinically effective in treating metastatic melanoma^{73,283}. Future studies will examine the contribution of CTLA-4 inhibition to the Δ PK-induced ICD, further elucidate the role of autophagy in Δ PK-induced tumor cell immunogenicity and verify their role in Δ PK-mediated inhibition of tumor growth.

C. Valproic acid induces neuronal cell death through a novel calpain-dependent necroptosis pathway

C1. Introduction

Valproic acid (VPA) is a histone deacetylase (HDAC) inhibitor used to treat epilepsy and mood disorders. Its utility as an anticonvulsant that blocks voltage-dependent sodium channels was originally supported by clinicians, but subsequently challenged due to its side-effects and HDAC-dependent and-independent toxicity^{186,187,284}. While protection was reported in some neurologic disease studies¹⁸³⁻¹⁸⁵, VPA-induced neurodegeneration was seen in both cultured neuronal cells and experimental animals^{188,189,195,198,199}. VPA reduced the proliferation of hippocampal neurons and caused cognitive impairment in intraperitoneally injected rats¹⁸⁹ and neonatal mice and rats given clinically relevant doses of anticonvulsant VPA therapy exhibited widespread apoptotic neurodegeneration in several brain regions^{198,200}. In humans, VPA has established teratogenic activity. It causes neural tube defects and increases the incidence of children with autism spectrum disorder (ASD), when taken during pregnancy^{191,192}. Developmental neurotoxicity was attributed to the generation of free radicals, oxidative stress¹⁹³ and caspase-dependent apoptosis^{194,195}, and the morphological changes seen in the brains of autistic children confirmed that VPA induces programmed cell death (PCD)¹⁹⁴. The pathways involved in the VPA-induced neurotoxicity are still poorly understood²⁰¹. Caspase-independent PCD was also reported²⁰², but its exact mechanism is still unknown. We report for the first time, that VPA induces a cascade of deleterious events, which contribute to neuronal cell death through an atypical calpain-dependent necroptosis pathway. The pathway involves early activation of c-Jun-N-terminal kinase 1 (JNK1) and increased expression of receptor-interacting protein 1 (RIP-1), and is followed by cleavage/nuclear translocation

of apoptosis-inducing-factor (AIF) and phosphorylation of the histone H2A family member H2AX as well as the altered balance between the death-inducing protein Smac/DIABLO and the protective protein XIAP.

C2 Results

C2.1. VPA has dose-dependent neurotoxic activity.

The ability of VPA to cause neuronal cell death was examined in neuronally differentiated PC12 cells, which are an established model for the study of neuronal cell life/death decisions^{285,286}. PC12 cells were differentiated by 4 days of culture with NGF and differentiation was confirmed by the formation of neurites and expression of the neuronal differentiation marker MAP-2. A first series of experiments to examine the effect of VPA on neuronal cells followed on previous findings that the levels of VPA in the serum from treated patients is generally 0.2–0.6 mM, but fetuses may be exposed to up to 5 times higher levels in maternal serum at term²⁸⁷. Accordingly, the neuronally differentiated PC12 cells were treated with VPA (0.05 to 5.0 mM) or mock-treated with PBS and examined for cell death by ethidium homodimer staining at 3 and 5 days post-treatment. Results are expressed as % dead cells normalized to the untreated cultures (% dead cells in VPA-treated cultures - % dead cells in the untreated cultures). The data summarized in Fig. 17A indicate that VPA caused dose-dependent cell death with relatively low numbers of dead cells ($\leq 20\%$) seen at 0.05-0.5mM and higher percentages (35-50%) seen at 1 and 5mM concentrations. Similar results were obtained by trypan blue staining. We used the 1mM concentration in the subsequent experiments, because: (i) the therapeutically relevant dose is lower than 5mM²⁸⁷, and (ii) previous studies using 1mM VPA reported contradictory cell type-dependent effects, with caspase-dependent

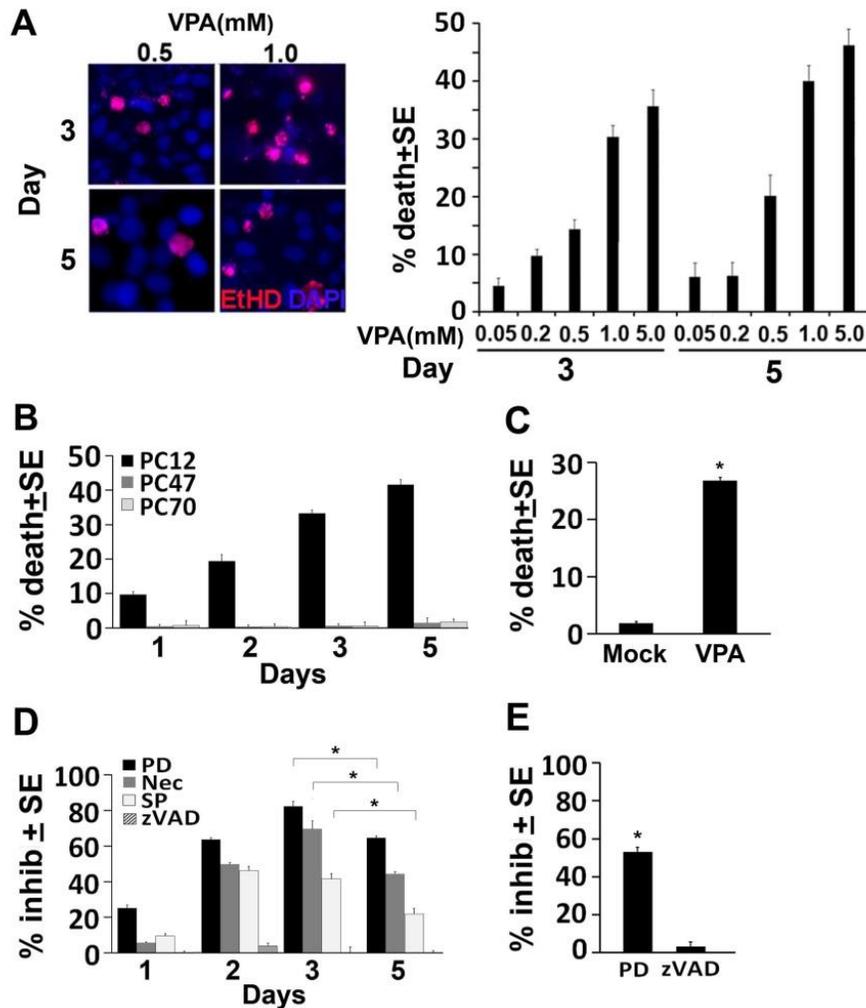


Figure 16: VPA induces neuronal cell death; inhibited by calpain, JNK and necroptosis inhibitors.

(A) PC12 cells neuronally differentiated, treated with VPA (0.05, 0.2, 0.5, 1, 5mM), and assayed for cell death by ethidium homodimer staining 3 and 5 days later. Dead cells (red staining) were counted in 4 quadrants and the % dead cells was calculated and normalized to untreated cells. The results are expressed as % death cells \pm SE. (B) PC12, PC70, and PC47 cells were differentiated as in (A). They were treated with VPA (1mM) and assayed for cell death by trypan blue exclusion 1, 2, 3, and 5 days later. Dead cells (blue staining) were counted in 4 quadrants and the % dead cells was calculated and normalized to untreated cells. The results are expressed as % trypan blue⁺ cells \pm SE. (C) Primary rat cortex neurons were mock or VPA (1mM)-treated and assayed for cell death by trypan blue staining 3 days post-treatment. Dead and live cells were counted as in (B) and the % dead cells was calculated and normalized to untreated cells. (D) PC12 cells cultured and differentiated as in (A) were treated with VPA (1mM) alone or with z-VAD-fmk (50 μ M), PD150606 (PD, 100 μ M), SP600125 (SP, 100 μ M) or Nec-1 (Nec, 50 μ M) and assayed for cell death by trypan blue exclusion at 1,2,3,and 5 days post-treatment.

Dead and live cells were counted as in (B) and the % dead cells was calculated and normalized to untreated cells. The results are expressed as % inhibition \pm SE calculated from the formula: $100 - ((\% \text{ death with inhibitors} / \% \text{ death without inhibitors}) \times 100)$. Maximal inhibitory levels were reached on day 3 post-treatment as determined by two-way ANOVA. (E) Primary rat cortex neuronal cells were treated with VPA (1mM) alone or in the presence of z-VAD-fmk (50 μ M) or PD150606 (PD, 100 μ M) and assayed for cell death by trypan blue 3 days post-treatment. The results are expressed as % inhibition \pm SE, calculated as in (D). (*P<0.01; **P<0.001, by two-way ANOVA, n=3).

apoptosis seen in non-neuronal cells^{190,288} and protection from cell death seen in neurons²⁸⁹. Significantly, VPA-induced cell death was also seen in similarly treated human neuroblastoma SK-N-SH cells (Fig. 18A) as measured by propidium iodide staining (Fig. 18B) and in primary rat cortex neuronal cultures ($26.8 \pm 0.54\%$ and $1.8 \pm 0.27\%$ cell death for VPA as compared to mock-treated cultures) (Fig 17C). Collectively, the data indicate that VPA induced cell death is not a technical artifact resulting from the use of PC12 cells or a specific assay and it extends to human neuronal cell lines and primary neuronal cultures. However, VPA-treated cells were negative for TUNEL staining (Fig. 18C), suggesting that death is not caused by apoptosis.

C2.2 Activated survival pathways block VPA-induced neurotoxicity.

To confirm the specificity of the VPA neurotoxic effect, PC12 cells stably transfected with the herpes simplex virus type 2 protein ICP10PK (PC47 and PC70) that have constitutively activated PI3-K/Akt and MEK/ERK survival pathways and resist death induced by various toxic stimuli^{210,290-293}, were studied in parallel. PC47 and PC70 cells were differentiated by 4 days of culture with NGF as described for PC12 cells and differentiation was again confirmed by the formation of neurites and expression of the neuronal differentiation marker MAP-2. Differentiated PC47, PC70 and PC12 cells were

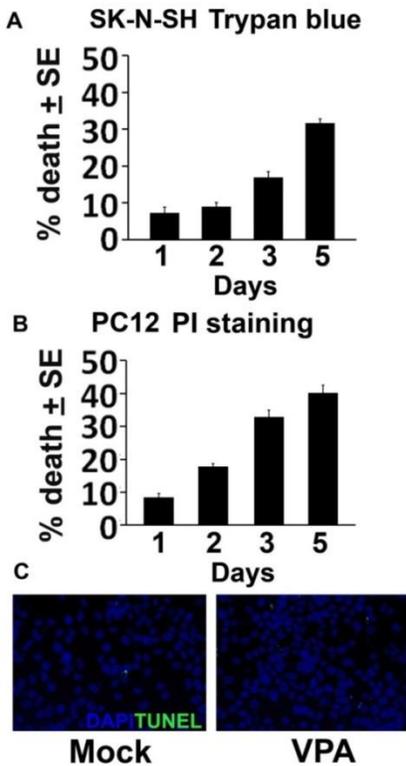


Figure 17: VPA induced cell death.

(A) Mock or VPA-treated SK-N-SH cells were assayed for cell death at 1,2,3, and 5 days post-treatment using trypan blue staining. Dead cells (identified by blue staining) and live cells were counted in four quadrants and the percentage of dead cells was calculated and normalized to untreated cells. The results are expressed as % death + cells \pm SE. VPA caused time-dependent cell death in SK-N-SH cells. (n=3) (B) PC12 cells were cultured on rat tail collagen-coated glass coverslips and neuronally differentiated for 4 days using NGF containing medium. They were treated with VPA (1mM) and assayed for cell death by propidium iodide (PI) staining at 1, 2, 3, and 5 days later. Dead cells (identified by red staining) and live cells were counted in four quadrants and the percentage of dead cells was calculated and normalized to untreated cells. The results are expressed as % death cells \pm SE. (n=3) (C) PC12 cells were cultured on rat tail collagen-coated glass coverslips and neuronally differentiated for 4 days using NGF containing medium. They were treated with VPA (1mM) and examined for apoptosis using TUNEL at 1, 2, 3, and 5 days post-treatment. Representative images from day 5 post-treatment are shown.

treated with VPA (1mM) and assayed for cell death by trypan blue staining at 1, 2, 3 and 5 days post treatment. Results are expressed as % dead cells normalized to the untreated cultures. VPA caused time-dependent cell death in PC12 cells that reached maximal

levels on days 3-5 post-treatment, but cell death was not seen in PC47 and PC70 cells (Fig. 17B), indicating that activated MEK/ERK and/or PI3-K/Akt survival pathways counteract the ability of VPA to induce cell death.

C2.3. Calpain, JNK, and necroptosis inhibitors block VPA-induced cell death.

Having seen that VPA-treated cultures are TUNEL negative, we wanted to better understand the mechanism of the VPA-induced cell death. Duplicate cultures of neuronally differentiated PC12 cells were treated with VPA (1mM) in the absence or presence of the pancaspase inhibitor z-VAD-fmk (100 μ M), the calpain inhibitor PD150606 (100 μ M), the JNK inhibitor SP600125 (10 μ M) or Necrostatin-1 (Nec-1; 50 μ M), a potent inhibitor of the RIP-1 kinase activity involved in necroptosis¹⁰⁵. They were examined for cell death by trypan blue staining at 1, 2, 3, and 5 days post-treatment and the results are expressed as % inhibition calculated as $100 - ((\% \text{ death with inhibitors} / \% \text{ death without inhibitors}) \times 100)$, normalized to untreated cultures. The data are summarized in Fig 17D. Consistent with the absence of TUNEL staining (Fig. 18), z-VAD-fmk did not interfere with the ability of VPA to induce cell death, suggesting that death is not caspase-dependent. This is also consistent with the absence of caspase activation as shown for caspases-3 and -8 in Fig. 19. However, cell death was inhibited in cultures that were given VPA together with PD150606, SP600125 or Nec-1, with maximal inhibitory levels seen on days 3 post-treatment. Maximal inhibition (relative to cultures given VPA alone) was seen for PD150606 [$63 \pm 0.97\%$ ($P= 0.003$) and $82.3 \pm 2.8\%$; ($P<0.001$) on days 2 and 3 post-treatment, respectively] followed by Nec-1 [$50 \pm 1.1\%$ ($P=0.014$) and $69.6 \pm 4.5\%$ ($P\leq 0.001$) on days 2 and 3 post-treatment,

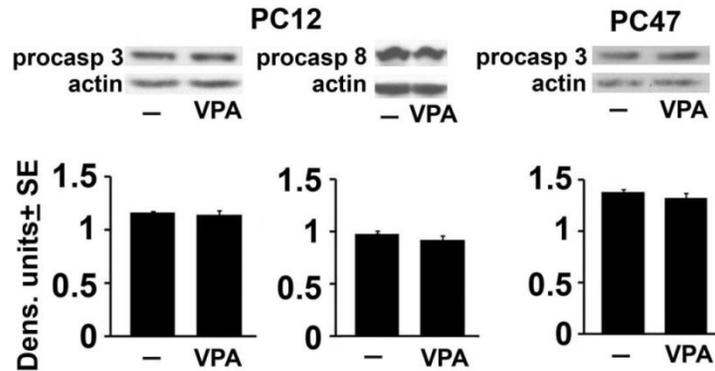


Figure 18: VPA does not activate caspases.

Neuronally differentiated PC12 and PC47 cells were either mock treated or treated with VPA (1mM). Cell extracts obtained 48 hrs post treatment were immunoblotted with antibodies to procaspase 8 or procaspase 3, the levels of which decrease upon activation. Membranes were stripped and re-probed for actin. Data were quantified using densitometric scanning and are expressed as densitometric units \pm SE. (n=3)

respectively]. The inhibitory levels seen for SP600125 were similar on days 2 and 3 post-treatment ($41.5 \pm 3.1\%$; $P < 0.01$), but inhibition was virtually absent at 5 days post-treatment. Collectively, the data indicate that VPA-induced cell death is through calpain and JNK activation and includes Nec-1 inhibitable necroptosis. We conclude that the VPA-induced cell death mechanism observed in PC12 cells is applicable to primary neuronal cultures, because PD150696, but not z-VAD-fmk, was also able to significantly ($P < 0.001$) inhibit cell death in VPA-treated primary neurons ($53.1 \pm 2.3\%$ inhibition) (Fig. 17E).

C2.4. Calpain is activated in VPA-treated PC12, but not PC47 cells.

Having seen that the VPA-induced death of neuronally differentiated PC12 cells is significantly reduced by treatment with PD150606, we wanted to confirm that VPA causes calpain activation. Protein extracts from neuronally differentiated PC12 and PC47

cells treated or not with VPA (1mM; 48hrs) were immunoblotted with antibody to the p28 calpain regulatory subunit, the disappearance of which is indicative of activation⁹⁵. Cells similarly treated with VPA in the absence or presence of PD150606 (100μM), Nec-1 (50μM) or SP600125 (10μM) were studied in parallel and the blots were stripped and re-probed with GAPDH antibody used as loading control. The expression levels were determined by densitometric scanning, as described in Materials and Methods and the results are summarized in Fig. 20. p28 was expressed in the mock-treated PC12 cells, but expression was virtually lost upon VPA treatment, indicative of calpain activation. Consistent with this interpretation, the levels of p28 in PC12 cells given VPA together with PD150606 were restored to those seen in the mock-treated cells, but this was not the case in cells given VPA together with SP600125 or Nec-1. The levels of p28 were not reduced in VPA-treated PC47 cells, indicating that VPA does not activate calpain in these cells and confirming that its ability to induce cell death is through calpain activation.

C2.5. Calpain-mediated JNK1 activation contributes to VPA-induced cell death.

JNKs have received considerable attention in the context of apoptosis-related neurodegeneration²⁹⁴, but their contribution to VPA-induced neurotoxicity, if any, is unclear. Following on the finding that SP600125 inhibits VPA-induced cell death in neuronally differentiated PC12 cells (Fig. 17), we wanted to better understand the role of JNK activation in the death process. Moreover, because PC12 cells do not express the JNK3 isoform²⁹⁵ that had been previously associated with apoptosis in neurons²⁹⁶, our system provides a unique opportunity to verify the differential, context-specific role of the JNK1/2 isoforms in neuronal cell death. Neuronally differentiated PC12 and PC47

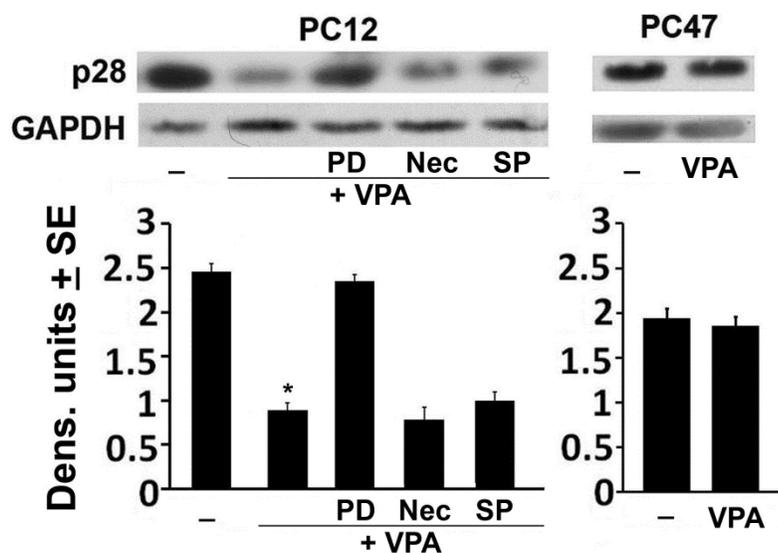


Figure 19: Calpain is activated in VPA-treated PC12, but not PC47 cells.

Neuronally differentiated PC12 and PC47 cells were mock- or VPA (1mM)-treated alone or in combination with PD, SP or Nec-1. Cell extracts obtained 48hrs post treatment were immunoblotted with antibody to the calpain p28 regulatory subunit. The blots were stripped and re-probed with antibody to GAPDH. Data were quantified by densitometric scanning and are expressed as densitometric units \pm SE. (*P<0.001, by two-way ANOVA, n=3).

cells were mock-treated or treated with VPA (1mM; 48hrs) and protein extracts were immunoblotted with an antibody that recognizes the phosphorylated (activated) JNK 1 and 2 isoforms (pJNK1 and pJNK2, respectively). The blots were stripped and re-probed with antibody to total JNK, used as control. Mock-treated PC12 cells had minimal levels of pJNK1 and pJNK2. VPA caused a significant increase in the levels of pJNK1, but not pJNK2, indicating that it has an isoform-specific activity. This activity is calpain-dependent, because the levels of pJNK1 were not increased in cells given VPA together with PD150606 (100 μ M), and the levels of pJNK1 in PC12 cells given VPA together with Nec-1 (50 μ M) were similar to those in cells given VPA alone. VPA did not increase pJNK1 levels in PC47 cells that resist cell death (Fig. 20A). Collectively, the data indicate that: (i) VPA specifically activates JNK1, (ii) JNK1 activation is calpain-

dependent and upstream of Nec-1 inhibitable necroptosis and (iii) JNK1 activation is associated with VPA-induced cell death.

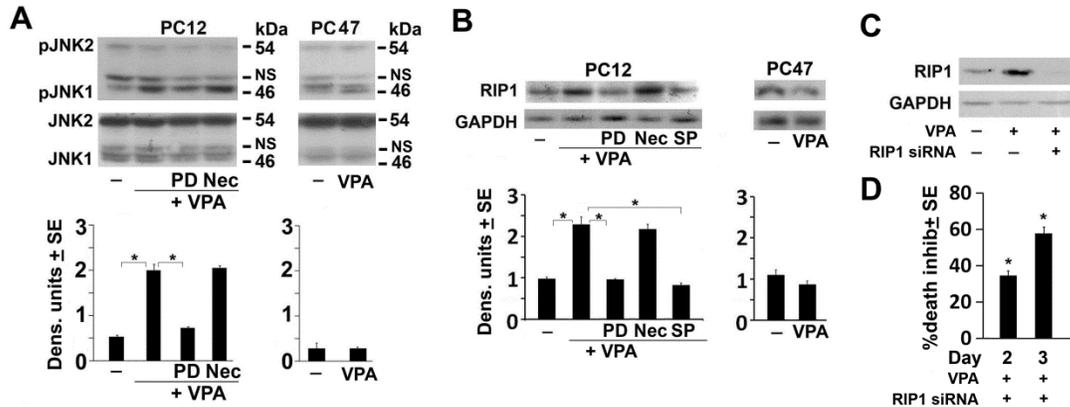


Figure 20: VPA activates JNK1, leading to increased RIP-1 expression.

(A) Neuronally differentiated PC12 and PC47 cells were mock- or VPA (1mM)-treated alone or in combination with PD or Nec as in Fig. 1B. Cell extracts obtained at 48hrs post treatment were immunoblotted with antibody to phosphorylated JNK1 (pJNK1, p46) and JNK2 (pJNK2, p54) and the blots were stripped and re-probed with antibody to total JNK1/2 or GAPDH. Data were quantified by densitometric scanning and the results are expressed as densitometric units \pm SE. NS = non-specific. (B) Duplicates of the PC12 and PC47 cultures were mock or VPA-treated alone or together with PD, SP or Nec as in Fig. 1B, and cell extracts collected at 48hrs post-treatment were immunoblotted with RIP-1 antibody. Blots were stripped and re-probed with antibody to GAPDH. (C) PC12 cells were differentiated and transfected with vehicle or RIP-1 siRNA as described in MM. Two days later, the cells were mock or VPA treated and cell extracts collected 2 days later were immunoblotted with RIP-1 antibody. The blots were stripped and re-probed with antibody to GAPDH. (D) Duplicates of similarly treated PC12 cultures were assayed for cell death using trypan blue staining at 2 and 3 days post VPA treatment. Dead cells (blue staining) were counted in 4 quadrants and the % was calculated and normalized to untreated cells. The results are expressed as % inhibition \pm SE calculated as in Fig 15D. (* $P < 0.001$ by two-way ANOVA, $n=3$).

C2.6. VPA induces RIP-1 expression through calpain/JNK1 activation.

Cell death activated when caspase-dependent apoptotic pathways are inhibited is also known as “necroptosis”. Recent studies have shown that necroptosis requires the serine-threonine kinases RIP-1 and RIP-3, and results from overproduction of reactive oxygen

species (ROS) and eventual mitochondrial dysfunction²⁹⁷⁻²⁹⁹. Nec-1 is a selective inhibitor of RIP1 kinase activity¹⁰⁵ and thereby, necroptosis.

Having seen that Nec-1 inhibits VPA-induced cell death in PC12 cultures, we wanted to better understand the relationship between the VPA-induced cell death and RIP-1. Neuronally differentiated PC12 cells were mock-treated or treated with VPA (1mM; 48 hrs) alone or together with PD150606 (100 μ M), SP600125 (10 μ M), or Nec-1 (50 μ M), and protein extracts were immunoblotted with RIP-1 antibody. The blots were stripped and re-probed with antibody to GAPDH used as loading control. The levels of RIP-1 were increased in the VPA-treated as compared to untreated PC12 cells, but increased expression was not seen in cell given VPA together with PD150606 or SP600125 (Fig. 20B). The levels of RIP-1 were not altered in PC12 cells given VPA together with Nec-1, which inhibits the RIP-1 kinase activity^{105,300} and VPA did not alter RIP-1 expression in PC47 cells (Fig. 21B) that resist VPA-induced cell death. Similar results were obtained at 72 hrs post treatment. The data indicate that VPA induces RIP-1 expression through a calpain/JNK1 pathway that is activated at 2-3 days post-treatment, and this increased expression contributes to the VPA-induced cell death.

While we did not examine the effect of VPA on RIP-1/RIP-3 complexation, our data confirm that inhibition of RIP-1 expression (and thereby kinase activity) causes a significant decrease in VPA-induced cell death. Specifically, neuronally differentiated PC12 cells were treated (2 days) with a RIP-1 siRNA-Lipofectamine complex, given VPA (1mM) and examined for cell death by trypan blue staining at 2 and 3 days later. Cell death was inhibited when VPA was given together with RIP-1 siRNA [34.6 ± 2.4 % and 57.8 ± 3.3 % inhibition on days 2 and 3, respectively ($P < 0.001$)] (Fig. 21C) and these

levels are similar to those seen for Nec-1 inhibition (Fig. 17C). The effect of the RIP-1 siRNA on cell death was due to RIP-1 knockdown, as confirmed by immunoblotting of cell extracts from duplicate cultures given VPA+ siRNA for 2 or 3-days (Fig 21D).

C2.7. VPA induces calpain-dependent AIF cleavage and nuclear translocation.

Mitochondrial release of several pro-apoptotic molecules is a determining factor for inducing caspase-dependent and independent cell death³⁰¹. We focused on apoptosis inducing factor (AIF) because it is cleaved by calpain to a 57kDa product (tAIF), which is released from the mitochondria⁹⁰ and translocates to the nucleus where it triggers DNA degradation³⁰² and provokes necroptosis³⁰³. Neuronally differentiated PC12 and PC47 cells were mock-treated or treated with VPA (1mM) alone or together with PD150606 (100 μ M). Total cell extracts were obtained at 72 hrs post-treatment and cytoplasmic and nuclear fractions were generated as described in Materials and Methods. They were immunoblotted with AIF antibody and the blots were stripped and re-probed with actin antibody used as loading control. The total cell extracts from the mock-treated PC12 cells had relatively low levels of a 57 kDa band that is consistent with tAIF⁹⁰, but the levels were significantly higher in the VPA-treated PC12 cells. The levels of tAIF in PC12 cells that received VPA together with PD150606 were similar to those in the untreated cells (Fig. 22A), indicating that VPA induces calpain-dependent AIF cleavage. Immunoblotting of the cytoplasmic and nuclear fractions from the untreated and VPA-treated PC12 cells confirmed that VPA causes a significant increase in the levels of tAIF and it translocates to the nucleus (Fig. 22B). Similar analysis of the cytoplasmic and nuclear fractions from the untreated and VPA-treated PC47 cells indicated that the levels

of tAIF were minimal in both fractions and they were not increased by treatment with VPA (Fig. 22B). It should be pointed out that tAIF was not seen in PC12 cells examined at 2 days post treatment with VPA, suggesting that AIF cleavage is a relatively delayed VPA-induced process (initiates on day 3 post-treatment).

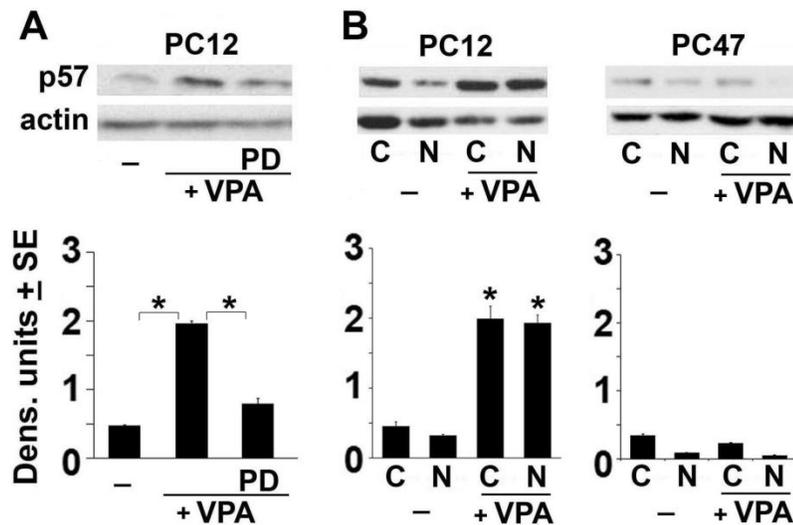


Figure 21 VPA induces AIF cleavage to a 57 kDa (tAIF) band and promotes its nuclear translocation.

(A) Neuronally differentiated PC12 cells were differentiated and mock- or VPA (1mM)-treated alone or in combination with PD as in Fig. 1B and whole cell extracts collected 72 hrs post-treatment were immunoblotted with AIF antibody. Data were quantified using densitometric scanning and are expressed as densitometric units ± SE. The levels of the 57kDa (tAIF) band were increased by VPA treatment and restored to those in mock-treated cells by PD treatment. (B) Neuronally differentiated PC12 and PC47 cells were mock- or VPA-treated as in Fig. 1 and cell extracts obtained 72 hrs post treatment were fractionated into nuclear (N) and cytoplasmic (C) fractions. The fractions were immunoblotted with AIF antibody and the blots were stripped and re-probed with antibody to β-actin (*P<0.001 by one-way ANOVA, n=3).

To further confirm the contribution of tAIF mitochondrial release and nuclear translocation to VPA-induced cell death, neuronally differentiated PC12 cells mock-treated or treated with VPA (1mM, 72hrs) alone or together with PD150606 (100 μM) were stained in double immunofluorescence with Alexa Fluor 488-labeled AIF antibody and the mitochondrial selective dye MitoTracker Red 580 in order to confirm its release

from the mitochondria and its nuclear translocation. The cells with nuclear staining were counted in 4 randomly selected fields (at least 250 cells) and the results are expressed as % cells with nuclear staining calculated relative to the total cell number determined by DAPI staining. AIF co-localized almost entirely with the mitochondrial stain in mock-treated cells, nuclear staining was seen in 19 ± 2.5 % of the cells in the VPA-treated cultures. PC12 cells given VPA together with PD150606 primarily evidenced AIF/mitochondrial co-localization confirming that calpain cleaves AIF, leading to its mitochondrial release and nuclear translocation (Fig. 23A). tAIF nuclear translocation was not seen in the untreated or VPA-treated PC47 cells.

C2.8. VPA treatment induces H2AX phosphorylation.

H2AX, a member of the histone H2A family has an SQE motif in its C-terminal tail that is phosphorylated at Ser139 (γ H2AX) and is associated with the generation of DNA double-strand breaks. Once in the cytosol, tAIF translocates to the nucleus where, in cooperation with γ H2AX, it provides the lethal DNA-degrading activity characteristic of AIF-mediated necroptosis^{112,304}. Having seen that VPA-induces AIF cleavage and nuclear translocation, we wanted to know whether this is accompanied by increased levels of γ H2AX. Duplicate PC12 cultures untreated or treated with VPA (1mM) were stained with γ H2AX antibody at 3 days post-treatment and the % γ H2AX staining cells was calculated as described for tAIF. Staining was seen in 21.1 ± 1.2 % of the VPA-treated cells at 3 days post-treatment, a significant increase over the minimal % of staining cells in the untreated cultures ($p < 0.01$) (Fig. 23B). γ H2AX staining was not seen in the untreated and VPA-treated PC47 cells (not shown). Significantly, γ H2AX staining

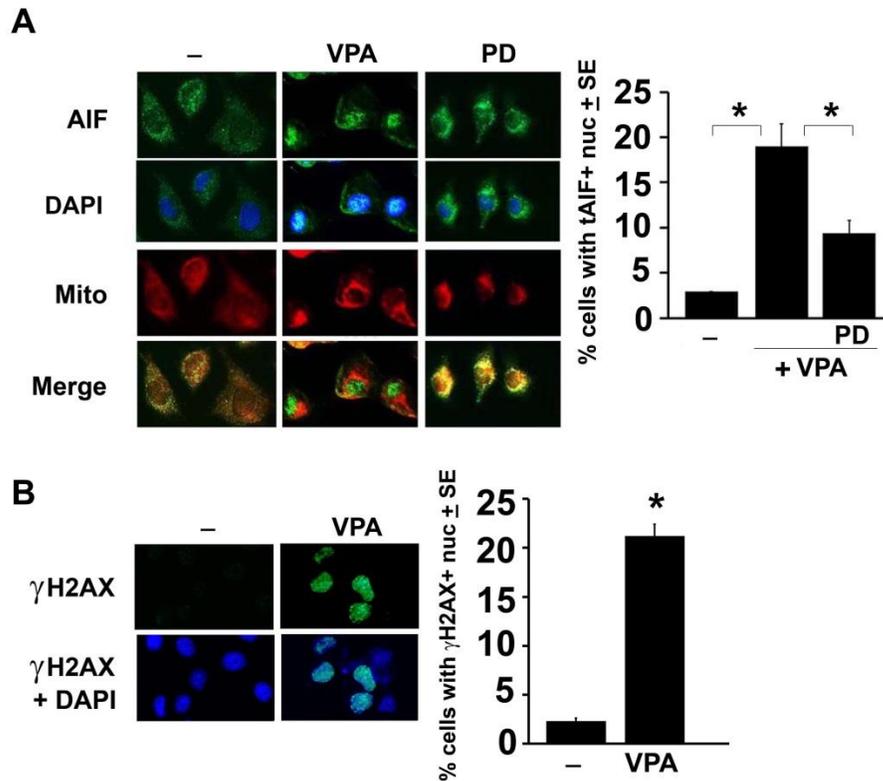


Figure 22: VPA induces tAIF mitochondrial release and increases levels of γ H2AX.

(A) Neuronally differentiated PC12 cells were mock- or VPA-treated alone or with PD as described in Fig. 1B. Coverslips obtained at 72hrs post-treatment were incubated with MitoTracker Red 580 and stained with AIF antibody (green), as described in MM. Cells with AIF nuclear staining were counted in 4 randomly chosen fields and the % staining cells was calculated relative to total cell numbers, identified by DAPI staining. Results are expressed as % cells with tAIF+ nuclear staining \pm SE. VPA caused a significant increase in the number of cells with AIF nuclear localization, inhibited by PD (* $P \leq 0.01$ by two-way ANOVA, $n=3$). (B) Duplicates of the mock- and VPA-treated PC12 cultures were stained with antibody to γ H2AX (green) and DAPI (blue) used to visualize the nuclei. Cells with γ H2AX nuclear staining were counted and the % calculated relative to total cell numbers as in (A). The results are expressed as % cells with γ H2AX + nuclear staining \pm SE. (* $P < 0.01$ by one-way ANOVA, $n=3$).

was not increased in cells given VPA for only 2 days, consistent with the interpretation that that γ H2AX upregulation, like AIF cleavage/nuclear translocation, is a relatively delayed VPA-induced death process (initiates on day 3 post-treatment). While we did not directly examine the interaction between nuclear tAIF and γ H2AX, the data indicate that

VPA induces H2AX phosphorylation, likely contributing to necroptosis through tAIF interaction^{112,304}.

C2.9. VPA alters the Smac/DIABLO vs XIAP balance.

Having seen that VPA treatment induces AIF cleavage and mitochondrial release, we wanted to know whether it is also associated with the release of other mitochondrial intermembrane proteins that modulate PCD. We focused on Smac/DIABLO, which inhibits the Inhibitors of apoptosis (IAP) proteins³⁰⁵ because its altered balance relative to the IAP proteins cIAP1 and cIAP2 induces necroptosis^{108,109}. Extracts from neuronally differentiated PC12 cells untreated or treated with VPA (1mM, 72hrs) in the absence or presence of PD150606 (100 μ M) were immunoblotted with Smac/DIABLO antibody and the blots were sequentially stripped and re-probed with antibodies to X-linked IAP (XIAP) or GAPDH (loading control). A significant increase in the levels of Smac/DIABLO was seen in the VPA-treated cells and it was accompanied by a marked decrease in the levels of XIAP (Fig 24A). PD150606 restored the Smac/DIABLO and XIAP levels to those seen in the untreated cells (Fig. 24B) and a similar effect was not seen in PC47 cells. While additional studies are needed in order to fully document the role played by Smac/DIABLO in VPA-induced cell death, our data indicate that VPA causes a calpain-dependent alteration in the Smac/DIABLO vs XIAP balance that is likely to contribute to cell death through the promotion of necroptosis.

C3: Discussion

VPA is a HDAC inhibitor, the primary indication of which is for the treatment of epilepsy and mood disorders^{178,179}, apparently due to its ability to enhance GABAergic

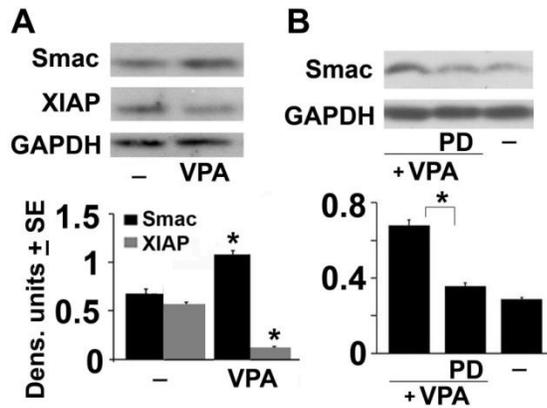


Figure 23: VPA increases Smac/DIABLO and decreases XIAP.

(A) Neuronally differentiated PC12 cells were mock- or VPA-treated as in Fig. 1 and cell extracts obtained 72 hrs post-treatment were immunoblotted with antibody to Smac/DIABLO. The blots were stripped and re-probed with antibody to XIAP followed by GAPDH. Data were quantified using densitometric scanning and are expressed as densitometric units \pm SE. (B) Duplicate cultures of PC12 cells were mock or VPA-treated alone or together with PD as in Fig. 1B and cells extracts were immunoblotted with Smac/DIABLO antibody. The blot was stripped and re-probed with antibody to GAPDH and the data were quantified by densitometric scanning. Results are expressed as densitometric units \pm SE. (* $P < 0.001$ by one or two-way ANOVA, $n = 3$).

neurotransmission through inhibition of the GABA degradative enzyme, GABA transaminase, modulate brain metabolism, decrease excitability, and affect voltage-gated sodium, potassium and calcium channels^{201,306}. While VPA was reported to have neuroprotective activity in some models of CNS injury and neurodegenerative disorders¹⁸³⁻¹⁸⁵, a growing body of evidence indicates that it causes HDAC-related and -unrelated neuronal cell death^{186,187}. Indeed, VPA was shown to exacerbate the death of cerebellar granule neurons¹⁸⁸, reduce the proliferation of hippocampal neurons resulting in cognitive impairments¹⁸⁹, and induce apoptosis in various neuronal cell populations^{186,190}. Rats and mice exposed to VPA *in utero* or shortly after birth present with behavioral and structural abnormalities similar to those observed in humans with ASD^{197,198}. In humans, VPA administration during pregnancy increases the incidence of

autism in the born children¹⁹² associated with widespread brain apoptosis^{194,198,200}. VPA was also shown to promote caspase-independent neuronal cell death albeit, by an as yet poorly understood mechanism²⁰². We report, for the first time, that VPA activates a previously unrecognized calpain-dependent necroptosis cascade that initiates with the activation of JNK1/RIP-1 signaling and is followed by AIF cleavage/nuclear translocation and H2AX phosphorylation as well as an altered Smac/DIABLO to XIAP balance, as schematically represented in Fig. 25. The following comments seem pertinent with respect to these findings.

Caspases are universally recognized as the main players in apoptosis^{307,308}. However, it is becoming increasingly evident that death can also be caused by other mechanisms, the relationship of which to apoptosis is still poorly understood. RIP-1, for example, is a core component of the cell death-inducing platform known as the ripoptosome, which has a critical role in regulating the switch from caspase-dependent apoptosis to necroptosis. RIP-1 is cleaved by activated caspase-8, thereby directing the cell to undergo apoptosis, but in the absence of caspase activation, RIP-1 can complex with and phosphorylate RIP-3 to initiate necroptosis. Calpains are Ca²⁺-dependent cysteine proteases that can also be activated by apoptotic stimuli resulting in the cleavage of multiple targets and the mitochondrial release of death-inducing proteins³⁰⁹. One of these is the calpain-cleaved AIF protein (tAIF) that translocates to the nucleus and in cooperation with γ H2AX, provokes DNA degradation and necroptosis^{112,304,310,311}.

Another one of the death-inducing proteins that are released from the mitochondria as a result of calpain activation is Smac/DIABLO that inhibits the anti-apoptotic cIAP proteins, thereby promoting necroptosis^{108,109}.

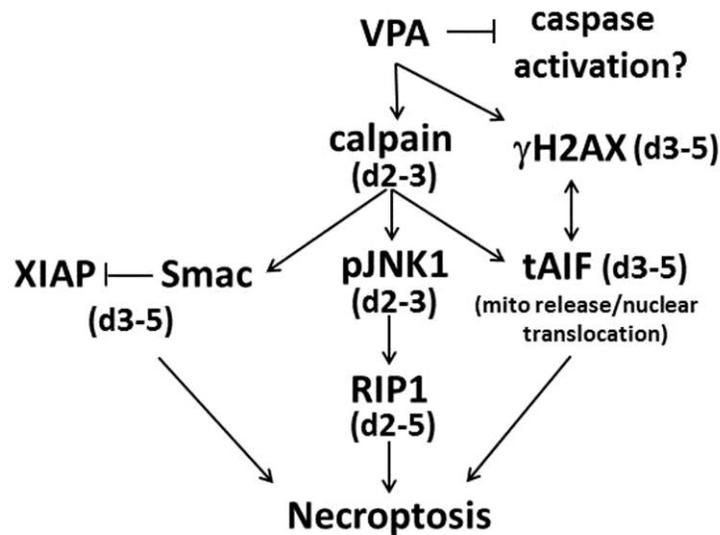


Figure 24: Schematic representation of VPA-induced neuronal cell death.

Our data confirm a VPA-induced necroptotic pathway that initiates with calpain activation and is accompanied by calpain-dependent activation of JNK1, which is responsible for increased RIP-1 expression. Calpain induces Smac/DIABLO expression as well as cleavage and nuclear translocation of AIF. VPA increases nuclear γ H2AX, which can complex with tAIF to promote chromatinolysis and necroptotic cell death. Smac/DIABLO increase is accompanied by reduced expression of XIAP, further contributing to necroptosis. These pathways are not activated in PC47 cells that have constitutively activated survival pathways. D= days post VPA-treatment.

We used neuronally differentiated PC12 cells, which are an established model of neuronal cell life/death choices to examine whether VPA causes cell death and define the mechanism responsible for neurotoxicity. PC12 cells modified to resist death-inducing stimuli through constitutive activation of the PI-3K/Akt and MEK/ERK survival pathways (PC47 and PC70) provide a well-defined cell culture system for the verification of neurotoxic mechanisms, and were studied in parallel. Neuronal differentiation was by exposure to NGF and it was confirmed by neurite formation and expression of the differentiation marker MAP-2. As schematically represented in Fig. 25, we found that VPA induced a time-dependent cascade of death signals the outcome of which was maximal levels of cell death on days 3-5 post-treatment. This was determined by different

assays including ethidium homodimer, trypan blue and propidium iodide staining and involved a cascade of death-inducing signals. However, TUNEL staining was negative (Fig. 18), caspases were not activated (Fig. 19) and the pancaspase inhibitor z-VAD-fmk did not inhibit cell death, indicating that death is not due to caspase-dependent apoptosis. By contrast, cell death was inhibited by the calpain inhibitor PD150606 and similar results were obtained in primary neurons in which cell death was also inhibited by the calpain but not pancaspase inhibitor. Significantly cell death was also inhibited by the JNK inhibitor SP600125, and immunoblotting experiments confirmed that VPA induces calpain and JNK1 activation, as respectively measured by loss of the p28 calpain regulatory subunit and a significant increase in the levels of phosphorylated JNK1 (pJNK1) relative to those in untreated cells. Both the loss of p28 and the increase in pJNK1 were seen at 2 days of VPA treatment, indicating that they are relatively early events in the VPA-induced cell death and they were not seen in PC47 cells that do not die in response to VPA treatment. Significantly, the levels of pJNK1 were not increased in cells given VPA together with the calpain inhibitor PD150606 and VPA did not increase the levels of pJNK2, indicating that VPA specifically activates JNK1 in a calpain-dependent mechanism and underscoring the differential contribution of the two JNK isoforms. While the JNK isoforms were previously reported to have distinct signaling pathways in neuronal cells^{312,313} and a different impact on behavioral parameters³¹⁴, the ability of calpain to specifically activate JNK1 thereby leading to cell death identifies a novel death regulatory process in neurons. JNK activation had previously been shown to occur upstream of calpain activation³¹⁵ or parallel to calpain activation during necrosis³¹⁶. The exact mechanism responsible for calpain-mediated JNK activation is still unclear.

Calpain could cleave JNK inhibitory proteins, such as JNK-interacting protein-1 (JIP-1), a scaffold protein and specific inhibitor of JNK, thereby inducing JNK activation. In addition, calpain activation can induce intracellular calcium overload³¹⁷ and it, in turn, can cause JNK phosphorylation³¹⁵. Indeed, calpains were previously shown to play a central role in the conversion of Ca^{+2} signals from the stressed endoplasmic reticulum to JNK activation³¹⁸. The VPA-induced calpain/JNK1 death pathway is not an artifact of the VPA dose, because at the same dose VPA caused caspase activation in non-neuronal cells^{190,288,319}. Although the exact mechanism of VPA-induced calpain activation is unclear, HDAC inhibition has previously been reported to cause calpain activation through a caspase 3 dependent mechanism³²⁰. However, since VPA-induced cell death in our model occurs independent of caspase activation, calpain may be activated through alternative HDACi dependent or independent effects.

The relationship between JNK activation and RIP-1, if any, is still unknown. We found increased expression of RIP-1, in the treated relative to untreated PC12 cultures as early as 2 days post-treatment. RIP-1 upregulation was inhibited by SP600125 indicating that it is JNK1-dependent. This is likely mediated by the activation of the AP-1 transcription factor, which regulates RIP-1 transcription³²¹. Consistent with this interpretation, Nec-1 did not inhibit the ability of VPA to upregulate RIP-1 expression. We do not believe that death involves the specific activation of TNF/Fas/CD40/CD30 receptor family members³²² that include the NGF-activated p75NGFR receptor in PC12 cells³²³ because the calpain/JNK1/RIP-1 pathway was also associated with cell death in the VPA-treated human neuroblastoma SK-N-SH cells. However, we conclude that increased RIP-1 expression is involved in the VPA-induced cell death, because: (i)

expression was blocked by the calpain inhibitor PD150606, which inhibits cell death, (ii) RIP-1 expression was not seen in PC47 cells that resist VPA-induced cell death, (iii) and knockdown of RIP-1 with specific siRNA inhibited cell death. Indeed, both the RIP-1 siRNA and Nec-1 inhibited cell death beginning on day 2 post-treatment and inhibition reached maximal levels one day later (day 3 post-treatment), likely reflecting the ability of Nec-1 to specifically inhibit RIP-1 kinase activity. Since RIP-1 is a caspase-8 substrate¹¹⁰, its increased levels in the VPA-treated PC12 cells are likely favored by the failure to induce caspase activation, potentially indicative of cell type and context specific effects of VPA-induced neurotoxicity.

Interestingly, beginning on day 3 post-treatment, the VPA-treated PC12 cells also evidenced calpain-dependent AIF cleavage and mitochondrial release of tAIF, with increased tAIF levels seen in both the cytoplasm and nuclear fractions relative to untreated cells. This increase was due to calpain-mediated mitochondrial release, as confirmed by staining with the mitochondrial selective dye MitoTracker Red 580, and it was blocked by treatment with the calpain inhibitor PD150606. Significantly, the tAIF nuclear translocation coincided temporally with VPA-induced γ H2AX increase, the association of which with tAIF is known to create a DNA-degrading complex that provokes chromatinolysis and cell death by necroptosis^{304,324,325}. AIF cleavage/nuclear translocation and γ H2AX increase were not seen in PC47 cells in which VPA does not induce cell death, but the mechanism of γ H2AX increase is still unclear and its interaction with tAIF remains to be documented. Consistent with the observed VPA-induced mitochondrial release of tAIF, VPA also increased the levels of Smac/DIABLO, another death associated mitochondrial protein that is released by activated calpain. This

was accompanied by the inhibition of the anti-apoptotic IAP protein XIAP, an altered balance associated with necroptosis, as previously reported for Smac mimetics^{108,109}. However, additional studies are needed in order to confirm the role of the Smac/DIABLO/XIAP balance in VPA-induced cell death.

The exact role of the activated survival pathways in inhibiting VPA-induced necroptosis is still unknown, but activated ERK and Akt were previously shown to play pivotal role in VPA-mediated neuroprotection¹⁸⁵. Additional studies designed to better understand the mechanism of VPA-induced H2AX phosphorylation, define the mechanisms responsible for the JNK1-mediated RIP-1 expression and the role of the Smac/DIABLO/XIAP balance in VPA-induced cell death are needed.

D. The histone deacetylase inhibitor valproic acid enhances the oncolytic activity of Δ PK in melanoma

D1: Introduction

Oncolytic viruses (OVs) are multi-functional cancer therapeutics that can be engineered to suit many different strategies. Genetic modifications can facilitate increased capacity for direct tumor killing and assist in the development of antitumor immune responses. However, administration of these modified OVs alone, has yet to induce successful regression of established tumors^{206,209}, potentially attributed to premature viral clearance by the host immune system, the presence of tumor-forming cancer stem cells, and the ability of tumors to adapt to therapeutic selective pressure. To overcome these barriers, many clinically established and novel chemotherapeutics have been used in combination with oncolytic virotherapy, showing synergistic effects that potentiate tumor killing^{326,327}. HDACis have been proposed as a new type of antitumor agent because they can induce cell cycle arrest and apoptosis in tumor cells and have anti-inflammatory properties that can suppress initial host response against the virus thereby allowing for more robust replication^{328,329}. Indeed, HDACi have been shown to enhance OV efficacy through initial suppression of immune cell recruitment, inhibition of inflammatory cell pathways within NK cells³³⁰, and inhibition of the type I IFN response, major components of the cellular innate antiviral response¹⁶⁸, identifying them as particularly attractive candidates for OV combination therapy.

Δ PK is an HSV-2 mutant deleted in the protein kinase domain of R1 (ICP10PK) which activates the Ras signaling pathway. ICP10PK is required for virus growth in normal

cells and its deletion confers tumor selectivity while keeping the R1 domain required for virus DNA replication intact. We have previously shown that Δ PK has strong oncolytic activity in melanoma cultures and xenografts through, in addition to virus replication, the activation of multiple PCD pathways that include apoptosis and autophagy¹⁴⁸. Studies done in collaboration with my colleague, Dr. Aric Colunga, also showed that Δ PK-lyses CSC-enriched cultures²¹⁶, an area of interest I pursued with respect to the mechanism of virus-induced lysis of the 3D CSC-enriched, cultures (Chapter 4A). Finally, my studies established that Δ PK induces multiple features of immunogenic cell death (Chapter 4B). Valproic acid (VPA), a HDACi used for the treatment of epilepsy and mood disorders, is currently in cancer therapy clinical trials both as a single agent and in combination therapy and has been shown to enhance the efficacy of an oHSV-1 in glioblastoma¹⁷⁷ as well as an oncolytic adenovirus³³¹. We showed that VPA induces the death of neuronal cells through a calpain-dependent necroptosis cascade³³². Because (i) Δ PK induces PCD through the activation of calpain and caspases -3 and -7 in melanoma, (ii) VPA induces a caspase-independent necroptotic cell death in neuronal cells, and (iii) melanoma is of neuronal lineage, we reasoned that VPA might increase the ability of Δ PK to kill melanoma cells by extending the cell death inducing mechanisms to include necroptosis. Although HDACis also have reported immunomodulatory effects³³³ that have to be considered for OV combination therapy, this preliminary *in vitro* study sought to first establish if, and by what mechanism, VPA can induce cytotoxicity in response to Δ PK infection. We report that co-treating Δ PK-infected melanoma cultures with VPA indeed results in a significant increase in cell death, however, it is related to cell-type specific

enhancement of caspase activation rather than the induction of the necroptotic pathway seen in neuronal cells.

D2: Results

D2.1 Valproic acid induces caspase-dependent cell death in melanoma

To determine whether VPA induces melanoma cell death, A2058 and A375 cultures were treated with 30 mM VPA, a dose previously established in *in vitro* cancer cultures¹⁷⁷, then assayed for cell death by trypan blue staining 24 and 48 hours later. To determine the mechanism of VPA-induced cell death, parallel cultures were treated with either the pancaspase inhibitor z-VAD-fmk (100 μ M), the calpain inhibitor PD150606 (100 μ M), or the necroptosis inhibitor necrostatin-1 (150 μ M). These inhibitors were selected based on reports that VPA can induce cell death through both caspase-dependent and independent mechanisms^{202,288} and our findings for VPA in neuronal cells³³². After 24h and 48 hours of treatment, cells were collected assayed for cell death using trypan blue. For A2058 cells, VPA induced cell death was $16.4 \pm 1.7\%$ at 24hr and it increased to $85.7 \pm 2.6\%$ at 48 hrs. For A375 cultures, VPA induced cell death was $14.3 \pm 2.4\%$ death at 24hrs which increased to $91.4 \pm 0.89\%$ at 48hrs. For both cell types, death was significantly inhibited at each time point by the addition of z-VAD-fmk, indicating that VPA-induced cell death in melanoma is caspase dependent and does not involve the activation of calpain or necroptosis (Fig 26).

D2.2 Combination of VPA and Δ PK enhances oncolysis

To determine whether VPA can increase the oncolytic activity of Δ PK, which is relatively low early in infection (24hrs) (Fig 14A), A2058 and A375 cultures were either

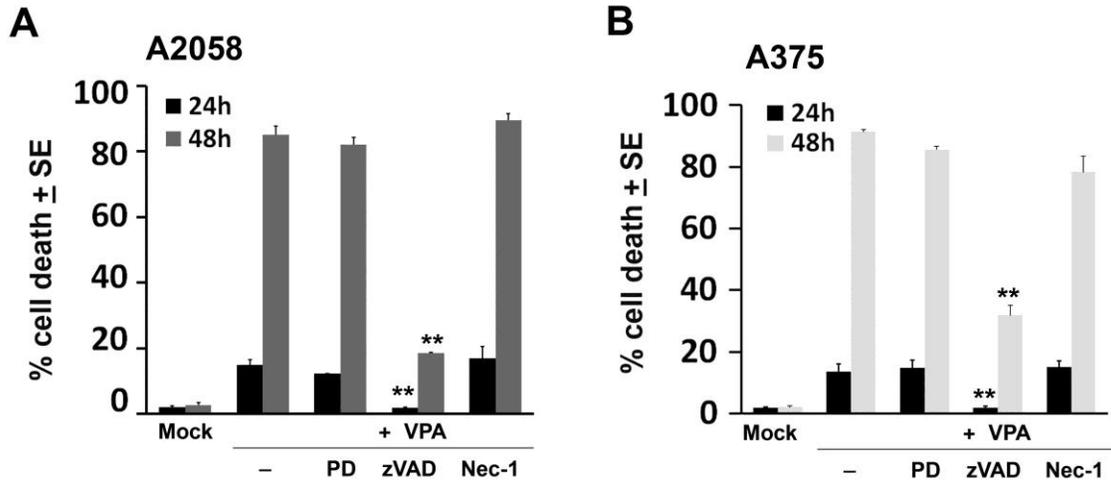


Figure 25: VPA-induced death is caspase dependent in melanoma

A2058 (A) and A375 (B) cultures were either mock or VPA-treated (30mM) in the absence or presence of the calpain inhibitor PD150606 (100μM), the pan caspase inhibitor zVAD-fmk (100μM), or the necroptosis inhibitor necrostatin-1(150μM). Cell were collected 24 and 48 hours after treatment and assayed for cell death using trypan blue staining. Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death ± SE. (**P<0.001 by two way ANOVA, n=3)

mock or ΔPK (moi=1)-infected in the absence or presence of VPA (30mM) and cells collected at 24h pi were assayed for cell death using trypan blue staining. ΔPK alone caused a 30.8 ± 1.1% and 18.9 ± 1.6% cell death in A2058 and A375, respectively. With VPA co-treatment, however, ΔPK induced cell death significantly increased to 50.3 ± 0.7% and 45.5 ± 2.1% in A2058 and A375, respectively (Fig 27, left panels).

To determine the mechanism of enhanced cell death, parallel cultures given both VPA and ΔPK were treated with z-VAD-fmk, PD150606, or necrostatin 1 and assayed for cell death at 24h. Cell death in both A2058 and A375 was inhibited most significantly through caspase inhibition (Fig 26, right panels), consistent with previous findings that ΔPK induces caspase activation¹⁴⁸, as well as our results demonstrating that VPA cell

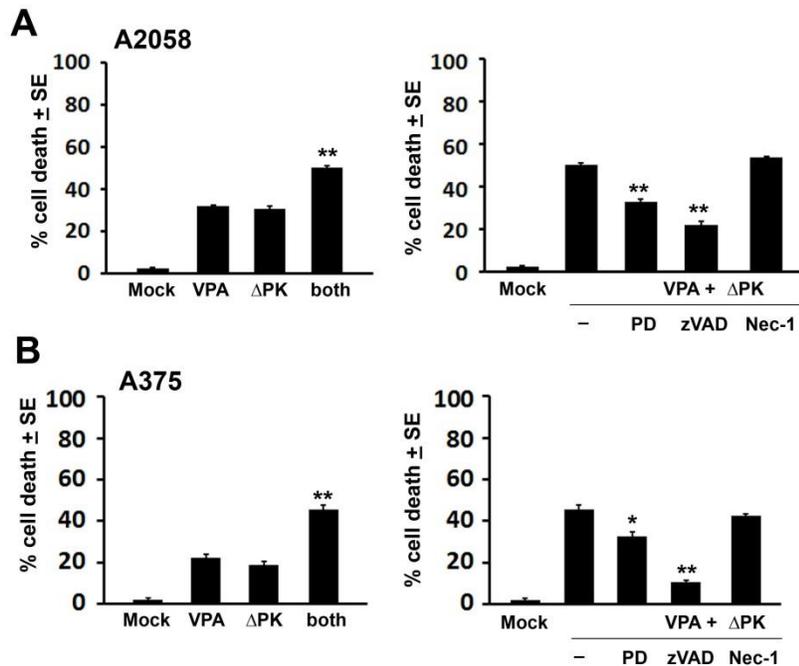


Figure 26: VPA enhances ΔPK-induced cell death through caspase activation

A2058 (A) and A375 (B) cultures were either mock-infected or infected with ΔPK(moi=1) in the absence or presence of VPA(30mM) then assayed for cell death 24h pi using trypan blue (left panels). Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death ± SE. Parallel cultures either mock-infected or ΔPK infected in the presence of VPA either the calpain inhibitor PD150606 (100μM), the pan caspase inhibitor zVAD-fmk (100μM), or the necroptosis inhibitor necrostatin-1(150 μM) were collected 24 hours pi and assayed for cell death as above (right panels). (*P<0.05, **P<0.001 by two way ANOVA, n=3)

death is caspase dependent (Fig 26). Cell death was also significantly inhibited through calpain inhibition, but this likely reflects only the contribution of ΔPK, because we showed that VPA-induced cell death does not involve calpain activation (Fig 26).

D2.3 Different caspases are activated by the ΔPK+VPA combination in distinct melanoma cells

To determine which caspases are involved in cell death induced by Δ PK and VPA combination treatment, and how they may differ between cell types, extracts from A2058 and A375 cultures either mock or VPA-treated (30mM), or infected with Δ PK (moi=1) in the absence or presence of VPA were collected at 24h pi and immunoblotted with antibodies against the initiator caspases 8 and 9, or the executioner caspase 3. Blots were stripped and re-probed with antibody to GAPDH as loading control. As shown in Fig 28A for A2058 cultures, combining VPA and Δ PK treatment enhances the activation of caspases 3 and 9 relative to the data seen for Δ PK alone (as evident by the disappearance of the procaspase form or increase in active form) Interestingly, while caspase-3 was also increasingly activated in A375 cultures given combination therapy as compared to Δ PK alone, the addition of VPA enhanced initiator caspase 8, rather than caspase 9 in these cells, demonstrating a cell type specificity of VPA-induced caspase enhancement (Fig 28B). While these two initiator caspases are activated through separate cellular pathways [caspase 9 via mitochondrial cell death (apoptosome) and caspase 8 through receptor initiated apoptosis (DISC)], these pathways appear to converge on the enhancement of caspase 3 activation in both melanoma cell lines. The similar levels of ZVAD-induced death inhibition in both cell types suggest that independent effects of caspase 8 or 9 outside of downstream caspase 3 activation, are unlikely to be involved in VPA-induced cell death.

D2.4 Valproic acid induces caspase-dependent cell death in breast cancer

Having seen that VPA induces enhances Δ PK oncolysis in a caspase-dependent manner in melanoma, we wanted to further investigate the utility of VPA for combination therapy in breast cancer cultures. We chose to study the breast cancer lines HS578T and

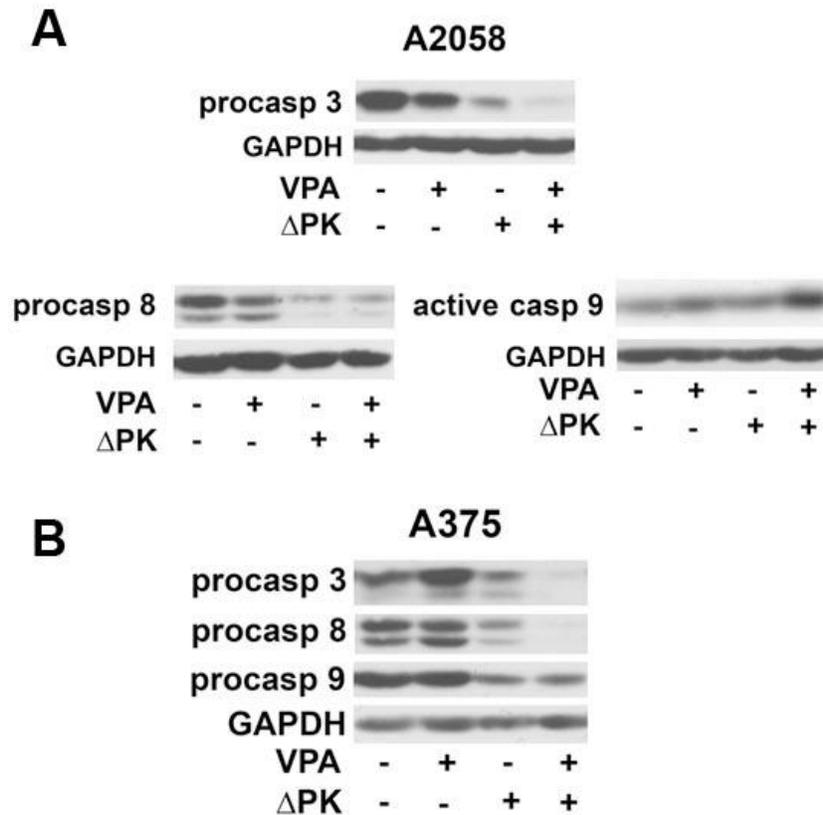


Figure 27: VPA enhances ΔPK-induced caspase activation in a cell type specific manner

A2058 (A) and A375 (B) cultures either mock-infected or infected with ΔPK(moi=1) in the absence or presence of VPA(30mM) were collected 24 h pi and protein extracts were immunoblotted with antibodies that recognize either the pro- or cleaved (active) form of caspases -3,-8,-9. Blots were stripped between each caspase immunoblot and lastly re-probed for GAPDH as loading control. (n=3)

MDA-MB-231. Both cell lines are triple negative for estrogen, progesterone, and human epidermal growth factor 2 receptors, but they differ in that HS578T cells have an activating H-Ras mutation while MDA-MB-231 cells have suppressive mutations in p53 and CDKN2A³³⁴, allowing us to also examine how these different mutations may impact ΔPK oncolysis and enhancement by VPA. To determine if VPA induces breast cancer cell death, and if so, by what mechanism, HS578T and MDA-MB-231 breast cancer

cultures were either mock or VPA-treated (30mM) in the absence or presence of the calpain inhibitor PD150606(100uM), the pan caspase inhibitor zVAD-fmk (100μM), or the necroptosis inhibitor necrostatin-1(150 μM). Cell were collected 24 and 48 hours after treatment and assayed for cell death using trypan blue staining. Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death + SE. In HS578T cells that have an activating Ras mutation, the ability of VPA to induce cell death was minimal at 24 hours ($6.5 \pm 0.4\%$) but it increased to $56.8 \pm 1.8\%$ at 48 hrs. At this time, cell death was significantly inhibited by both zVAD and PD150606 treatment, indicative of the involvement of caspase and calpain activation in VPA-induced cell death. In MDA-MB-231 cultures which have suppressive p53 and CDKN2A mutations, VPA induced cell death was $34.6 \pm 3.1\%$ at 24hrs and it increased to $93.4 \pm 1.4\%$ at 48hrs. At 24 hours, cell death was inhibited by both zVAD and Nec-1 treatment, but by 48 hours only zVAD significantly inhibited death, suggesting an early involvement of necroptotic death pathways that were overpowered by caspase activation by 48 hours (Fig 29). These results demonstrate that even among the same tumor type, VPA can function through different pathways and though not confirmed, this is likely related to the differing molecular signatures between the cell types. For example, the low levels of VPA-induced death seen in HS578T cells may be due to the inability of VPA to dramatically counteract the overactive proliferative pathways induced by the H-Ras mutation, as also suggested by our findings in the ICP10PK constitutively expressing cells (chapter 4B) whereas the higher levels of death seen in MDA-MB-231 cells could reflect the ability of VPA to perform better in cells with mutations affecting cell cycle regulation.

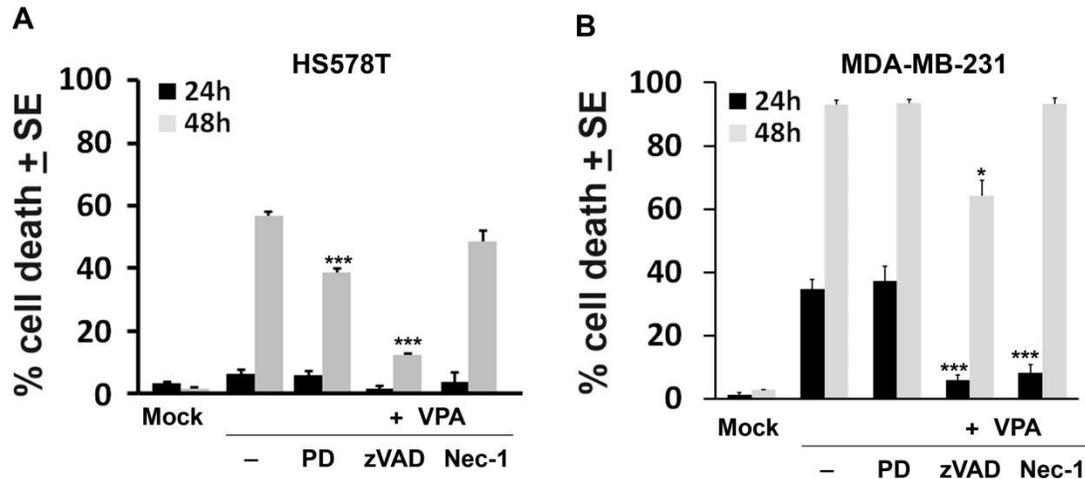


Figure 28: VPA induces cell death in breast cancer cultures

HS578T (A) and MDA-MB-231 (B) cultures were either mock or VPA-treated (30mM) in the absence or presence of the calpain inhibitor PD150606(100uM), the pan caspase inhibitor zVAD-fmk (100uM), or the necroptosis inhibitor necrostatin-1(150 μ M). Cell were collected 24 and 48 hours after treatment and assayed for cell death using trypan blue staining. Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death \pm SE. (**P<0.001 by two way ANOVA, n=3)

D2.5 Combination of VPA and Δ PK enhances cell death in MDA-MB-231, but not

HS578T

To determine whether VPA can increase the oncolytic activity of Δ PK in breast cancer, HS578t and MDA-MB-231 cultures were either mock or Δ PK (moi=1)- infected in the absence or presence of VPA (30mM) and cells collected at 24h pi were assayed for cell death using trypan blue staining. Δ PK alone caused a $48.0 \pm 1.8\%$ and $35.4 \pm 2.2\%$ cell death in HS578T and MDA-MB-231, respectively but VPA co-treatment did not increase Δ PK induced cell death in HS578T cells while increasing it in MDA-MB-231 to $66.5 \pm 4.2\%$ (Fig 30), demonstrating both a tumor- and cell- type specificity of VPA's ability to enhance Δ PK. Enhancement in MDA-MB-231 cells likely reflects the ability of

VPA to induce a necroptotic death pathway that is distinct from the known oncolytic mechanisms of Δ PK, whereas in HS578T cells VPA-induced death only involved calpain and caspase activation, both of which are also activated by Δ PK¹⁴⁸.

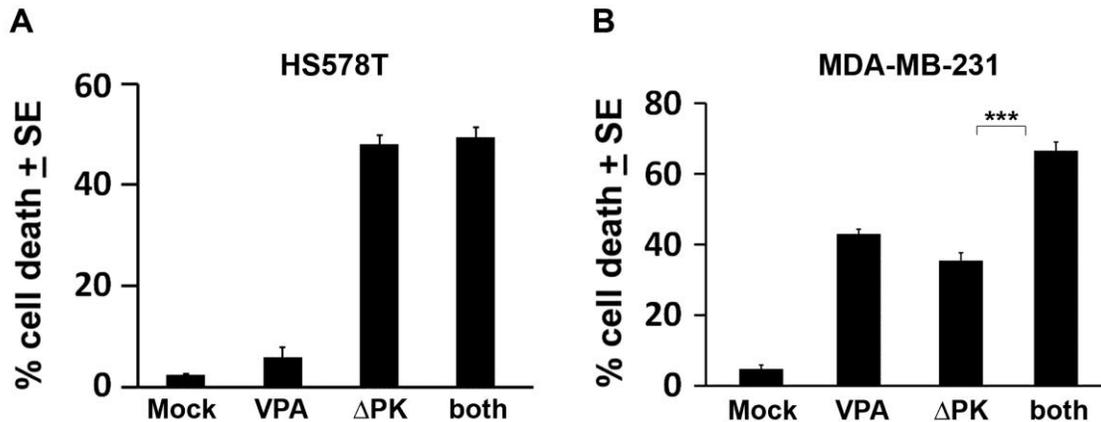


Figure 29: Δ PK enhancement by VPA cell-type specific for breast cancer

HS578T (A) and MDA-MB-231 (B) cultures were either mock-infected or infected with Δ PK(moi=1) in the absence or presence of VPA(30mM) then assayed for cell death 24h pi using trypan blue (left panels). Dead cells (blue staining) were counted in 4 quadrants and the % dead cells were calculated. The results are expressed as % death \pm SE. (***)P<0.001 by two way ANOVA, n=3)

D3. Discussion of results

The salient feature of the data presented in this report is the finding that VPA co-treatment enhances the oncolytic activity of Δ PK through cell type specific activation of caspases. The following comments seem pertinent to these findings.

Oncolytic viruses, which replicate selectively and induce cell death in tumor cells, are a promising therapeutic for the treatment of human malignances, however, as a stand-alone therapeutic they have not been shown to induce complete, long-term regression of established tumors *in vivo*²⁰⁹. Chemotherapeutic agents, therefore, are increasingly sought for combination therapy and offer the advantage of potentiating the cytotoxic

mechanisms of OV while also acting to remove barriers to successful oncolytic virotherapy, such as host immune response, which may increase viral replication and persistence and thereby extend the duration of the therapeutic effect. HDACi have been proposed as a new type of antitumor agent due to their ability to induce cell cycle arrest and apoptosis in tumor cells. HDACi are a promising class of adjuvant agents for oncolytic virotherapy because several have been reported to upregulate the transcription of virally delivered transgenes³³⁵ as well as suppress the activation of IFN-responsive antiviral genes^{176,336}, thereby improving OV efficacy. Valproic acid (VPA), a HDACi used for the treatment of epilepsy and mood disorders, is currently in cancer therapy clinical trials both as a single agent and in combination therapy and has been shown to enhance efficacy of an oHSV-1 in a model of glioma through increased viral propagation *in vivo* and increased animal survival¹⁷⁷. We have shown that VPA induces cell death in neuronal cultures through a novel form of calpain-dependent necroptosis³³², that differs from the caspase-dependent death induced by Δ PK, highlighting a potential opportunity for increased cell death through combinatorial therapy in melanoma cells, which are of neuronal lineage.

We found that in contrast to the mode of cell death induced in neuronal cultures, VPA-induced melanoma cell death was not inhibited by inhibition of either calpain or necroptosis, identifying a different mechanism of action than that seen in neuronal cells. We focused on the executioner caspase 3, which is activated by Δ PK, as well as the initiator caspases 8 and 9, and found that VPA enhanced caspase activation in a cell-type specific manner. In both melanoma cell types, Δ PK activated all of the studied caspases. However, VPA co-treatment only enhanced activation of caspases 3/9 in A2058 cells and

caspases 3/8 in A375 cultures. The explanation for the differential caspase enhancement by VPA is currently unclear, but is consistent with independent reports that HDAC inhibition is cell type dependent³³⁷. The failure of VPA to induce necroptosis in the Δ PK-treated melanoma cells, is likely related to the ability of Δ PK to activate caspase 8 and is consistent with the inability of Nec-1 to inhibit cell death since activated caspase 8 negatively regulates necroptosis by cleaving RIP1, thereby preventing formation of the necrosome¹¹⁰.

We further examined the potential role of VPA in combination therapy by examining its action in two breast cancer cell lines with different mutational profiles. We found that in HS578T cultures, VPA induced cell death was calpain and caspase dependent, but adding VPA to Δ PK infected cultures did not increase Δ PK oncolysis at 24 hours. A possible interpretation of this finding is that because VPA-induced cell death in HS578T cultures is very low at 24 hours and HS578T cells have an activating H-Ras mutation that favors Δ PK replication, Δ PK simply outperformed VPA at 24hours and adding VPA could not enhance cell death. In MDA-MB-231 cultures, VPA-induced cell death was associated with necroptosis and caspase activation at 24 hours but by 48hours, only caspase activation was involved. Unlike HS578T, VPA significantly enhanced Δ PK oncolysis in MDA-MD-231 cultures, showing that not only does VPA induce cell death by cell-type specific mechanisms, but enhancement of Δ PK also differs between cell types. These differential effects of VPA may be attributed in part to the varying degrees of HDAC deregulation in each cancer cell line which would translate to varying effects achieved by HDAC inhibition³³⁸, although the specific differences in HDAC activity

between the cell lines is unclear. These differences in mode of action should be considered for future therapeutic and research application of HDAC inhibitors.

In addition to their use as cytotoxic chemotherapeutic drugs, HDACis are also under investigation as anti-inflammatory and immunosuppressive drugs. Although their immunomodulatory functions can enhance the spread and antitumor effects of OV by impeding the type I IFN response¹⁶⁸ and inhibiting inflammatory cell pathways within NK cells³³⁰, it is unclear whether such a benefit would be at the expense of the optimal development of antitumor immunity that may be required to synergize and/or sustain virus-induced tumor regression. For example, we have shown that Δ PK exerts its oncolytic activity, in part, through the induction of multiple features of immunogenic cell death, including the TLR2/NF κ B dependent secretion of pro-inflammatory cytokines, that *in vivo*, would likely function to enhance the elicitation of an anti-tumor immune response (Section B). It is possible that VPA, having immunosuppressive properties, may reduce the level of pro-inflammatory cytokine release induced by Δ PK, thereby limiting anti-tumor immunity and outweighing any benefit of increased cytotoxicity with combination therapy. The timing of VPA treatment may be a critical aspect affecting its utility for OV combination therapy. A recent study demonstrated that the HDACi, MS-275, when given during the booster phase with an oncolytic vesicular stomatitis virus was able to suppress the primary immune response against the virus thereby enhancing the secondary response against the tumor antigen. However, co-administration of MS-275 with the OV attenuated the anti-tumor immune response³³⁹. VPA pre-treatment, rather than co-treatment was also shown to enhance replication of an oHSV-1 *in vivo*, attributed to the reduction of anti-viral immunity¹⁷⁷.

Although VPA shows promise *in vitro* as a combination agent for Δ PK by increasing cytotoxicity, the impact of VPA on other oncolytic mechanism of Δ PK, especially those that involve immune modulation, remains to be investigated. Future studies should address the effect of VPA pretreatment and/or co-treatment on host anti-viral immune response, Δ PK replication, and the degree of anti-tumor immunity elicited by Δ PK in the presence or absence of VPA treatment using an immunocompetent *in vivo* model, such as subcutaneous injection of B16 melanoma into C57BL/6 mice, to better understand whether VPA could increase the therapeutic efficacy of Δ PK.

Chapter 5: General Discussion and Future Directions

The studies presented in this dissertation began with the investigation of the impact of Δ PK infection on CSC-enriched cultures and how inflammatory mechanisms may play a role in Δ PK oncolysis. The studies went on to examine the mechanism of neuronal cell death induced by VPA, a potential candidate for combination therapy with Δ PK, and concluded by determining whether VPA can increase the efficacy of Δ PK. The salient features of the data are that: (i) Δ PK prevents growth of CSC-enriched anchorage independent cultures and lyses these cultures through calpain-dependent clearance of the autophagy protein p62/SQSTM1, (ii) Δ PK oncolysis includes several features of immunogenic cell death, (iii) VPA induces a novel calpain-dependent necroptotic form of cell death in neuronal cells, and (iv) the addition of VPA to Δ PK infection in melanoma increases Δ PK -induced cell death through the enhancement of caspase activation. The following comments seem pertinent with respect to these findings.

Carcinogenesis, the process by which normal cells become malignant, occurs through the accumulation of mutations that dysregulate intracellular signaling pathways and lead to unchecked growth and proliferation. Tumor cell heterogeneity as a result of DNA instability promotes the development of tumor cells with greater proliferative capacity and invasive potential. As a result, treatment methods that address a singular therapeutic strategy may be insufficient to completely eliminate tumor growth. Oncolytic viruses are a promising cancer therapeutic strategy because they are multi-mechanistic. They work through direct tumor cell lysis via replication and the induction of PCD pathways, cell death due to anti-angiogenesis and vasculature targeting, and the elicitation of adaptive

anti-tumor immunity. OV_s have been developed from virtually all virus platforms, each with varying modes of cell death and respective advantages and disadvantages. Several factors still limit OV efficacy, however, including the presence of tumor-forming CSC that are resistant to therapy, and the immunosuppressive tumor microenvironment that makes eliciting a tumor specific immune response challenging. Even if the viral platform has the ability to eliminate CSC and combat the immunosuppressive tumor milieu, the majority of OV_s have not been studied for these properties.

As discussed below, ΔPK is a particularly promising therapeutic because it has the potential to overcome all of these aforementioned barriers. We found that ΔPK infection induced calpain-dependent autophagic cell death of CSC-enriched melanoma 3D spheroid cultures. ΔPK was shown to lyse 3D spheroid cultures at low titers (0.1 pfu/cell) and without the development of resistance²¹⁶. In addition, adherent melanoma cultures infected with ΔPK were unable to grow in anchorage-independent conditions, demonstrating that ΔPK abrogates 3D growth potential. Interestingly, unlike the caspase-dependent cell death previously reported in adherent melanoma cultures¹⁴⁸, ΔPK eradication of anchorage-independent growth and 3D spheroid lysis occurred independently of caspase activation, and instead was dependent upon activation of calpain and autophagy. These mechanistic differences underscore the importance of using therapeutic approaches (be they drugs or OV_s) that use distinct oncolytic mechanisms which are specifically adjusted to regular tumor cells vs CSC. The different mechanisms needed to kill CSC relative to the rest of the tumor cells potentially reflect an increased expression of anti-apoptotic proteins, such as survivin and Bcl-2, in CSC and while this remains to be established, the fact that ΔPK can use distinct mechanisms in order to lyse

the CSC underscores its strong therapeutic potential. The calpain-mediated autophagic death induced by Δ PK was associated with the calpain-dependent clearance of the autophagy associated protein p62/SQSTM1. To the extent of our knowledge, this is the first report that calpain activation induces anti-tumor activity through p62/SQSTM1 degradation. The documentation of CSC lysis by Δ PK agrees with previous *in vivo* findings that some xenograft-bearing animals treated with Δ PK remained tumor free for at least a year¹⁴⁸, supporting a role of CSC-lysis in the strong *in vivo* therapeutic activity of Δ PK.

The various mechanisms through which OV_s are capable of lysing cancer cells can result in the release of TAAs, proinflammatory cytokines, chemokines, and other danger signals, in a process known as immunogenic cell death, and can facilitate immune cell recruitment and activation within tumors. In particular, activation and maturation of DCs and other APCs allow for efficient cross-presentation to T cells, and subsequent initiation of antitumor immune responses³⁴⁰. Although originally thought to depend mostly on direct viral lysis of tumor cells, it has become increasingly clear that the elicitation of an anti-tumor immune response is a critical aspect of OV-induced cell death²⁰⁸. However, due to immunosuppressive regulatory factors within the tumor, OV_s induce only weak tumor-specific immune responses. It is important therefore, that in addition to inducing inflammation that can promote anti-tumor immunity, OV_s should also be able to eliminate mediators of immunosuppression, which may blunt the formation of a pro-inflammatory response. Efforts to enhance the anti-tumor immune response after virus infection mostly focus on arming OV_s with proinflammatory cytokines or co-stimulatory molecules²³⁶. Several OV_s expressing single cytokines have demonstrated clinical

therapeutic benefits, but this approach typically results in reduced viral replication due to premature viral clearance³⁴¹. Furthermore, the use of a single cytokine could result in the stimulation of only one step of the complex kinetic process of innate to adaptive immune response. We have demonstrated that Δ PK, without the addition of any transgene cassettes, not only promotes a Th1-biased microenvironment, but it also inhibits Th2-based immunosuppressive conditions and induces anti-tumor immune surveillance mechanisms. Δ PK accomplishes this by (i) upregulating the pro-inflammatory cytokines TNF- α , GM-CSF and IL-1 β through activation of the TLR2/NF κ B pathway and pyroptosis, (ii) the JNK/c-Jun dependent inhibition of the anti-inflammatory and immunosuppressive cytokine IL-10 and the resulting upregulation of MICA, the ligand for the activating NKG2D receptor expressed on NK and cytotoxic T cells, and (iii) the inhibition of the negative immune checkpoint regulator CTLA-4. The expression of CTLA-4 on several solid tumors, including melanoma, has been recently demonstrated⁵⁴. Because the majority of CTLA-4 studies have focused on its expression and function as it relates to T cells, the exact contribution of the melanoma-expressed CTLA-4 is still unclear. Nevertheless, CTLA-4 blockade has been shown to induce tumor regression⁵⁶ and be clinically effective against metastatic melanoma^{73,283}, supporting the hypothesis that downregulation of its expression by Δ PK may help combat tumor immunosuppression.

Upregulation of MICA by Δ PK is particularly relevant because NK cells are important mediators of the innate immune response against tumors and MICA downregulation is one of the mechanisms by which tumors can avoid immune detection. In melanoma, downregulation of MICA resulted in reduced lysis of tumor cells by lymphocyte-

activated killer cells⁵², and MICA overexpression has been shown to increase *in vitro* NK-mediated cell lysis and reduce tumor growth and metastasis *in vivo*³⁴², underscoring the role of MICA in tumorigenesis. We demonstrate that MICA upregulation is the result of the ability of Δ PK to inhibit the tumor immunosuppressive milieu as evidenced by the fact that it results from the inhibition of IL-10 secretion.

Cell death induced by Δ PK is, by definition, immunogenic. Although the impact of Δ PK on anti-tumor immune modulation remains to be investigated *in vivo*, it is likely that Δ PK -induced melanoma tumor cell lysis, the inhibition of the immunosuppressive milieu, and the secretion of multiple proinflammatory cytokines that can recruit and expand antitumor effector cells such as macrophages and NK cells while inhibiting T regs, are likely to promote tumor antigen cross-presentation and the induction of adaptive antitumor immune responses. The development of a specific anti-tumor immune response could also facilitate the lysis of tumor cells in distant locations not necessarily infected by the virus. To test this, an interesting future experiment would be to generate tumors in two separate locations in a syngeneic immunocompetent model of melanoma, and see if inoculating one of the tumors with Δ PK could also eradicate/decrease the volume of the untreated tumor. Additionally, tumor cells could be re-introduced into animals that have already undergone previous tumor treatment with Δ PK to determine if a specific anti-tumor immune response was developed and if it could prevent the formation of new tumors. The most widely used syngeneic model is the B16 melanoma cell line, originally derived from a chemically-induced melanoma arising in C57BL/6J mice³⁴³. However, it must be pointed out that while this model is good for studying immune response, its major disadvantage is the use of murine melanoma cell lines, which are limited in their

range and are not a good match for the human counterparts, since mouse melanoma cells differ from human melanoma cells in several important respects, such as their mutational profile³⁴⁴. The B16 model is also limited due to it being derived from an inbred mouse strain with little genetic diversity.

While the development of tumor specific adaptive immunity is critical for efficacious OV therapy, the premature clearance of virus by host innate anti-viral immune responses is a barrier to therapy that must be considered. The first line of defense against viral infection is the innate immune cells that patrol, detect, and rapidly eliminate foreign invaders and innate cell recruitment and activation have been shown to be deleterious to OV efficacy³⁴⁵. To combat this, various combination therapies between OVs and anti-inflammatory drug agents are being investigated. Of particular promise for combination therapy are HDACis, which in addition to having anti-inflammatory properties, can also increase the cytotoxicity of OVs³⁴⁶. We chose to investigate the ability of the HDACi VPA to enhance Δ PK efficacy, focusing initially on the potential cytotoxic effects of the drug rather than its potential to dampen host anti-viral immune responses, which would require investigation in an immunocompetent *in vivo* model.

VPA is an HDACi with well-documented neuroprotective effects and is indicated for the treatment of epilepsy and mood disorders. However, several others have reported neurotoxic effects of VPA. This dichotomy in the literature was of particular interest to us because we proposed to use VPA to enhance Δ PK in melanoma cultures, which are of neuronal lineage. Therefore, we first wanted to investigate the mechanism of action of VPA in neuronal cultures. We found that in our model, VPA induced neuronal cell death through a novel calpain-dependent necroptotic pathway. We reasoned that adding VPA

would therefore enhance Δ PK-induced cell death, which was previously shown to be dependent on caspase activation. We found that while VPA did enhance Δ PK, it was through enhanced caspase activation rather than the induction of the necroptotic pathway observed in neuronal cultures. Even among the two melanoma cultures studied, addition of VPA resulted in the enhancement of different initiator caspases, with caspase 9 and caspase 8 being enhanced for A2058 and A375 cultures, respectively. However, this differential caspase activation was unlikely to impact cellular death pathways outside of downstream executioner caspase activation, as caspase 3 was enhanced in both cell lines and zVAD has similar effects on death inhibition. The potential of VPA for combination therapy with Δ PK was further investigated in two breast cancer cultures and we found that while caspase activation was involved in VPA-induced death in both cell types, HS578T cell death also involved calpain activation while an early necroptotic pathway was involved in early killing of MDA-MB-231 cells. Furthermore, VPA only enhanced Δ PK oncolysis in MDA-MB-231, not HS578T, cultures, further highlighting cell-type specific differences of VPA in combination with OV that should be considered when pursuing VPA treatment in additional cell types, tumor models, and/or OV platforms. The explanation of this varying response to VPA remains unclear, but is likely related to varying degrees of HDAC deregulation and therefore differential impacts of HDAC inhibition between the cell types³³⁷, and could potentially be related to the type and degree of genetic alterations in each cell type. These preliminary studies confirm the initial hypothesis that VPA can enhance the cytotoxic effects of Δ PK in melanoma, and support further investigation into combination therapy with VPA *in vivo*.

An additional aspect of Δ PK therapy in combination with VPA that remains to be investigated is the modulation of the host antiviral innate immune response. VPA has been reported to reduce the host cellular IFN response as well as the recruitment of NK cells and macrophages into tumors and the accompanying NK cell-mediated cytotoxicity³³⁰. Interestingly, tumor infiltration by immune cells rebounded robustly at later time points, assuaging concerns that reduced lymphocyte infiltration may negatively impact the development of a potent anti-tumor response. It appears that the timing of combination therapy between HDACis and OV plays an important role in successful treatment. For example, the HDACi MS-275 was initially found to compromise the induction of anti-tumor immunity when co-administered with an oncolytic VSV, however, this effect was avoided when MS-275 was administered at the boosting phase, and in fact resulted in suppression of the primary immune response against the virus and enhancement of the secondary response against the tumor antigen³³⁹. To test this, an immunocompetent mouse model would need to be used to examine the effect of VPA co-treatment on host antiviral responses, such as IFN-transcription, as well as Δ PK replication *in vivo*. Additional experiments to test time-dependent differences in VPA co-treatment should be conducted to find an optimal regimen. Most likely, pre-treatment of animals with VPA prior to Δ PK infection would be needed to help reduce host anti-viral response and then VPA treatment should be stopped shortly after Δ PK infection as not to interfere with the ability of the virus to induce ICD. However, the effect of such a regimen on the caspase-mediated enhancement of the Δ PK lytic potential seen in our studies is unclear. Because our data indicate that potentiation occurs early in Δ PK infection, it seems reasonable to suggest that VPA pre-treatment or early treatment post

Δ PK (viz, within 24hrs) may have beneficial effects. It would also be interesting to examine the effect of VPA on Δ PK -induced features of ICD, such as the production of inflammatory cytokines and the increase in the immune surveillance protein MICA. VPA has also been reported to enhance the expression of MICA in various cancer models³⁴⁷, supporting the idea that combination therapy with VPA may assist in the development of anti-tumor immune responses. However, due to its anti-inflammatory nature, it is possible that VPA in addition to inhibiting the host anti-viral immune response could also inhibit some of the immunostimulatory effects of Δ PK. Such a negative aspect of combination therapy might be rectifiable through accurate timing of the treatment.

Ultimately VPA may not significantly enhance the efficacy of Δ PK *in vivo*, in which case the ability of other HDACis or cytotoxic/anti-inflammatory drugs to potentially synergize with Δ PK may have to be investigated. However, when considering a chemotherapeutic drug agent for combination therapy with OV it is important to keep in mind that biological pathways that OVs manipulate to support their replication are similar to those utilized by cancer cells to become increasingly malignant and targeting certain pathways with chemotherapy could therefore compromise the replicative capacity of OVs. Conflicts between virus-enabled therapeutic strategies and drug-enabled therapeutic strategies may limit the extent to which the two can be combined. We also cannot exclude the possibility that Δ PK alone may have a better therapeutic outcome than when in combination with chemotherapeutics, which does not seem unreasonable considering its multi-mechanistic nature. In fact, administration of an OV that can mimic the effects

of combinatorial therapy by inducing diverse anti-tumor effects at the relatively lower toxicity of a single agent therapy may be the most favorable outcome.

Although combination therapy with anti-inflammatory HDACis may be able to dampen host anti-viral immune responses without interfering with the development of anti-tumor immunity, the adaptive immune response against viral antigens remains a potential barrier to virotherapy, and a particularly relevant factor in the host immune response against virus is the method of virus delivery. The majority of people are infected with at least one strain of HSV (asymptotically), therefore the presence of circulating antibodies against the virus may limit oncolytic delivery, particularly if administered intravenously. Although Δ PK does have an intact ICP47, a gene responsible for immune evasion through the inhibition of viral peptide presentation on MHC Class I, the presence of ICP47 does not interfere with the utility of Δ PK as a therapeutic HSV-2 vaccine¹⁵⁰, therefore in the context of virotherapy, we must conclude that ICP47 does not play a role in preventing immune detection. However, it is recognized that individuals can be infected with multiple strains of HSV virus, demonstrating that pre-existing immunity is unlikely to impede OV efficacy. Indeed, studies using oHSV showed that prior immunity to HSV did not inhibit anti-tumor efficacy of the OV in murine tumors^{348,349}. Furthermore, a study using an oHSV-1 on a model of intracranial metastatic melanoma reported that viral therapy induced a tumor-specific cytotoxic and proliferative T cell response without an increase of viral-neutralizing antibodies in the serum, although this may be the result of decreased levels of B cells present in intracranial tumors³⁵⁰. Admittedly, the most clinically successful oHSVs have been administered intratumorally, suggesting that although prior exposure to HSV would not preclude efficacy of Δ PK, the

most efficacious outcome will likely be in tumor models that allow for direct injection of OVs into the tumor mass. The combination of direct tumor injection along with the documented ability of repeat HSV administration without the impedance of the host anti-viral immunity, will allow for the option of retreatment without a reduction in efficacy.

Collectively, this thesis work demonstrates the multi-mechanistic properties of Δ PK, evident in its ability to eradicate anchorage independent growth of melanoma cultures and induce multiple features of immunogenic cell, and highlights the therapeutic potential of combining Δ PK with the HDACi VPA.

Chapter 6: Literature Cited

- 1 Knudson, A. G. Two genetic hits (more or less) to cancer. *Nature reviews. Cancer* **1**, 157-162, doi:10.1038/35101031 (2001).
- 2 Lowenstein, E. J. *et al.* The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70**, 431-442 (1992).
- 3 McCormick, F. ras GTPase activating protein: signal transmitter and signal terminator. *Cell* **56**, 5-8 (1989).
- 4 Bos, J. L. ras oncogenes in human cancer: a review. *Cancer research* **49**, 4682-4689 (1989).
- 5 Steelman, L. S. *et al.* JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* **18**, 189-218, doi:10.1038/sj.leu.2403241 (2004).
- 6 Chang, F. *et al.* Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* **17**, 1263-1293, doi:10.1038/sj.leu.2402945 (2003).
- 7 Saadeddin, A., Babaei-Jadidi, R., Spencer-Dene, B. & Nateri, A. S. The links between transcription, beta-catenin/JNK signaling, and carcinogenesis. *Molecular cancer research : MCR* **7**, 1189-1196, doi:10.1158/1541-7786.MCR-09-0027 (2009).
- 8 Weston, C. R. & Davis, R. J. The JNK signal transduction pathway. *Current opinion in cell biology* **19**, 142-149, doi:10.1016/j.ceb.2007.02.001 (2007).
- 9 Liu, J. & Lin, A. Role of JNK activation in apoptosis: a double-edged sword. *Cell research* **15**, 36-42, doi:10.1038/sj.cr.7290262 (2005).
- 10 Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M. & Karin, M. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* **354**, 494-496, doi:10.1038/354494a0 (1991).
- 11 Eferl, R. & Wagner, E. F. AP-1: a double-edged sword in tumorigenesis. *Nature reviews. Cancer* **3**, 859-868, doi:10.1038/nrc1209 (2003).
- 12 Mingo-Sion, A. M., Marietta, P. M., Koller, E., Wolf, D. M. & Van Den Berg, C. L. Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. *Oncogene* **23**, 596-604, doi:10.1038/sj.onc.1207147 (2004).
- 13 Zarubin, T. & Han, J. Activation and signaling of the p38 MAP kinase pathway. *Cell research* **15**, 11-18, doi:10.1038/sj.cr.7290257 (2005).
- 14 Zohn, I. E., Symons, M., Chrzanowska-Wodnicka, M., Westwick, J. K. & Der, C. J. Mas oncogene signaling and transformation require the small GTP-binding protein Rac. *Molecular and cellular biology* **18**, 1225-1235 (1998).
- 15 Greenberg, A. K. *et al.* Selective p38 activation in human non-small cell lung cancer. *American journal of respiratory cell and molecular biology* **26**, 558-564, doi:10.1165/ajrcmb.26.5.4689 (2002).
- 16 Salh, B., Marotta, A., Wagey, R., Sayed, M. & Pelech, S. Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. *International journal of cancer. Journal international du cancer* **98**, 148-154 (2002).

- 17 Uzgare, A. R., Kaplan, P. J. & Greenberg, N. M. Differential expression and/or
activation of P38MAPK, erk1/2, and jnk during the initiation and progression of
prostate cancer. *The Prostate* **55**, 128-139, doi:10.1002/pros.10212 (2003).
- 18 O'Gorman, D. M. & Cotter, T. G. Molecular signals in anti-apoptotic survival
pathways. *Leukemia* **15**, 21-34 (2001).
- 19 Rafiee, P. *et al.* Human esophageal microvascular endothelial cells respond to
acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and
Hsp27. *American journal of physiology. Cell physiology* **291**, C931-945,
doi:10.1152/ajpcell.00474.2005 (2006).
- 20 McCubrey, J. A. *et al.* Roles of the Raf/MEK/ERK pathway in cell growth,
malignant transformation and drug resistance. *Biochimica et biophysica acta*
1773, 1263-1284, doi:10.1016/j.bbamcr.2006.10.001 (2007).
- 21 Vivanco, I. *et al.* Identification of the JNK signaling pathway as a functional
target of the tumor suppressor PTEN. *Cancer cell* **11**, 555-569,
doi:10.1016/j.ccr.2007.04.021 (2007).
- 22 Jerant, A. F., Johnson, J. T., Sheridan, C. D. & Caffrey, T. J. Early detection and
treatment of skin cancer. *American family physician* **62**, 357-368, 375-356, 381-
352 (2000).
- 23 Lin, J. Y. & Fisher, D. E. Melanocyte biology and skin pigmentation. *Nature* **445**,
843-850, doi:10.1038/nature05660 (2007).
- 24 Herlyn, M. *et al.* Primary melanoma cells of the vertical growth phase:
similarities to metastatic cells. *Journal of the National Cancer Institute* **74**, 283-
289 (1985).
- 25 Fecher, L. A., Cummings, S. D., Keefe, M. J. & Alani, R. M. Toward a molecular
classification of melanoma. *Journal of clinical oncology : official journal of the
American Society of Clinical Oncology* **25**, 1606-1620,
doi:10.1200/JCO.2006.06.0442 (2007).
- 26 Omholt, K., Platz, A., Kanter, L., Ringborg, U. & Hansson, J. NRAS and BRAF
mutations arise early during melanoma pathogenesis and are preserved throughout
tumor progression. *Clinical cancer research : an official journal of the American
Association for Cancer Research* **9**, 6483-6488 (2003).
- 27 Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-
954, doi:10.1038/nature00766 (2002).
- 28 Ascierto, P. A. *et al.* The role of BRAF V600 mutation in melanoma. *Journal of
translational medicine* **10**, 85, doi:10.1186/1479-5876-10-85 (2012).
- 29 Robertson, G. P. Functional and therapeutic significance of Akt deregulation in
malignant melanoma. *Cancer metastasis reviews* **24**, 273-285,
doi:10.1007/s10555-005-1577-9 (2005).
- 30 Stahl, J. M. *et al.* Deregulated Akt3 activity promotes development of malignant
melanoma. *Cancer research* **64**, 7002-7010, doi:10.1158/0008-5472.CAN-04-
1399 (2004).
- 31 Wu, H., Goel, V. & Haluska, F. G. PTEN signaling pathways in melanoma.
Oncogene **22**, 3113-3122, doi:10.1038/sj.onc.1206451 (2003).
- 32 Gober, M. D., Smith, C. C., Ueda, K., Toretsky, J. A. & Aurelian, L. Forced
expression of the H11 heat shock protein can be regulated by DNA methylation

- and trigger apoptosis in human cells. *The Journal of biological chemistry* **278**, 37600-37609, doi:10.1074/jbc.M303834200 (2003).
- 33 Cui, X. Y. *et al.* HSPB8 is methylated in hematopoietic malignancies and overexpression of HSPB8 exhibits antileukemia effect. *Experimental hematology* **40**, 14-21, doi:10.1016/j.exphem.2011.09.004 (2012).
- 34 Smith, C. C. *et al.* Restored expression of the atypical heat shock protein H11/HspB8 inhibits the growth of genetically diverse melanoma tumors through activation of novel TAK1-dependent death pathways. *Cell death & disease* **3**, e371, doi:10.1038/cddis.2012.108 (2012).
- 35 Smith, C. C., Li, B., Liu, J., Lee, K. S. & Aurelian, L. The Levels of H11/HspB8 DNA methylation in human melanoma tissues and xenografts are a critical molecular marker for 5-Aza-2'-deoxycytidine therapy. *Cancer investigation* **29**, 383-395, doi:10.3109/07357907.2011.584588 (2011).
- 36 Bollino, D. & Aurelian, L. HSPB8 (heat shock 22kDa protein 8). *Atlas Genet Cytogenet Oncol Haematol* **18**, 907-918 (2014).
- 37 Serrano, M., Hannon, G. J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-707, doi:10.1038/366704a0 (1993).
- 38 Bishop, J. N., Harland, M. & Bishop, D. T. The genetics of melanoma. *British journal of hospital medicine* **67**, 299-304, doi:10.12968/hmed.2006.67.6.21288 (2006).
- 39 Kemp, J. A., Arora, S. & Fawaz, K. Recurrent acute pancreatitis as a manifestation of Wegener's granulomatosis. *Digestive diseases and sciences* **35**, 912-915 (1990).
- 40 Schatton, T. & Frank, M. H. Cancer stem cells and human malignant melanoma. *Pigment cell & melanoma research* **21**, 39-55, doi:10.1111/j.1755-148X.2007.00427.x (2008).
- 41 Fang, D. *et al.* A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer research* **65**, 9328-9337, doi:10.1158/0008-5472.CAN-05-1343 (2005).
- 42 Dou, J. *et al.* Isolation and identification of cancer stem-like cells from murine melanoma cell lines. *Cellular & molecular immunology* **4**, 467-472 (2007).
- 43 Schatton, T. *et al.* Identification of cells initiating human melanomas. *Nature* **451**, 345-349, doi:10.1038/nature06489 (2008).
- 44 Klein, W. M. *et al.* Increased expression of stem cell markers in malignant melanoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **20**, 102-107, doi:10.1038/modpathol.3800720 (2007).
- 45 Gajewski, T. F. Identifying and overcoming immune resistance mechanisms in the melanoma tumor microenvironment. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 2326s-2330s, doi:10.1158/1078-0432.CCR-05-2517 (2006).
- 46 Fujii, S., Shimizu, K., Hemmi, H. & Steinman, R. M. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunological reviews* **220**, 183-198, doi:10.1111/j.1600-065X.2007.00561.x (2007).

- 47 Dissemond, J. *et al.* Downregulation of tapasin expression in progressive human malignant melanoma. *Archives of dermatological research* **295**, 43-49, doi:10.1007/s00403-003-0393-8 (2003).
- 48 Ferrone, S. & Marincola, F. M. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunology today* **16**, 487-494 (1995).
- 49 Burke, S., Lakshmikanth, T., Colucci, F. & Carbone, E. New views on natural killer cell-based immunotherapy for melanoma treatment. *Trends in immunology* **31**, 339-345, doi:10.1016/j.it.2010.06.003 (2010).
- 50 Ostrand-Rosenberg, S. Immune surveillance: a balance between protumor and antitumor immunity. *Current opinion in genetics & development* **18**, 11-18, doi:10.1016/j.gde.2007.12.007 (2008).
- 51 Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727-729 (1999).
- 52 Serrano, A. E. *et al.* Interleukin 10 decreases MICA expression on melanoma cell surface. *Immunology and cell biology* **89**, 447-457, doi:10.1038/icb.2010.100 (2011).
- 53 Perez, V. L. *et al.* Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* **6**, 411-417 (1997).
- 54 Contardi, E. *et al.* CTLA-4 is constitutively expressed on tumor cells and can trigger apoptosis upon ligand interaction. *International journal of cancer. Journal international du cancer* **117**, 538-550, doi:10.1002/ijc.21155 (2005).
- 55 Shah, K. V., Chien, A. J., Yee, C. & Moon, R. T. CTLA-4 is a direct target of Wnt/beta-catenin signaling and is expressed in human melanoma tumors. *The Journal of investigative dermatology* **128**, 2870-2879, doi:10.1038/jid.2008.170 (2008).
- 56 Leach, D. R., Krummel, M. F. & Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **271**, 1734-1736 (1996).
- 57 Koon, H. B. & Atkins, M. B. Update on therapy for melanoma: opportunities for patient selection and overcoming tumor resistance. *Expert review of anticancer therapy* **7**, 79-88, doi:10.1586/14737140.7.1.79 (2007).
- 58 La Porta, C. A. Drug resistance in melanoma: new perspectives. *Current medicinal chemistry* **14**, 387-391 (2007).
- 59 Cheng, Y., Zhang, G. & Li, G. Targeting MAPK pathway in melanoma therapy. *Cancer metastasis reviews* **32**, 567-584, doi:10.1007/s10555-013-9433-9 (2013).
- 60 Finn, L., Markovic, S. N. & Joseph, R. W. Therapy for metastatic melanoma: the past, present, and future. *BMC medicine* **10**, 23, doi:10.1186/1741-7015-10-23 (2012).
- 61 Flaherty, K. T. *et al.* Inhibition of mutated, activated BRAF in metastatic melanoma. *The New England journal of medicine* **363**, 809-819, doi:10.1056/NEJMoa1002011 (2010).
- 62 Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *The New England journal of medicine* **364**, 2507-2516, doi:10.1056/NEJMoa1103782 (2011).

- 63 Hauschild, A. *et al.* Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **380**, 358-365, doi:10.1016/S0140-6736(12)60868-X (2012).
- 64 Flaherty, K. T. *et al.* Improved survival with MEK inhibition in BRAF-mutated melanoma. *The New England journal of medicine* **367**, 107-114, doi:10.1056/NEJMoa1203421 (2012).
- 65 Flaherty, K. T. *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *The New England journal of medicine* **367**, 1694-1703, doi:10.1056/NEJMoa1210093 (2012).
- 66 Smith, K. A. Interleukin-2: inception, impact, and implications. *Science* **240**, 1169-1176 (1988).
- 67 Atkins, M. B. *et al.* High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **17**, 2105-2116 (1999).
- 68 Herndon, T. M. *et al.* U.S. Food and Drug Administration Approval: peginterferon-alfa-2b for the adjuvant treatment of patients with melanoma. *The oncologist* **17**, 1323-1328, doi:10.1634/theoncologist.2012-0123 (2012).
- 69 Rosenberg, S. A. *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 4550-4557, doi:10.1158/1078-0432.CCR-11-0116 (2011).
- 70 Boni, A. *et al.* Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer research* **70**, 5213-5219, doi:10.1158/0008-5472.CAN-10-0118 (2010).
- 71 Besser, M. J. *et al.* Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 2646-2655, doi:10.1158/1078-0432.CCR-10-0041 (2010).
- 72 Sharpe, A. H. Mechanisms of costimulation. *Immunological reviews* **229**, 5-11, doi:10.1111/j.1600-065X.2009.00784.x (2009).
- 73 Hodi, F. S. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine* **363**, 711-723, doi:10.1056/NEJMoa1003466 (2010).
- 74 Gajewski, T. F., Schreiber, H. & Fu, Y. X. Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology* **14**, 1014-1022, doi:10.1038/ni.2703 (2013).
- 75 Topalian, S. L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* **366**, 2443-2454, doi:10.1056/NEJMoa1200690 (2012).
- 76 Wolchok, J. D. *et al.* Nivolumab plus ipilimumab in advanced melanoma. *The New England journal of medicine* **369**, 122-133, doi:10.1056/NEJMoa1302369 (2013).

- 77 Ribas, A., Hodi, F. S., Callahan, M., Kondo, C. & Wolchok, J. Hepatotoxicity with combination of vemurafenib and ipilimumab. *The New England journal of medicine* **368**, 1365-1366, doi:10.1056/NEJMc1302338 (2013).
- 78 Shtivelman, E. *et al.* Pathways and therapeutic targets in melanoma. *Oncotarget* **5**, 1701-1752 (2014).
- 79 Sharpe, J. C., Arnoult, D. & Youle, R. J. Control of mitochondrial permeability by Bcl-2 family members. *Biochimica et biophysica acta* **1644**, 107-113, doi:10.1016/j.bbamcr.2003.10.016 (2004).
- 80 Cohen, G. M. Caspases: the executioners of apoptosis. *The Biochemical journal* **326 (Pt 1)**, 1-16 (1997).
- 81 Rai, N. K., Tripathi, K., Sharma, D. & Shukla, V. K. Apoptosis: a basic physiologic process in wound healing. *The international journal of lower extremity wounds* **4**, 138-144, doi:10.1177/1534734605280018 (2005).
- 82 Salvesen, G. S. & Riedl, S. J. Caspase mechanisms. *Advances in experimental medicine and biology* **615**, 13-23, doi:10.1007/978-1-4020-6554-5_2 (2008).
- 83 Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews. Molecular cell biology* **9**, 47-59, doi:10.1038/nrm2308 (2008).
- 84 Riedl, S. J. & Salvesen, G. S. The apoptosome: signalling platform of cell death. *Nature reviews. Molecular cell biology* **8**, 405-413, doi:10.1038/nrm2153 (2007).
- 85 LaCasse, E. C. *et al.* IAP-targeted therapies for cancer. *Oncogene* **27**, 6252-6275, doi:10.1038/onc.2008.302 (2008).
- 86 Wilson, N. S., Dixit, V. & Ashkenazi, A. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nature immunology* **10**, 348-355, doi:10.1038/ni.1714 (2009).
- 87 Tait, S. W. & Green, D. R. Caspase-independent cell death: leaving the set without the final cut. *Oncogene* **27**, 6452-6461, doi:10.1038/onc.2008.311 (2008).
- 88 Duprez, L., Wirawan, E., Vanden Berghe, T. & Vandenabeele, P. Major cell death pathways at a glance. *Microbes and infection / Institut Pasteur* **11**, 1050-1062, doi:10.1016/j.micinf.2009.08.013 (2009).
- 89 Vahsen, N. *et al.* AIF deficiency compromises oxidative phosphorylation. *The EMBO journal* **23**, 4679-4689, doi:10.1038/sj.emboj.7600461 (2004).
- 90 Cao, G. *et al.* Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 9278-9293, doi:10.1523/JNEUROSCI.2826-07.2007 (2007).
- 91 Yuste, V. J. *et al.* Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. *Cell death and differentiation* **12**, 1445-1448, doi:10.1038/sj.cdd.4401687 (2005).
- 92 Susin, S. A. *et al.* Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446, doi:10.1038/17135 (1999).
- 93 Ye, H. *et al.* DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nature structural biology* **9**, 680-684, doi:10.1038/nsb836 (2002).
- 94 Li, L. Y., Luo, X. & Wang, X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95-99, doi:10.1038/35083620 (2001).

- 95 Goll, D. E., Thompson, V. F., Li, H., Wei, W. & Cong, J. The calpain system. *Physiological reviews* **83**, 731-801, doi:10.1152/physrev.00029.2002 (2003).
- 96 Wolf, B. B. *et al.* Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* **94**, 1683-1692 (1999).
- 97 Ruiz-Vela, A., Gonzalez de Buitrago, G. & Martinez, A. C. Implication of calpain in caspase activation during B cell clonal deletion. *The EMBO journal* **18**, 4988-4998, doi:10.1093/emboj/18.18.4988 (1999).
- 98 Nakagawa, T. & Yuan, J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *The Journal of cell biology* **150**, 887-894 (2000).
- 99 Chua, B. T., Guo, K. & Li, P. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *The Journal of biological chemistry* **275**, 5131-5135 (2000).
- 100 Reimertz, C., Kogel, D., Lankiewicz, S., Poppe, M. & Prehn, J. H. Ca(2+)-induced inhibition of apoptosis in human SH-SY5Y neuroblastoma cells: degradation of apoptotic protease activating factor-1 (APAF-1). *Journal of neurochemistry* **78**, 1256-1266 (2001).
- 101 Cao, X., Deng, X. & May, W. S. Cleavage of Bax to p18 Bax accelerates stress-induced apoptosis, and a cathepsin-like protease may rapidly degrade p18 Bax. *Blood* **102**, 2605-2614, doi:10.1182/blood-2003-01-0211 (2003).
- 102 Galluzzi, L. *et al.* Programmed necrosis from molecules to health and disease. *International review of cell and molecular biology* **289**, 1-35, doi:10.1016/B978-0-12-386039-2.00001-8 (2011).
- 103 Vandenabeele, P., Galluzzi, L., Vanden Berghe, T. & Kroemer, G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nature reviews. Molecular cell biology* **11**, 700-714, doi:10.1038/nrm2970 (2010).
- 104 Yuan, J. & Kroemer, G. Alternative cell death mechanisms in development and beyond. *Genes & development* **24**, 2592-2602, doi:10.1101/gad.1984410 (2010).
- 105 Degterev, A. *et al.* Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature chemical biology* **4**, 313-321, doi:10.1038/nchembio.83 (2008).
- 106 Geserick, P. *et al.* Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. *The Journal of cell biology* **187**, 1037-1054, doi:10.1083/jcb.200904158 (2009).
- 107 Feoktistova, M. *et al.* cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Molecular cell* **43**, 449-463, doi:10.1016/j.molcel.2011.06.011 (2011).
- 108 McComb, S. *et al.* cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. *Cell death and differentiation* **19**, 1791-1801, doi:10.1038/cdd.2012.59 (2012).
- 109 Steinhart, L., Belz, K. & Fulda, S. Smac mimetic and demethylating agents synergistically trigger cell death in acute myeloid leukemia cells and overcome apoptosis resistance by inducing necroptosis. *Cell death & disease* **4**, e802, doi:10.1038/cddis.2013.320 (2013).

- 110 Lin, Y., Devin, A., Rodriguez, Y. & Liu, Z. G. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes & development* **13**, 2514-2526 (1999).
- 111 Moubarak, R. S. *et al.* Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. *Molecular and cellular biology* **27**, 4844-4862, doi:10.1128/MCB.02141-06 (2007).
- 112 Baritaud, M., Boujrad, H., Lorenzo, H. K., Krantic, S. & Susin, S. A. Histone H2AX: The missing link in AIF-mediated caspase-independent programmed necrosis. *Cell cycle* **9**, 3166-3173 (2010).
- 113 Miao, E. A. *et al.* Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nature immunology* **11**, 1136-1142, doi:10.1038/ni.1960 (2010).
- 114 Bergsbaken, T., Fink, S. L. & Cookson, B. T. Pyroptosis: host cell death and inflammation. *Nature reviews. Microbiology* **7**, 99-109, doi:10.1038/nrmicro2070 (2009).
- 115 Tricarico, P. M. *et al.* Mevalonate kinase deficiency and neuroinflammation: balance between apoptosis and pyroptosis. *International journal of molecular sciences* **14**, 23274-23288, doi:10.3390/ijms141223274 (2013).
- 116 Fink, S. L. & Cookson, B. T. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cellular microbiology* **8**, 1812-1825, doi:10.1111/j.1462-5822.2006.00751.x (2006).
- 117 Das, G., Shrivage, B. V. & Baehrecke, E. H. Regulation and function of autophagy during cell survival and cell death. *Cold Spring Harbor perspectives in biology* **4**, doi:10.1101/cshperspect.a008813 (2012).
- 118 Clarke, P. G. & Puyal, J. Autophagic cell death exists. *Autophagy* **8**, 867-869, doi:10.4161/auto.20380 (2012).
- 119 Mizushima, N. Autophagy in protein and organelle turnover. *Cold Spring Harbor symposia on quantitative biology* **76**, 397-402, doi:10.1101/sqb.2011.76.011023 (2011).
- 120 Itakura, E., Kishi, C., Inoue, K. & Mizushima, N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Molecular biology of the cell* **19**, 5360-5372, doi:10.1091/mbc.E08-01-0080 (2008).
- 121 Pattingre, S., Espert, L., Biard-Piechaczyk, M. & Codogno, P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* **90**, 313-323, doi:10.1016/j.biochi.2007.08.014 (2008).
- 122 Pattingre, S. *et al.* Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927-939, doi:10.1016/j.cell.2005.07.002 (2005).
- 123 Mizushima, N. *et al.* Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *Journal of cell science* **116**, 1679-1688 (2003).
- 124 Tooze, S. A. *et al.* Assessing Mammalian autophagy. *Methods in molecular biology* **1270**, 155-165, doi:10.1007/978-1-4939-2309-0_12 (2015).

- 125 Zitvogel, L., Kepp, O. & Kroemer, G. Immune parameters affecting the efficacy of chemotherapeutic regimens. *Nature reviews. Clinical oncology* **8**, 151-160, doi:10.1038/nrclinonc.2010.223 (2011).
- 126 Tesniere, A. *et al.* Molecular characteristics of immunogenic cancer cell death. *Cell death and differentiation* **15**, 3-12, doi:10.1038/sj.cdd.4402269 (2008).
- 127 Locher, C. *et al.* Desirable cell death during anticancer chemotherapy. *Annals of the New York Academy of Sciences* **1209**, 99-108, doi:10.1111/j.1749-6632.2010.05763.x (2010).
- 128 Biederer, C., Ries, S., Brandts, C. H. & McCormick, F. Replication-selective viruses for cancer therapy. *Journal of molecular medicine* **80**, 163-175, doi:10.1007/s00109-001-0295-1 (2002).
- 129 Sobol, P. T. *et al.* Adaptive antiviral immunity is a determinant of the therapeutic success of oncolytic virotherapy. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 335-344, doi:10.1038/mt.2010.264 (2011).
- 130 Workenhe, S. T. *et al.* Immunogenic HSV-mediated oncolysis shapes the antitumor immune response and contributes to therapeutic efficacy. *Molecular therapy : the journal of the American Society of Gene Therapy* **22**, 123-131, doi:10.1038/mt.2013.238 (2014).
- 131 Guo, Z. S., Liu, Z. & Bartlett, D. L. Oncolytic Immunotherapy: Dying the Right Way is a Key to Eliciting Potent Antitumor Immunity. *Frontiers in oncology* **4**, 74, doi:10.3389/fonc.2014.00074 (2014).
- 132 Coukos, G. *et al.* Oncolytic herpes simplex virus-1 lacking ICP34.5 induces p53-independent death and is efficacious against chemotherapy-resistant ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6**, 3342-3353 (2000).
- 133 Breitbach, C. J. *et al.* Oncolytic vaccinia virus disrupts tumor-associated vasculature in humans. *Cancer research* **73**, 1265-1275, doi:10.1158/0008-5472.CAN-12-2687 (2013).
- 134 Liu, T. C., Hwang, T., Park, B. H., Bell, J. & Kirn, D. H. The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 1637-1642, doi:10.1038/mt.2008.143 (2008).
- 135 Murphy, K. M., Travers, P. & Walport, M. *Janeway's Immunobiology*. 7th edn, (Garland Publishing, 2008).
- 136 Whitley, R., Kimberlin, D. W. & Prober, C. G. in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (eds A. Arvin *et al.*) (2007).
- 137 Rajcani, J., Andrea, V. & Ingeborg, R. Peculiarities of herpes simplex virus (HSV) transcription: an overview. *Virus genes* **28**, 293-310 (2004).
- 138 Tang, S. *et al.* An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 10931-10936, doi:10.1073/pnas.0801845105 (2008).
- 139 Gober, M. D., Wales, S. Q., Hunter, J. C., Sharma, B. K. & Aurelian, L. Stress up-regulates neuronal expression of the herpes simplex virus type 2 large subunit

- of ribonucleotide reductase (R1; ICP10) by activating activator protein 1. *Journal of neurovirology* **11**, 329-336, doi:10.1080/13550280591002423 (2005).
- 140 Agarwalla, P. K. & Aghi, M. K. Oncolytic herpes simplex virus engineering and preparation. *Methods in molecular biology* **797**, 1-19, doi:10.1007/978-1-61779-340-0_1 (2012).
- 141 Chung, T. D., Wymer, J. P., Smith, C. C., Kulka, M. & Aurelian, L. Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *Journal of virology* **63**, 3389-3398 (1989).
- 142 Smith, C. C., Nelson, J., Aurelian, L., Gober, M. & Goswami, B. B. Ras-GAP binding and phosphorylation by herpes simplex virus type 2 RR1 PK (ICP10) and activation of the Ras/MEK/MAPK mitogenic pathway are required for timely onset of virus growth. *Journal of virology* **74**, 10417-10429 (2000).
- 143 Smith, C. C., Peng, T., Kulka, M. & Aurelian, L. The PK domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is required for immediate-early gene expression and virus growth. *Journal of virology* **72**, 9131-9141 (1998).
- 144 Kanai, R. *et al.* Effect of gamma34.5 deletions on oncolytic herpes simplex virus activity in brain tumors. *Journal of virology* **86**, 4420-4431, doi:10.1128/JVI.00017-12 (2012).
- 145 Mace, A. T., Ganly, I., Soutar, D. S. & Brown, S. M. Potential for efficacy of the oncolytic Herpes simplex virus 1716 in patients with oral squamous cell carcinoma. *Head & neck* **30**, 1045-1051, doi:10.1002/hed.20840 (2008).
- 146 Aurelian, L., Laing, J. M. & Lee, K. S. H11/HspB8 and Its Herpes Simplex Virus Type 2 Homologue ICP10PK Share Functions That Regulate Cell Life/Death Decisions and Human Disease. *Autoimmune diseases* **2012**, 395329, doi:10.1155/2012/395329 (2012).
- 147 Nagano, S., Perentes, J. Y., Jain, R. K. & Boucher, Y. Cancer cell death enhances the penetration and efficacy of oncolytic herpes simplex virus in tumors. *Cancer research* **68**, 3795-3802, doi:10.1158/0008-5472.CAN-07-6193 (2008).
- 148 Colunga, A. G., Laing, J. M. & Aurelian, L. The HSV-2 mutant DeltaPK induces melanoma oncolysis through nonredundant death programs and associated with autophagy and pyroptosis proteins. *Gene therapy* **17**, 315-327, doi:10.1038/gt.2009.126 (2010).
- 149 Gyotoku, T., Ono, F. & Aurelian, L. Development of HSV-specific CD4+ Th1 responses and CD8+ cytotoxic T lymphocytes with antiviral activity by vaccination with the HSV-2 mutant ICP10DeltaPK. *Vaccine* **20**, 2796-2807 (2002).
- 150 Aurelian, L. Herpes simplex virus type 2 vaccines: new ground for optimism? *Clinical and diagnostic laboratory immunology* **11**, 437-445, doi:10.1128/CDLI.11.3.437-445.2004 (2004).
- 151 Ren, X. W. *et al.* A tumor-specific conditionally replicative adenovirus vector expressing TRAIL for gene therapy of hepatocellular carcinoma. *Cancer gene therapy* **13**, 159-168, doi:10.1038/sj.cgt.7700868 (2006).
- 152 Yang, G. *et al.* Antitumor effects of a dual cancer-specific oncolytic adenovirus on colorectal cancer and. *Experimental and therapeutic medicine* **9**, 327-334, doi:10.3892/etm.2014.2086 (2015).

- 153 van Beusechem, V. W., van den Doel, P. B., Grill, J., Pinedo, H. M. & Gerritsen, W. R. Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer research* **62**, 6165-6171 (2002).
- 154 Hermiston, T. W. & Kuhn, I. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer gene therapy* **9**, 1022-1035, doi:10.1038/sj.cgt.7700542 (2002).
- 155 Yamada, S. *et al.* Oncolytic herpes simplex virus expressing yeast cytosine deaminase: relationship between viral replication, transgene expression, prodrug bioactivation. *Cancer gene therapy* **19**, 160-170, doi:10.1038/cgt.2011.70 (2012).
- 156 Chase, M., Chung, R. Y. & Chiocca, E. A. An oncolytic viral mutant that delivers the CYP2B1 transgene and augments cyclophosphamide chemotherapy. *Nature biotechnology* **16**, 444-448, doi:10.1038/nbt0598-444 (1998).
- 157 Schepelmann, S. *et al.* Suicide gene therapy of human colon carcinoma xenografts using an armed oncolytic adenovirus expressing carboxypeptidase G2. *Cancer research* **67**, 4949-4955, doi:10.1158/0008-5472.CAN-07-0297 (2007).
- 158 Fu, X., Nakamori, M., Tao, L., Amato, R. & Zhang, X. Antitumor effects of two newly constructed oncolytic herpes simplex viruses against renal cell carcinoma. *International journal of oncology* **30**, 1561-1567 (2007).
- 159 Chen, H. H. *et al.* Active adenoviral vascular penetration by targeted formation of heterocellular endothelial-epithelial syncytia. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 67-75, doi:10.1038/mt.2010.209 (2011).
- 160 Fu, X., Tao, L. & Zhang, X. An HSV-2-based oncolytic virus deleted in the PK domain of the ICP10 gene is a potent inducer of apoptotic death in tumor cells. *Gene therapy* **14**, 1218-1225, doi:10.1038/sj.gt.3302971 (2007).
- 161 Hersey, P. & Gallagher, S. Intralesional immunotherapy for melanoma. *Journal of surgical oncology* **109**, 320-326, doi:10.1002/jso.23494 (2014).
- 162 Cripe, T. P. *et al.* Phase 1 Study of Intratumoral Pexa-Vec (JX-594), an Oncolytic and Immunotherapeutic Vaccinia Virus, in Pediatric Cancer Patients. *Molecular therapy : the journal of the American Society of Gene Therapy* **23**, 602-608, doi:10.1038/mt.2014.243 (2015).
- 163 Muroya, M., Chang, K., Uchida, K., Bougaki, M. & Yamada, Y. Analysis of cytotoxicity induced by proinflammatory cytokines in the human alveolar epithelial cell line A549. *Bioscience trends* **6**, 70-80 (2012).
- 164 Han, Z. Q. *et al.* Development of a second-generation oncolytic Herpes simplex virus expressing TNFalpha for cancer therapy. *The journal of gene medicine* **9**, 99-106, doi:10.1002/jgm.999 (2007).
- 165 Opyrchal, M., Aderca, I. & Galanis, E. Phase I clinical trial of locoregional administration of the oncolytic adenovirus ONYX-015 in combination with mitomycin-C, doxorubicin, and cisplatin chemotherapy in patients with advanced sarcomas. *Methods in molecular biology* **542**, 705-717, doi:10.1007/978-1-59745-561-9_35 (2009).
- 166 Jha, B. K., Dong, B., Nguyen, C. T., Polyakova, I. & Silverman, R. H. Suppression of antiviral innate immunity by sunitinib enhances oncolytic virotherapy. *Molecular therapy : the journal of the American Society of Gene Therapy* **21**, 1749-1757, doi:10.1038/mt.2013.112 (2013).

- 167 Bose, A. *et al.* Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. *International journal of cancer. Journal international du cancer* **129**, 2158-2170, doi:10.1002/ijc.25863 (2011).
- 168 Nguyen, T. L. *et al.* Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14981-14986, doi:10.1073/pnas.0803988105 (2008).
- 169 Marks, P. A., Richon, V. M. & Rifkind, R. A. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *Journal of the National Cancer Institute* **92**, 1210-1216 (2000).
- 170 Gluzak, M. A. & Seto, E. Histone deacetylases and cancer. *Oncogene* **26**, 5420-5432, doi:10.1038/sj.onc.1210610 (2007).
- 171 Kim, H. J. & Bae, S. C. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American journal of translational research* **3**, 166-179 (2011).
- 172 Marks, P. A., Richon, V. M., Miller, T. & Kelly, W. K. Histone deacetylase inhibitors. *Advances in cancer research* **91**, 137-168, doi:10.1016/S0065-230X(04)91004-4 (2004).
- 173 Fouladi, M. Histone deacetylase inhibitors in cancer therapy. *Cancer investigation* **24**, 521-527, doi:10.1080/07357900600814979 (2006).
- 174 Dion, L. D. *et al.* Amplification of recombinant adenoviral transgene products occurs by inhibition of histone deacetylase. *Virology* **231**, 201-209, doi:10.1006/viro.1997.8538 (1997).
- 175 Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. & Townes, T. M. Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5798-5803 (1997).
- 176 Nusinzon, I. & Horvath, C. M. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14742-14747, doi:10.1073/pnas.2433987100 (2003).
- 177 Otsuki, A. *et al.* Histone deacetylase inhibitors augment antitumor efficacy of herpes-based oncolytic viruses. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 1546-1555, doi:10.1038/mt.2008.155 (2008).
- 178 Leng, Y. *et al.* Synergistic neuroprotective effects of lithium and valproic acid or other histone deacetylase inhibitors in neurons: roles of glycogen synthase kinase-3 inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 2576-2588, doi:10.1523/JNEUROSCI.5467-07.2008 (2008).
- 179 Chiu, C. T., Wang, Z., Hunsberger, J. G. & Chuang, D. M. Therapeutic potential of mood stabilizers lithium and valproic acid: beyond bipolar disorder. *Pharmacological reviews* **65**, 105-142, doi:10.1124/pr.111.005512 (2013).
- 180 Mesdjian, E. *et al.* Sodium valproate: kinetic profile and effects on GABA levels in various brain areas of the rat. *Progress in neuro-psychopharmacology & biological psychiatry* **6**, 223-233 (1982).

- 181 Zeise, M. L., Kasparow, S. & Zieglgansberger, W. Valproate suppresses N-methyl-D-aspartate-evoked, transient depolarizations in the rat neocortex in vitro. *Brain research* **544**, 345-348 (1991).
- 182 VanDongen, A. M., VanErp, M. G. & Voskuyl, R. A. Valproate reduces excitability by blockage of sodium and potassium conductance. *Epilepsia* **27**, 177-182 (1986).
- 183 Carriere, C. H., Kang, N. H. & Niles, L. P. Neuroprotection by valproic acid in an intrastriatal rotenone model of Parkinson's disease. *Neuroscience* **267**, 114-121, doi:10.1016/j.neuroscience.2014.02.028 (2014).
- 184 Chen, S., Wu, H., Klebe, D., Hong, Y. & Zhang, J. Valproic Acid: A New Candidate of Therapeutic Application for the Acute Central Nervous System Injuries. *Neurochemical research*, doi:10.1007/s11064-014-1241-2 (2014).
- 185 Zhang, C. *et al.* Neuroprotective and anti-apoptotic effects of valproic acid on adult rat cerebral cortex through ERK and Akt signaling pathway at acute phase of traumatic brain injury. *Brain research*, doi:10.1016/j.brainres.2014.01.051 (2014).
- 186 Han, B. R., You, B. R. & Park, W. H. Valproic acid inhibits the growth of HeLa cervical cancer cells via caspase-dependent apoptosis. *Oncology reports* **30**, 2999-3005, doi:10.3892/or.2013.2747 (2013).
- 187 Shah, R. D. *et al.* Sodium valproate potentiates staurosporine-induced apoptosis in neuroblastoma cells via Akt/survivin independently of HDAC inhibition. *Journal of cellular biochemistry* **114**, 854-863, doi:10.1002/jcb.24422 (2013).
- 188 Jin, N., Kovacs, A. D., Sui, Z., Dewhurst, S. & Maggirwar, S. B. Opposite effects of lithium and valproic acid on trophic factor deprivation-induced glycogen synthase kinase-3 activation, c-Jun expression and neuronal cell death. *Neuropharmacology* **48**, 576-583, doi:10.1016/j.neuropharm.2004.11.010 (2005).
- 189 Umka, J. *et al.* Valproic acid reduces spatial working memory and cell proliferation in the hippocampus. *Neuroscience* **166**, 15-22, doi:10.1016/j.neuroscience.2009.11.073 (2010).
- 190 Wang, C. *et al.* Valproic acid induces apoptosis in differentiating hippocampal neurons by the release of tumor necrosis factor-alpha from activated astrocytes. *Neuroscience letters* **497**, 122-127, doi:10.1016/j.neulet.2011.04.044 (2011).
- 191 Arndt, T. L., Stodgell, C. J. & Rodier, P. M. The teratology of autism. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* **23**, 189-199, doi:10.1016/j.ijdevneu.2004.11.001 (2005).
- 192 Christensen, J. *et al.* Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *Jama* **309**, 1696-1703, doi:10.1001/jama.2013.2270 (2013).
- 193 Chaudhary, S. & Parvez, S. An in vitro approach to assess the neurotoxicity of valproic acid-induced oxidative stress in cerebellum and cerebral cortex of young rats. *Neuroscience* **225**, 258-268, doi:10.1016/j.neuroscience.2012.08.060 (2012).
- 194 Sheikh, A. M. *et al.* Cathepsin D and apoptosis related proteins are elevated in the brain of autistic subjects. *Neuroscience* **165**, 363-370, doi:10.1016/j.neuroscience.2009.10.035 (2010).

- 195 Fujiki, R., Sato, A., Fujitani, M. & Yamashita, T. A proapoptotic effect of valproic acid on progenitors of embryonic stem cell-derived glutamatergic neurons. *Cell death & disease* **4**, e677, doi:10.1038/cddis.2013.205 (2013).
- 196 Sheikh, A. M. *et al.* BDNF-Akt-Bcl2 antiapoptotic signaling pathway is compromised in the brain of autistic subjects. *Journal of neuroscience research* **88**, 2641-2647, doi:10.1002/jnr.22416 (2010).
- 197 Ingram, J. L., Peckham, S. M., Tisdale, B. & Rodier, P. M. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicology and teratology* **22**, 319-324 (2000).
- 198 Yochum, C. L., Dowling, P., Reuhl, K. R., Wagner, G. C. & Ming, X. VPA-induced apoptosis and behavioral deficits in neonatal mice. *Brain research* **1203**, 126-132, doi:10.1016/j.brainres.2008.01.055 (2008).
- 199 Tung, E. W. & Winn, L. M. Valproic acid increases formation of reactive oxygen species and induces apoptosis in postimplantation embryos: a role for oxidative stress in valproic acid-induced neural tube defects. *Molecular pharmacology* **80**, 979-987, doi:10.1124/mol.111.072314 (2011).
- 200 Bittigau, P., Sifringer, M. & Ikonomidou, C. Antiepileptic drugs and apoptosis in the developing brain. *Annals of the New York Academy of Sciences* **993**, 103-114; discussion 123-104 (2003).
- 201 Johannessen, C. U. Mechanisms of action of valproate: a commentary. *Neurochemistry international* **37**, 103-110 (2000).
- 202 Forgione, N. & Tropepe, V. Histone deacetylase inhibition promotes Caspase-independent cell death of ventral midbrain neurons. *Molecular and cellular neurosciences* **48**, 117-128, doi:10.1016/j.mcn.2011.06.012 (2011).
- 203 Duenas-Gonzalez, A. *et al.* Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. *Cancer treatment reviews* **34**, 206-222, doi:10.1016/j.ctrv.2007.11.003 (2008).
- 204 White, M. C. & Frampton, A. R., Jr. The histone deacetylase inhibitor valproic acid enhances equine herpesvirus type 1 (EHV-1)-mediated oncolysis of human glioma cells. *Cancer gene therapy* **20**, 88-93, doi:10.1038/cgt.2012.89 (2013).
- 205 Breitbach, C. J. *et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* **477**, 99-102, doi:10.1038/nature10358 (2011).
- 206 Russell, S. J., Peng, K. W. & Bell, J. C. Oncolytic virotherapy. *Nature biotechnology* **30**, 658-670, doi:10.1038/nbt.2287 (2012).
- 207 Breitbach, C. J. *et al.* Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Molecular therapy : the journal of the American Society of Gene Therapy* **15**, 1686-1693, doi:10.1038/sj.mt.6300215 (2007).
- 208 Chiocca, E. A. & Rabkin, S. D. Oncolytic viruses and their application to cancer immunotherapy. *Cancer immunology research* **2**, 295-300, doi:10.1158/2326-6066.CIR-14-0015 (2014).
- 209 Vaha-Koskela, M. J., Heikkila, J. E. & Hinkkanen, A. E. Oncolytic viruses in cancer therapy. *Cancer letters* **254**, 178-216, doi:10.1016/j.canlet.2007.02.002 (2007).

- 210 Wales, S. Q., Laing, J. M., Chen, L. & Aurelian, L. ICP10PK inhibits calpain-dependent release of apoptosis-inducing factor and programmed cell death in response to the toxin MPP+. *Gene therapy* **15**, 1397-1409, doi:10.1038/gt.2008.88 (2008).
- 211 Sano, M., Nishiyama, K. & Kitajima, S. A nerve growth factor-dependent protein kinase that phosphorylates microtubule-associated proteins in vitro: possible involvement of its activity in the outgrowth of neurites from PC12 cells. *Journal of neurochemistry* **55**, 427-435 (1990).
- 212 Courtenay, V. D. A soft agar colony assay for Lewis lung tumour and B16 melanoma taken directly from the mouse. *British journal of cancer* **34**, 39-45 (1976).
- 213 Visvader, J. E. & Lindeman, G. J. Cancer stem cells: current status and evolving complexities. *Cell stem cell* **10**, 717-728, doi:10.1016/j.stem.2012.05.007 (2012).
- 214 Fukunaga-Kalabis, M., Roesch, A. & Herlyn, M. From cancer stem cells to tumor maintenance in melanoma. *The Journal of investigative dermatology* **131**, 1600-1604, doi:10.1038/jid.2011.159 (2011).
- 215 Dufau, I. *et al.* Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer. *BMC cancer* **12**, 15, doi:10.1186/1471-2407-12-15 (2012).
- 216 Colunga, A., Bollino, D., Schech, A. & Aurelian, L. Calpain-dependent clearance of the autophagy protein p62/SQSTM1 is a contributor to DeltaPK oncolytic activity in melanoma. *Gene therapy* **21**, 371-378, doi:10.1038/gt.2014.6 (2014).
- 217 Komatsu, M., Kageyama, S. & Ichimura, Y. p62/SQSTM1/A170: physiology and pathology. *Pharmacological research : the official journal of the Italian Pharmacological Society* **66**, 457-462, doi:10.1016/j.phrs.2012.07.004 (2012).
- 218 Wakimoto, H. *et al.* Human glioblastoma-derived cancer stem cells: establishment of invasive glioma models and treatment with oncolytic herpes simplex virus vectors. *Cancer research* **69**, 3472-3481, doi:10.1158/0008-5472.CAN-08-3886 (2009).
- 219 Mahller, Y. Y. *et al.* Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. *PloS one* **4**, e4235, doi:10.1371/journal.pone.0004235 (2009).
- 220 Pressey, J. G. *et al.* CD133 marks a myogenically primitive subpopulation in rhabdomyosarcoma cell lines that are relatively chemoresistant but sensitive to mutant HSV. *Pediatric blood & cancer* **60**, 45-52, doi:10.1002/pbc.24117 (2013).
- 221 Kanai, R., Wakimoto, H., Martuza, R. L. & Rabkin, S. D. A novel oncolytic herpes simplex virus that synergizes with phosphoinositide 3-kinase/Akt pathway inhibitors to target glioblastoma stem cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 3686-3696, doi:10.1158/1078-0432.CCR-10-3142 (2011).
- 222 Perkins, D., Pereira, E. F., Gober, M., Yarowsky, P. J. & Aurelian, L. The herpes simplex virus type 2 R1 protein kinase (ICP10 PK) blocks apoptosis in hippocampal neurons, involving activation of the MEK/MAPK survival pathway. *Journal of virology* **76**, 1435-1449 (2002).

- 223 Yee, S. B. *et al.* zVAD-fmk, unlike BocD-fmk, does not inhibit caspase-6 acting on 14-3-3/Bad pathway in apoptosis of p815 mastocytoma cells. *Experimental & molecular medicine* **38**, 634-642, doi:10.1038/emm.2006.75 (2006).
- 224 Rodriguez-Enfedaque, A. *et al.* zVAD-fmk upregulates caspase-9 cleavage and activity in etoposide-induced cell death of mouse embryonic fibroblasts. *Biochimica et biophysica acta* **1823**, 1343-1352, doi:10.1016/j.bbamcr.2012.05.013 (2012).
- 225 Kim, J. W., Ho, W. J. & Wu, B. M. The role of the 3D environment in hypoxia-induced drug and apoptosis resistance. *Anticancer research* **31**, 3237-3245 (2011).
- 226 Gong, C. *et al.* Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. *Oncogene* **32**, 2261-2272, 2272e 2261-2211, doi:10.1038/onc.2012.252 (2013).
- 227 Qu, X. *et al.* Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *The Journal of clinical investigation* **112**, 1809-1820, doi:10.1172/JCI20039 (2003).
- 228 Komatsu, M. *et al.* The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature cell biology* **12**, 213-223, doi:10.1038/ncb2021 (2010).
- 229 Valencia, T. *et al.* Metabolic reprogramming of stromal fibroblasts through p62-mTORC1 signaling promotes inflammation and tumorigenesis. *Cancer cell* **26**, 121-135, doi:10.1016/j.ccr.2014.05.004 (2014).
- 230 Duran, A. *et al.* The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. *Cancer cell* **13**, 343-354, doi:10.1016/j.ccr.2008.02.001 (2008).
- 231 Yeh, L. Y. *et al.* miR-372 inhibits p62 in head and neck squamous cell carcinoma in vitro and in vivo. *Oncotarget* (2015).
- 232 Parkhitko, A. *et al.* Tumorigenesis in tuberous sclerosis complex is autophagy and p62/sequestosome 1 (SQSTM1)-dependent. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 12455-12460, doi:10.1073/pnas.1104361108 (2011).
- 233 Inoue, D. *et al.* Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma. *Cancer science* **103**, 760-766, doi:10.1111/j.1349-7006.2012.02216.x (2012).
- 234 Rolland, P. *et al.* The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. *Endocrine-related cancer* **14**, 73-80, doi:10.1677/erc.1.01312 (2007).
- 235 Bridle, B. W. *et al.* Potentiating cancer immunotherapy using an oncolytic virus. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 1430-1439, doi:10.1038/mt.2010.98 (2010).
- 236 Thorne, S. H. Immunotherapeutic potential of oncolytic vaccinia virus. *Immunologic research* **50**, 286-293, doi:10.1007/s12026-011-8211-4 (2011).
- 237 Workenhe, S. T. & Mossman, K. L. Oncolytic virotherapy and immunogenic cancer cell death: sharpening the sword for improved cancer treatment strategies. *Molecular therapy : the journal of the American Society of Gene Therapy* **22**, 251-256, doi:10.1038/mt.2013.220 (2014).

- 238 Wolf, A. *et al.* Identification and functional characterization of novel phosphorylation sites in TAK1-binding protein (TAB) 1. *PloS one* **6**, e29256, doi:10.1371/journal.pone.0029256 (2011).
- 239 Machida, N. *et al.* Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. *The Journal of biological chemistry* **279**, 15711-15714, doi:10.1074/jbc.C300542200 (2004).
- 240 Han, J. *et al.* Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *The Journal of biological chemistry* **271**, 2886-2891 (1996).
- 241 Vallabhapurapu, S. *et al.* Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. *Nature immunology* **9**, 1364-1370, doi:10.1038/ni.1678 (2008).
- 242 Ma, Y. *et al.* Tumor necrosis factor is dispensable for the success of immunogenic anticancer chemotherapy. *Oncoimmunology* **2**, e24786, doi:10.4161/onci.24786 (2013).
- 243 Tian, H. *et al.* Cellular immunotherapy using irradiated lung cancer cell vaccine co-expressing GM-CSF and IL-18 can induce significant antitumor effects. *BMC cancer* **14**, 48, doi:10.1186/1471-2407-14-48 (2014).
- 244 Roncarolo, M. G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* **212**, 28-50, doi:10.1111/j.0105-2896.2006.00420.x (2006).
- 245 Chen, L. *et al.* Cotransfection with IL-10 and TGF-beta1 into immature dendritic cells enhances immune tolerance in a rat liver transplantation model. *American journal of physiology. Gastrointestinal and liver physiology* **306**, G575-581, doi:10.1152/ajpgi.00283.2013 (2014).
- 246 Oltmanns, U., Issa, R., Sukkar, M. B., John, M. & Chung, K. F. Role of c-jun N-terminal kinase in the induced release of GM-CSF, RANTES and IL-8 from human airway smooth muscle cells. *British journal of pharmacology* **139**, 1228-1234, doi:10.1038/sj.bjp.0705345 (2003).
- 247 Angel, P. & Karin, M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et biophysica acta* **1072**, 129-157 (1991).
- 248 Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology* **2**, 675-680, doi:10.1038/90609 (2001).
- 249 Kurt-Jones, E. A. *et al.* Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1315-1320, doi:10.1073/pnas.0308057100 (2004).
- 250 Kim, M. *et al.* Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes. *Journal of immunology* **188**, 4158-4170, doi:10.4049/jimmunol.1103450 (2012).
- 251 Harris, J. Autophagy and cytokines. *Cytokine* **56**, 140-144, doi:10.1016/j.cyto.2011.08.022 (2011).

- 252 Crisan, T. O. *et al.* Inflammasome-independent modulation of cytokine response by autophagy in human cells. *PloS one* **6**, e18666, doi:10.1371/journal.pone.0018666 (2011).
- 253 Li, Y. *et al.* Efficient cross-presentation depends on autophagy in tumor cells. *Cancer research* **68**, 6889-6895, doi:10.1158/0008-5472.CAN-08-0161 (2008).
- 254 Uhl, M. *et al.* Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. *Cell death and differentiation* **16**, 991-1005, doi:10.1038/cdd.2009.8 (2009).
- 255 Seglen, P. O. & Gordon, P. B. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1889-1892 (1982).
- 256 Kim, C. *et al.* Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. *Nature communications* **4**, 1562, doi:10.1038/ncomms2534 (2013).
- 257 Sutterwala, F. S., Haasken, S. & Cassel, S. L. Mechanism of NLRP3 inflammasome activation. *Annals of the New York Academy of Sciences* **1319**, 82-95, doi:10.1111/nyas.12458 (2014).
- 258 Fernandes-Alnemri, T. *et al.* The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell death and differentiation* **14**, 1590-1604, doi:10.1038/sj.cdd.4402194 (2007).
- 259 Senovilla, L. *et al.* Immunosurveillance against cancer-associated hyperploidy. *Oncotarget* **3**, 1270-1271 (2012).
- 260 Lauerova, L. *et al.* Malignant melanoma associates with Th1/Th2 imbalance that coincides with disease progression and immunotherapy response. *Neoplasma* **49**, 159-166 (2002).
- 261 Zou, W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nature reviews. Cancer* **5**, 263-274, doi:10.1038/nrc1586 (2005).
- 262 DeNardo, D. G., Andreu, P. & Coussens, L. M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer metastasis reviews* **29**, 309-316, doi:10.1007/s10555-010-9223-6 (2010).
- 263 Ruffell, B., DeNardo, D. G., Affara, N. I. & Coussens, L. M. Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine & growth factor reviews* **21**, 3-10, doi:10.1016/j.cytogfr.2009.11.002 (2010).
- 264 Hirvonen, M. *et al.* Immunological effects of a TNF-alpha armed oncolytic adenovirus. *Human gene therapy*, doi:10.1089/hum.2014.069 (2015).
- 265 Kaufman, H. L., Ruby, C. E., Hughes, T. & Slingsluff, C. L., Jr. Current status of granulocyte-macrophage colony-stimulating factor in the immunotherapy of melanoma. *Journal for immunotherapy of cancer* **2**, 11, doi:10.1186/2051-1426-2-11 (2014).
- 266 Parviainen, S. *et al.* GM-CSF-armed vaccinia virus induces an antitumor immune response. *International journal of cancer. Journal international du cancer* **136**, 1065-1072, doi:10.1002/ijc.29068 (2015).

- 267 Angelova, A. L. *et al.* Complementary induction of immunogenic cell death by oncolytic parvovirus H-1PV and gemcitabine in pancreatic cancer. *Journal of virology* **88**, 5263-5276, doi:10.1128/JVI.03688-13 (2014).
- 268 Zamarin, D. *et al.* Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. *Science translational medicine* **6**, 226ra232, doi:10.1126/scitranslmed.3008095 (2014).
- 269 Mortha, A. *et al.* Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* **343**, 1249288, doi:10.1126/science.1249288 (2014).
- 270 Buelens, C. *et al.* Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. *European journal of immunology* **27**, 756-762, doi:10.1002/eji.1830270326 (1997).
- 271 de Waal Malefyt, R. *et al.* Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *The Journal of experimental medicine* **174**, 915-924 (1991).
- 272 Errington, F. *et al.* Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. *Gene therapy* **15**, 1257-1270, doi:10.1038/gt.2008.58 (2008).
- 273 Moretta, A. *et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annual review of immunology* **19**, 197-223, doi:10.1146/annurev.immunol.19.1.197 (2001).
- 274 Chanteux, H., Guisset, A. C., Pilette, C. & Sibille, Y. LPS induces IL-10 production by human alveolar macrophages via MAPKs- and Sp1-dependent mechanisms. *Respiratory research* **8**, 71, doi:10.1186/1465-9921-8-71 (2007).
- 275 Norkina, O. *et al.* Acute alcohol activates STAT3, AP-1, and Sp-1 transcription factors via the family of Src kinases to promote IL-10 production in human monocytes. *Journal of leukocyte biology* **82**, 752-762, doi:10.1189/jlb.0207099 (2007).
- 276 Dobrova, Z. G., Miteva, L. D. & Stanilova, S. A. The inhibition of JNK and p38 MAPKs downregulates IL-10 and differentially affects c-Jun gene expression in human monocytes. *Immunopharmacology and immunotoxicology* **31**, 195-201, doi:10.1080/08923970802626276 (2009).
- 277 Armeanu, S. *et al.* Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer research* **65**, 6321-6329, doi:10.1158/0008-5472.CAN-04-4252 (2005).
- 278 Skov, S. *et al.* Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. *Cancer research* **65**, 11136-11145, doi:10.1158/0008-5472.CAN-05-0599 (2005).
- 279 Inoue, H. & Tani, K. Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments. *Cell death and differentiation* **21**, 39-49, doi:10.1038/cdd.2013.84 (2014).

- 280 Aravalli, R. N., Hu, S., Rowen, T. N., Palmquist, J. M. & Lokensgard, J. R. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. *Journal of immunology* **175**, 4189-4193 (2005).
- 281 Hornung, V. & Latz, E. Critical functions of priming and lysosomal damage for NLRP3 activation. *European journal of immunology* **40**, 620-623, doi:10.1002/eji.200940185 (2010).
- 282 Teft, W. A., Kirchhof, M. G. & Madrenas, J. A molecular perspective of CTLA-4 function. *Annual review of immunology* **24**, 65-97, doi:10.1146/annurev.immunol.24.021605.090535 (2006).
- 283 Revicki, D. A. *et al.* Health related quality of life outcomes for unresectable stage III or IV melanoma patients receiving ipilimumab treatment. *Health and quality of life outcomes* **10**, 66, doi:10.1186/1477-7525-10-66 (2012).
- 284 Chateauvieux, S., Morceau, F., Dicato, M. & Diederich, M. Molecular and therapeutic potential and toxicity of valproic acid. *Journal of biomedicine & biotechnology* **2010**, doi:10.1155/2010/479364 (2010).
- 285 Francois, F., Godinho, M. J., Dragunow, M. & Grimes, M. L. A population of PC12 cells that is initiating apoptosis can be rescued by nerve growth factor. *Molecular and cellular neurosciences* **18**, 347-362, doi:10.1006/mcne.2001.1035 (2001).
- 286 Valavanis, C. *et al.* Model cell lines for the study of apoptosis in vitro. *Methods in cell biology* **66**, 417-436 (2001).
- 287 Ornoy, A. Valproic acid in pregnancy: how much are we endangering the embryo and fetus? *Reproductive toxicology* **28**, 1-10, doi:10.1016/j.reprotox.2009.02.014 (2009).
- 288 Dragunow, M. *et al.* Valproic acid induces caspase 3-mediated apoptosis in microglial cells. *Neuroscience* **140**, 1149-1156, doi:10.1016/j.neuroscience.2006.02.065 (2006).
- 289 Kanai, H., Sawa, A., Chen, R. W., Leeds, P. & Chuang, D. M. Valproic acid inhibits histone deacetylase activity and suppresses excitotoxicity-induced GAPDH nuclear accumulation and apoptotic death in neurons. *The pharmacogenomics journal* **4**, 336-344, doi:10.1038/sj.tpj.6500269 (2004).
- 290 Perkins, D., Pereira, E. F. & Aurelian, L. The herpes simplex virus type 2 R1 protein kinase (ICP10 PK) functions as a dominant regulator of apoptosis in hippocampal neurons involving activation of the ERK survival pathway and upregulation of the antiapoptotic protein Bag-1. *Journal of virology* **77**, 1292-1305 (2003).
- 291 Gober, M. D., Laing, J. M., Thompson, S. M. & Aurelian, L. The growth compromised HSV-2 mutant DeltaRR prevents kainic acid-induced apoptosis and loss of function in organotypic hippocampal cultures. *Brain research* **1119**, 26-39, doi:10.1016/j.brainres.2006.08.078 (2006).
- 292 Laing, J. M. *et al.* Intranasal administration of the growth-compromised HSV-2 vector DeltaRR prevents kainate-induced seizures and neuronal loss in rats and mice. *Molecular therapy : the journal of the American Society of Gene Therapy* **13**, 870-881, doi:10.1016/j.ymthe.2005.12.013 (2006).

- 293 Golembewski, E. K., Wales, S. Q., Aurelian, L. & Yarowsky, P. J. The HSV-2 protein ICP10PK prevents neuronal apoptosis and loss of function in an in vivo model of neurodegeneration associated with glutamate excitotoxicity. *Experimental neurology* **203**, 381-393, doi:10.1016/j.expneurol.2006.08.022 (2007).
- 294 Coffey, E. T. Nuclear and cytosolic JNK signalling in neurons. *Nature reviews. Neuroscience* **15**, 285-299, doi:10.1038/nrn3729 (2014).
- 295 Mielke, K., Damm, A., Yang, D. D. & Herdegen, T. Selective expression of JNK isoforms and stress-specific JNK activity in different neural cell lines. *Brain research. Molecular brain research* **75**, 128-137 (2000).
- 296 Waetzig, V. & Herdegen, T. A single c-Jun N-terminal kinase isoform (JNK3-p54) is an effector in both neuronal differentiation and cell death. *The Journal of biological chemistry* **278**, 567-572, doi:10.1074/jbc.M207391200 (2003).
- 297 Festjens, N., Vanden Berghe, T., Cornelis, S. & Vandenabeele, P. RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell death and differentiation* **14**, 400-410, doi:10.1038/sj.cdd.4402085 (2007).
- 298 Cho, Y. S. *et al.* Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137**, 1112-1123, doi:10.1016/j.cell.2009.05.037 (2009).
- 299 Vandenabeele, P., Declercq, W., Van Herreweghe, F. & Vanden Berghe, T. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Science signaling* **3**, re4, doi:10.1126/scisignal.3115re4 (2010).
- 300 Han, W., Xie, J., Fang, Y., Wang, Z. & Pan, H. Nec-1 Enhances Shikonin-Induced Apoptosis in Leukemia Cells by Inhibition of RIP-1 and ERK1/2. *International journal of molecular sciences* **13**, 7212-7225, doi:10.3390/ijms13067212 (2012).
- 301 Xiong, S., Mu, T., Wang, G. & Jiang, X. Mitochondria-mediated apoptosis in mammals. *Protein & cell* **5**, 737-749, doi:10.1007/s13238-014-0089-1 (2014).
- 302 Sevrioukova, I. F. Apoptosis-inducing factor: structure, function, and redox regulation. *Antioxidants & redox signaling* **14**, 2545-2579, doi:10.1089/ars.2010.3445 (2011).
- 303 Delavallee, L., Cabon, L., Galan-Malo, P., Lorenzo, H. K. & Susin, S. A. AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. *IUBMB life* **63**, 221-232, doi:10.1002/iub.432 (2011).
- 304 Pasupuleti, N., Leon, L., Carraway, K. L., 3rd & Gorin, F. 5-Benzylglycinylnilamide kills proliferating and nonproliferating malignant glioma cells through caspase-independent necroptosis mediated by apoptosis-inducing factor. *The Journal of pharmacology and experimental therapeutics* **344**, 600-615, doi:10.1124/jpet.112.200519 (2013).
- 305 Morizane, Y., Honda, R., Fukami, K. & Yasuda, H. X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO. *Journal of biochemistry* **137**, 125-132, doi:10.1093/jb/mvi029 (2005).
- 306 Loscher, W. Basic pharmacology of valproate: a review after 35 years of clinical use for the treatment of epilepsy. *CNS drugs* **16**, 669-694 (2002).

- 307 Green, D. R. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* **102**, 1-4 (2000).
- 308 Danial, N. N. & Korsmeyer, S. J. Cell death: critical control points. *Cell* **116**, 205-219 (2004).
- 309 Storr, S. J., Carragher, N. O., Frame, M. C., Parr, T. & Martin, S. G. The calpain system and cancer. *Nature reviews. Cancer* **11**, 364-374, doi:10.1038/nrc3050 (2011).
- 310 Cabon, L. *et al.* BID regulates AIF-mediated caspase-independent necroptosis by promoting BAX activation. *Cell death and differentiation* **19**, 245-256, doi:10.1038/cdd.2011.91 (2012).
- 311 Autheman, D. *et al.* Clostridium perfringens beta-toxin induces necrostatin-inhibitable, calpain-dependent necrosis in primary porcine endothelial cells. *PLoS one* **8**, e64644, doi:10.1371/journal.pone.0064644 (2013).
- 312 Haeusgen, W., Boehm, R., Zhao, Y., Herdegen, T. & Waetzig, V. Specific activities of individual c-Jun N-terminal kinases in the brain. *Neuroscience* **161**, 951-959, doi:10.1016/j.neuroscience.2009.04.014 (2009).
- 313 Zhao, Y. *et al.* The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. *Molecular and cellular neurosciences* **49**, 300-310, doi:10.1016/j.mcn.2011.12.005 (2012).
- 314 Reinecke, K., Herdegen, T., Eminel, S., Aldenhoff, J. B. & Schiffelholz, T. Knockout of c-Jun N-terminal kinases 1, 2 or 3 isoforms induces behavioural changes. *Behavioural brain research* **245**, 88-95, doi:10.1016/j.bbr.2013.02.013 (2013).
- 315 Huang, Y. *et al.* Endoplasmic reticulum stress-induced hepatic stellate cell apoptosis through calcium-mediated JNK/P38 MAPK and Calpain/Caspase-12 pathways. *Molecular and cellular biochemistry* **394**, 1-12, doi:10.1007/s11010-014-2073-8 (2014).
- 316 Douglas, D. L. & Baines, C. P. PARP1-mediated necrosis is dependent on parallel JNK and Ca(2+)-calpain pathways. *Journal of cell science* **127**, 4134-4145, doi:10.1242/jcs.128009 (2014).
- 317 Lai, T. W., Zhang, S. & Wang, Y. T. Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Progress in neurobiology* **115**, 157-188, doi:10.1016/j.pneurobio.2013.11.006 (2014).
- 318 Tan, Y. *et al.* Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *The Journal of biological chemistry* **281**, 16016-16024, doi:10.1074/jbc.M601299200 (2006).
- 319 Stockhausen, M. T., Sjolund, J., Manetopoulos, C. & Axelson, H. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. *British journal of cancer* **92**, 751-759, doi:10.1038/sj.bjc.6602309 (2005).
- 320 El-Khoury, V. *et al.* The histone deacetylase inhibitor MGCD0103 induces apoptosis in B-cell chronic lymphocytic leukemia cells through a mitochondria-mediated caspase activation cascade. *Molecular cancer therapeutics* **9**, 1349-1360, doi:10.1158/1535-7163.MCT-09-1000 (2010).
- 321 Christofferson, D. E. *et al.* A novel role for RIP1 kinase in mediating TNF α production. *Cell death & disease* **3**, e320, doi:10.1038/cddis.2012.64 (2012).

- 322 Hirsch, B., von der Wall, E., Hummel, M. & Durkop, H. RIP1 expression is necessary for CD30-mediated cell death induction in anaplastic large-cell lymphoma cells. *Laboratory investigation; a journal of technical methods and pathology* **93**, 677-689, doi:10.1038/labinvest.2013.50 (2013).
- 323 Mahadeo, D., Kaplan, L., Chao, M. V. & Hempstead, B. L. High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *The Journal of biological chemistry* **269**, 6884-6891 (1994).
- 324 Artus, C. *et al.* AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX. *The EMBO journal* **29**, 1585-1599, doi:10.1038/emboj.2010.43 (2010).
- 325 Baritaud, M. *et al.* AIF-mediated caspase-independent necroptosis requires ATM and DNA-PK-induced histone H2AX Ser139 phosphorylation. *Cell death & disease* **3**, e390, doi:10.1038/cddis.2012.120 (2012).
- 326 Wennier, S. T., Liu, J. & McFadden, G. Bugs and drugs: oncolytic virotherapy in combination with chemotherapy. *Current pharmaceutical biotechnology* **13**, 1817-1833 (2012).
- 327 Ottolino-Perry, K., Diallo, J. S., Lichty, B. D., Bell, J. C. & McCart, J. A. Intelligent design: combination therapy with oncolytic viruses. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 251-263, doi:10.1038/mt.2009.283 (2010).
- 328 Chang, H. M. *et al.* Induction of interferon-stimulated gene expression and antiviral responses require protein deacetylase activity. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 9578-9583, doi:10.1073/pnas.0400567101 (2004).
- 329 Mehnert, J. M. & Kelly, W. K. Histone deacetylase inhibitors: biology and mechanism of action. *Cancer journal* **13**, 23-29, doi:10.1097/PPO.0b013e31803c72ba (2007).
- 330 Alvarez-Breckenridge, C. A. *et al.* The histone deacetylase inhibitor valproic acid lessens NK cell action against oncolytic virus-infected glioblastoma cells by inhibition of STAT5/T-BET signaling and generation of gamma interferon. *Journal of virology* **86**, 4566-4577, doi:10.1128/JVI.05545-11 (2012).
- 331 Watanabe, Y. *et al.* Enhanced antitumor efficacy of telomerase-specific oncolytic adenovirus with valproic acid against human cancer cells. *Cancer gene therapy* **19**, 767-772, doi:10.1038/cgt.2012.57 (2012).
- 332 Bollino, D., Balan, I. & Aurelian, L. Valproic acid induces neuronal cell death through a novel calpain-dependent necroptosis pathway. *Journal of neurochemistry*, doi:10.1111/jnc.13029 (2015).
- 333 Licciardi, P. V., Ververis, K., Tang, M. L., El-Osta, A. & Karagiannis, T. C. Immunomodulatory effects of histone deacetylase inhibitors. *Current molecular medicine* **13**, 640-647 (2013).
- 334 Chavez, K. J., Garimella, S. V. & Lipkowitz, S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast disease* **32**, 35-48, doi:10.3233/BD-2010-0307 (2010).
- 335 Yamano, T. *et al.* Amplification of transgene expression in vitro and in vivo using a novel inhibitor of histone deacetylase. *Molecular therapy : the journal of the*

- American Society of Gene Therapy* **1**, 574-580, doi:10.1006/mthe.2000.0074 (2000).
- 336 Vlasakova, J. *et al.* Histone deacetylase inhibitors suppress IFN α -induced up-regulation of promyelocytic leukemia protein. *Blood* **109**, 1373-1380, doi:10.1182/blood-2006-02-003418 (2007).
- 337 Gotfryd, K. *et al.* Cell type-specific anti-cancer properties of valproic acid: independent effects on HDAC activity and Erk1/2 phosphorylation. *BMC cancer* **10**, 383, doi:10.1186/1471-2407-10-383 (2010).
- 338 Ropero, S. & Esteller, M. The role of histone deacetylases (HDACs) in human cancer. *Molecular oncology* **1**, 19-25, doi:10.1016/j.molonc.2007.01.001 (2007).
- 339 Bridle, B. W. *et al.* HDAC inhibition suppresses primary immune responses, enhances secondary immune responses, and abrogates autoimmunity during tumor immunotherapy. *Molecular therapy : the journal of the American Society of Gene Therapy* **21**, 887-894, doi:10.1038/mt.2012.265 (2013).
- 340 Edukulla, R. *et al.* Antitumoral immune response by recruitment and expansion of dendritic cells in tumors infected with telomerase-dependent oncolytic viruses. *Cancer research* **69**, 1448-1458, doi:10.1158/0008-5472.CAN-08-1160 (2009).
- 341 Banaszynski, L. A., Sellmyer, M. A., Contag, C. H., Wandless, T. J. & Thorne, S. H. Chemical control of protein stability and function in living mice. *Nature medicine* **14**, 1123-1127, doi:10.1038/nm.1754 (2008).
- 342 Pich, C. *et al.* Statins Reduce Melanoma Development and Metastasis through MICA Overexpression. *Frontiers in immunology* **4**, 62, doi:10.3389/fimmu.2013.00062 (2013).
- 343 Fidler, I. J. & Nicolson, G. L. Organ selectivity for implantation survival and growth of B16 melanoma variant tumor lines. *Journal of the National Cancer Institute* **57**, 1199-1202 (1976).
- 344 Castle, J. C. *et al.* Exploiting the mutanome for tumor vaccination. *Cancer research* **72**, 1081-1091, doi:10.1158/0008-5472.CAN-11-3722 (2012).
- 345 Alvarez-Breckenridge, C. A. *et al.* NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors. *Nature medicine* **18**, 1827-1834, doi:10.1038/nm.3013 (2012).
- 346 Grant, S. & Dai, Y. Histone deacetylase inhibitors and rational combination therapies. *Advances in cancer research* **116**, 199-237, doi:10.1016/B978-0-12-394387-3.00006-9 (2012).
- 347 Shi, P. *et al.* Valproic acid sensitizes pancreatic cancer cells to natural killer cell-mediated lysis by upregulating MICA and MICB via the PI3K/Akt signaling pathway. *BMC cancer* **14**, 370, doi:10.1186/1471-2407-14-370 (2014).
- 348 Chahlavi, A., Rabkin, S., Todo, T., Sundaresan, P. & Martuza, R. Effect of prior exposure to herpes simplex virus 1 on viral vector-mediated tumor therapy in immunocompetent mice. *Gene therapy* **6**, 1751-1758, doi:10.1038/sj.gt.3301003 (1999).
- 349 Liu, B. L. *et al.* ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene therapy* **10**, 292-303, doi:10.1038/sj.gt.3301885 (2003).
- 350 Miller, C. G. & Fraser, N. W. Requirement of an integrated immune response for successful neuroattenuated HSV-1 therapy in an intracranial metastatic melanoma

model. *Molecular therapy : the journal of the American Society of Gene Therapy*
7, 741-747 (2003).