BNIP3 MUTATIONS IN BREAST CANCER

By

Nicolle Bristow

A thesis
Submitted to the faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
of Masters of Science

Department of Biochemistry and Medical Genetics
University of Manitoba
Winnipeg, Manitoba
Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

BNIP3 MUTATIONS IN BREAST CANCER

BY

Nicolle Bristow

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

For my parents

ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisor, Dr. Spencer Gibson for providing me with the opportunity to work in his lab first as a summer student, followed by a project student and finally as a graduate student. His guidance, advice and support were invaluable and allowed me to learn a great deal under his supervision.

I would like to thank my committee members, Dr. David Eisenstat and Dr. Dan Gietz, for their time, guidance and advice.

My success in completing this project is due in large part to the support and encouragement of my friends in the Gibson/Johnston lab and within MICB. Thanks to Liz, Bonnie, Meghan, Teralee, Paula, Brenda, Michelle, Francisco, Eileen and Nichola.

Lastly, I would like to thank my family and friends outside of science for their unconditional support, encouragement and confidence for I would never have made it this far without you.

ABSTRACT

BNIP3 (Bcl-2/adenovirus E1B Ninteen Kilodalton Interacting Protein) is a pro-cell death member of the Bcl-2 family of proteins. Its expression is induced by the transcription factor Hypoxia Inducible Factor-1 (HIF-1) under conditions of low oxygen (hypoxia). We found a mutation in the BNIP3 gene in an ovarian cancer cell line, SkOv3, which results in BNIP3 protein truncation and elimination of its functional transmembrane domain. This mutant protein inhibits hypoxia-induced cell death endogenously in SkOv3 cells, as well as in stably or transiently transfected HEK293 and MCF-7 cells. It has been shown that BNIP3 expression is increased in hypoxic regions of breast tumors where it correlates with worsened prognosis. This over-expression of BNIP3 is paradoxical since BNIP3 induces death, yet these tumor cells remain viable. The presence of mutations in the BNIP3 gene in breast tumors could provide an explanation as to why BNIP3 fails to induce cell death in viable tumor cells. To investigate the potential role of BNIP3 mutations in breast cancer, tumor samples from the Manitoba Breast Tumor Bank were screened for mutations in the BNIP3 gene. Mutations were found in 23% of tumors screened. Four different mutations were observed, all of which resulted in the elimination of BNIP3 protein's transmembrane domain and were able to inhibit hypoxia-induced cell death when transfected into HEK293 cells. Immuno-fluorescent staining revealed BNIP3 protein expression in tumors containing mutations in the BNIP3 gene and this expression correlates with the hypoxia-marker Glut-1. Overall, these results indicate that BNIP3 is mutated in a subset of breast tumors and these mutations may render breast cancer cells more resistant to hypoxia-induced cell death contributing to disease progression.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α	alpha
β	beta
Δ	delta
Δψm	mitochondrial membrane potential
κ	kappa
λ	lambda
ψ	psi
μ	micro
μg	microgram
μL	microlitre
μM	micromolar
%	percent
°C	degree Celsius
AI	aromatase inhibitors
AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor 1
ARD1	Arrest-defective 1 protein
ARNT	aryl hydrocarbon nuclear translocator
Asn	asparagine
ATM	ataxia telangiectasia mutated
Atg	Autophagy-related genes
ATP	adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BCS	bovine calf serum
BH1-4	Bcl-2 homology domain 1-4
Bid	BH3-interacting domain death agonist
BNIP	Bcl-2 E1B-19kDa interacting protein
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer type 2 susceptibility protein
BSA	bovine serum albumin
Ca	calcium
CAIX	carbonic anhydrase IX
CARD	caspase activation and recruitment domain
CBP	creb-binding protein
CD	conserved domain
cDNA	complimentary DNA
C.elegans	*Caenorhabditis elegans*
CHK2	checkpoint homology 2
cm	centimetre
CO_2	carbon dioxide
CREB	cAMP response element-binding protein
Cul2	cullin 2
DCIS	ductal carcinoma *in situ*

dH_20	distilled water
ddH_20	deionized distilled water
DAPI	4',6-diamidino-2-phenylindole
DD	death domain
DED	death-effector domain
DISC	death-initiating signaling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DR4	death receptor 4
DR5	death receptor 5
ECL	enhanced chemiluminescence reagent
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EndoG	endonuclease G
ER	estrogen receptor
ERE	estrogen response element
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
FIH-1	factor inhibiting HIF-1
FITC	fluorescein isothiocyanate
g	gram
GBM	glioblastoma multiforme
Glut-1	glucose transporter 1
HA	hemaglutinin
H_20	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HDAC	histone deacetylase
HEK	human embryonic kidney
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt
Hg	mercury
HIF	hypoxia inducible factor
HRE	hypoxia response element
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis proteins
IGF	insulin growth factor
K	potassium
kDa	kilodalton
L	litre
LB	Luria Bertani
Lys	lysine
M	molar
mA	milliampere
MAPK	mitogen-activated protein kinase

mg	milligram
mm	millimeter
mM	millimolar
mL	millilitre
mRNA	messenger RNA
Na	sodium
NaCl	sodium chloride
NaF	sodium fluoride
NF-κB	nuclear factor kappa B
Nix	BNIP3-like protein X
nm	nanometre
NO	nitric oxide
ODDD	oxygen dependent degradation domain
PAS	PER-ARNT-SIM
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween 20
PCD	programmed cell death
PCR	polymerase chain reaction
PEST	prolyl, glutamic acid, serine, threonine rich domain
PHD	proyl hydroxylase
pmol	picomole
PR	progesterone receptor
Pro	proline
pVHL	von Hippel Lindau protein
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphoinositide phosphotase
PT pore	permeability transition pore
RCC	renal cell carcinoma
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	rotations per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SERM	selective estrogen receptor modulator
SMAC	second mitochondrial-derived activator of caspases
SSCP	single stranded conformation polymorphism
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20
tBid	truncated Bid
TE	Tris-EDTA buffer
TEMED	N,N,N,N;-tetramethylethylenediamine
TGFα	transforming growth factor alpha
TM	transmembrane domain
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor

TRADD	TNF-R associated death domain protein
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Tris	Tris(hydroxymethyl)aminomethane
U	units
V	volts
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
v/v	volume/volume
w/v	weight/volume
x	times

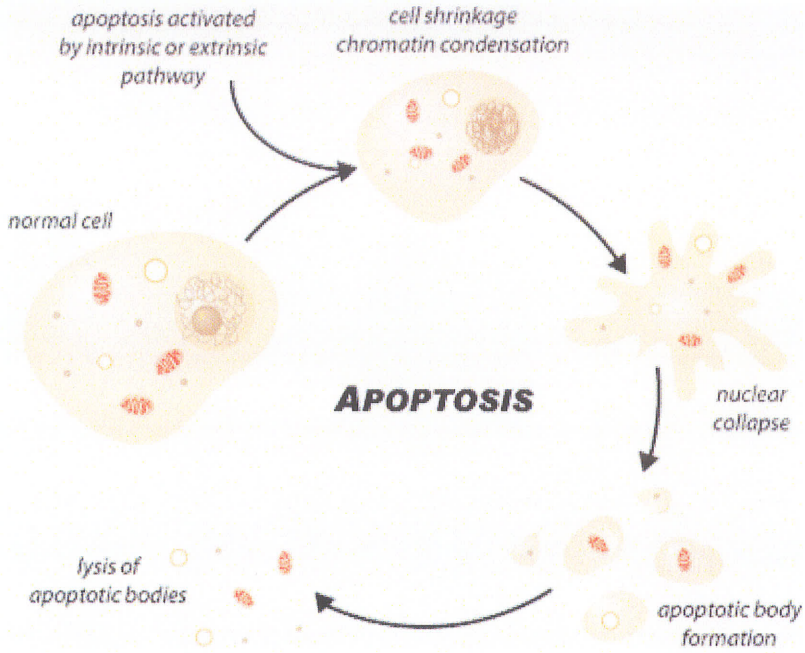
1. INTRODUCTION

1.1 Cell death

Cell death is an essential part of life for multicellular organisms. It is a normal process that is important in both development and homeostasis. It is tightly regulated as either too much or too little can lead to pathological defects, including auto-immune and degenerative diseases and cancer. Cell death has been subdivided into several different categories, namely apoptosis (Type I), autophagy (Type II) and necrosis (Type III). Apoptosis is the most well-characterized type of cell death and many human tumors carry mutations that inactivate the apoptotic pathway, which allows tumor cells to continue growing beyond normal homeostasic limits (1). The boundaries between the different types of cell death, however, are not clearly defined and can often be overlapping. For example, mitochondrial dysfunction is a well characterized change in apoptosis; however, it is not unique to apoptosis. Cells dying from autophagy and necrosis also display mitochondrial permeability transition (2). In addition, some molecules appear to be involved in multiple forms of cell death. Bcl-2, Bcl-X_L, or Bax proteins are involved in apoptosis, but can also influence other types of death (discussed in more detail below) (2). Lastly, if one type of cell death is blocked, other forms of cell death may be used to compensate, ensuring cell death. It has been shown that cells may initiate apoptosis, but if this pathway is blocked the cell can succumb to alternate death pathways (2). In summary, cell death is an essential, although complex process. Each of the types of cell death will be discussed in more detail below.

1.1.2 Apoptosis

The term apoptosis was coined in 1972 by Kerr et al. (3) and is alternatively referred to as programmed cell death I (PCDI). It refers to a highly regulated, conserved, energy dependent cell death that leads to elimination of the cells without activation of an inflammatory response. It is characterized by a particular pattern of morphologic changes (Figure 1) including cell shrinkage, DNA fragmentation, chromatin condensation, membrane blebbing, rounding of the cell, externalization of phosphatidylserine and fragmentation of the cell into apoptotic bodies (3-6). Signaling for apoptosis can occur through multiple independent pathways and can be initiated either from inside (intrinsic or mitochondrial pathway) or outside (extrinsic or death receptor pathway) of the cell. The apoptotic pathways contain a number of amplification steps and positive feedback loops that ensure a cell will either fully commit to or completely abstain from apoptosis (7). There are four main groups involved in the regulation of apoptosis including: caspases, adaptor proteins, members of the tumor necrosis factor (TNF) superfamily and the Bcl-2 (B cell lymphoma 2) protein family. Considerable insight into the mechanism of apoptosis is derived from genetic studies in *Caenorhabditis elegans (C. elegans)*. The 4 genes involved in apoptosis in *C. elegans* are CED-3, CED-4, Egl-1 and CED-9 (4). CED-3 is homologous to mammalian caspases; CED-4 is homologous to the adaptor molecule Apaf-1 which initiates the proteolytic cascade; Egl-1 is a pro-apoptotic BH3-only Bcl-2 family member ; and CED-9 is homologous to Bcl-2, which can prevent apoptosis by binding to pro-apoptotic members (8). Under survival conditions, CED3 is bound to CED9. When cells undergo apoptosis, CED3 releases CED9 and binds to CED4 to induce death.



(http://www.scq.ubc.ca/wp-content/uploads/2006/07/Apoptosis.gif)

Figure 1. Schematic illustrating the morphological changes in a cell undergoing apoptosis.

1.1.2.1 Caspases

The two main apoptotic pathways are the intrinsic pathway which is mediated through the mitochondria, and the extrinsic pathway which involves signaling through membrane bound death receptors (Figure 2). All signaling pathways converge on a common family of cysteine proteases, called caspases, which are central to the morphological changes ultimately resulting in cellular destruction (4). Caspases are highly conserved thorough evolution (7) with at least 14 identified in mammals (4). Some caspases contribute to cell death (caspases 2, 3, 6, 7, 8, 9, and 10), while others are involved in cytokine processing and inflammation. Caspases that participate in cell death can be further divided into an initiator group (2, 8, 9 and 10) and an effector group (3, 6 and 7) (9). Initiator caspases (8

and 9) cause a cascade of increasing caspase activity by processing and activating the effector caspases (4). Caspase 8 is the initiator caspase for the extrinsic apoptotic pathway, while caspase 9 is the initiator caspase of the intrinsic pathway (7). Caspases recognize tetrapeptide motifs and have individual substrate specificities that are determined by the amino acid sequence upstream of the cleavage site (4). Caspases are expressed as inactive proenzymes with very low intrinsic activity and become activated by proteolytic cleavage. There are three general mechanisms of caspase activation: (1) cleavage by an upstream caspase (caspases 3, 6 and 7); (2) induced proximity (caspases 2 and 8); (3) association with a regulatory subunit (caspase 9) (7). Caspases are integral to apoptosis and are often termed the "executioners" of apoptosis.

1.1.2.2 Intrinsic apoptotic pathway

The mitochondria is a double membrane organelle responsible for energy production within the cell and is essential in regulating the life and death of a cell. The intrinsic or mitochondrial apoptotic pathway is induced in response to extracellular cues, such as growth factor withdrawal, as well as intracellular insults such as DNA damage (7). This pathway involves the release of apoptotic proteins (cytochrome c, second mitochondrial-derived activator of caspases (SMAC), apoptosis-inducing factor (AIF) and endonuclease G (EndoG)) from the mitochondria (10). The release of proteins from the mitochondria is mediated by the Bcl-2 family of proteins (discussed below). Cytochrome c and SMAC are sequestered in the inter-membrane mitochondrial space. SMAC binds to inhibitor of apoptosis proteins (IAP), which normally inhibit caspase activity. The release of SMAC from the mitochondria allows it to inhibit IAP, preventing IAP from

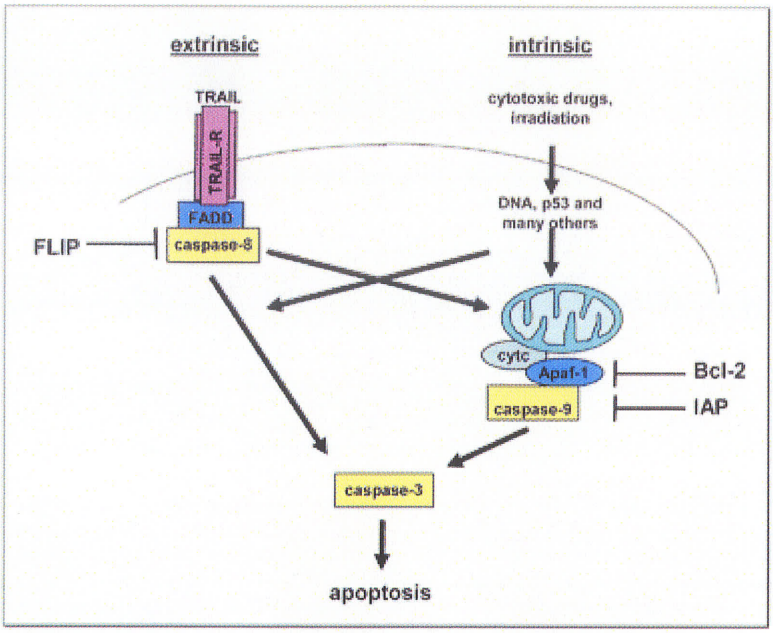
blocking caspase activity and therefore allowing apoptosis to proceed. Release of cytochrome c leads to the formation of the apoptosome, which is a complex involving cytochrome c, apoptosis protease activating factor 1 (Apaf-1), and procaspase-9 (9). This leads to the activation of caspase 9, which in turn activates downstream caspases leading to apoptosis. Two other proteins released from the mitochondria are apoptosis-inducing factor (AIF) and endonuclease G (EndoG). Both AIF and EndoG translocate to the nucleus upon release from the mitochondria where they induce caspase-independent chromatin condensation and DNA fragmentation (11).

The mitochondrial respiratory chain produces energy which is stored as an electrochemical gradient called the mitochondrial transmembrane potential ($\Delta\psi m$). $\Delta\psi m$ is required for mitochondrial function, and its disruption is a key event in apoptosis. The permeability transition (PT) pore is a multi-protein complex that acts as a voltage sensor, and participates in the maintenance of $\Delta\psi m$ (12). Bcl-2 family members are able to modulate the activity of the PT pore and hence $\Delta\psi m$. There is disagreement about how proteins get out of the mitochondria, but most agree that the loss of $\Delta\psi m$ leads to the permeability of the outer mitochondrial membrane, leading to apoptosis induction (13). Mitochondrial respiration produces reactive oxygen species (ROS) which can play a role in furthering the apoptotic process. ROS can trigger apoptosis via the mitochondrial PT pore without apparent contribution of pro-apoptotic Bcl-2 family proteins (14).

1.1.2.3 Extrinsic apoptotic pathway

Members of the tumor necrosis factor (TNF) superfamily are pleiotropic and can lead to proliferation, survival, differentiation and death, depending on the cell type and the other signals received by the cell. Members of the family that induce apoptosis include Fas (CD95 or APO-1), TNF receptor 1 (TNFR1), death receptor 4 (DR4) and death receptor 5 (DR5) (4). These receptors are usually membrane-anchored trimers, and are activated by ligands, such as Fas-ligand (FasL) or TRAIL/APO-2L (TNF-related apoptosis inducing ligand) binding to the receptors (4). The extrinsic apoptotic pathway is triggered by binding of a ligand to one of these death receptor trimers. Adaptor proteins then link the death receptors to their downstream signaling targets. The main adaptor proteins involved in apoptosis include Fas-associating death domain protein (FADD) and TNF-R1-associated death domain protein (TRADD) (4). The associations between the adaptor proteins and the death receptors are characteristically mediated by interactions between domains known as death domains (DD). When an adaptor protein binds to a death receptor, it allows the recruitment, aggregation and activation of caspases (4). The adaptor proteins associate with caspases through their mutual death effector domains (DED) and the caspase recruitment domains (CARD) (4). The formation of a complex called the death-inducing signaling complex, or DISC, which is comprised of a death receptor trimer, adaptor proteins and procaspase-8 triggers the caspase cascade of the extrinsic apoptotic pathway (9). Procaspase-8 is activated by induced proximity in the DISC (7) and can activate the caspase cascade, but it can also cleave Bid, a pro-apoptotic Bcl-2 family member, which in turn activates the mitochondrial apoptotic pathway. The

two apoptotic pathways converge on caspase 3 activation and the mitochondria with cross talk between the two pathways provided by Bid (7).



(http://www.gsf.de/agv/images/jeremias2.gif)

Figure 2. The intrinsic and extrinsic apoptotic pathways.

1.1.2.4 Bcl-2 family proteins

The key function of Bcl-2 family members in apoptosis seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome c, from the mitochondrial intermembrane compartment (7). Many members of the family have a conserved C-terminal transmembrane domain that localizes proteins to the outer mitochondrial membrane (4). The family can be divided into pro-apoptotic and anti-apoptotic members. The fate of a cell largely depends on the balance between these pro- and anti-apoptotic proteins, as these proteins can heterodimerize and titrate one another's function (8).

Knockout studies have shown that loss of anti-apoptotic genes can lead to abnormal apoptosis in a tissue specific manner while loss of pro-apoptotic genes can result in increased cellular proliferation (8, 15). The importance of the balance between the two sub-groups is highlighted by the fact that most anti-apoptotic members are likely to be oncogenes while the pro-apoptotic members are probably tumor suppressors (15). There are at least 15 Bcl-2 family members (Figure 3) that have been identified in mammalian cells (8, 16). They are divided into sub-families based on their function and the presence of one or more conserved motifs called Bcl-2 homology (BH) domains, of which there are four (BH1-BH4).

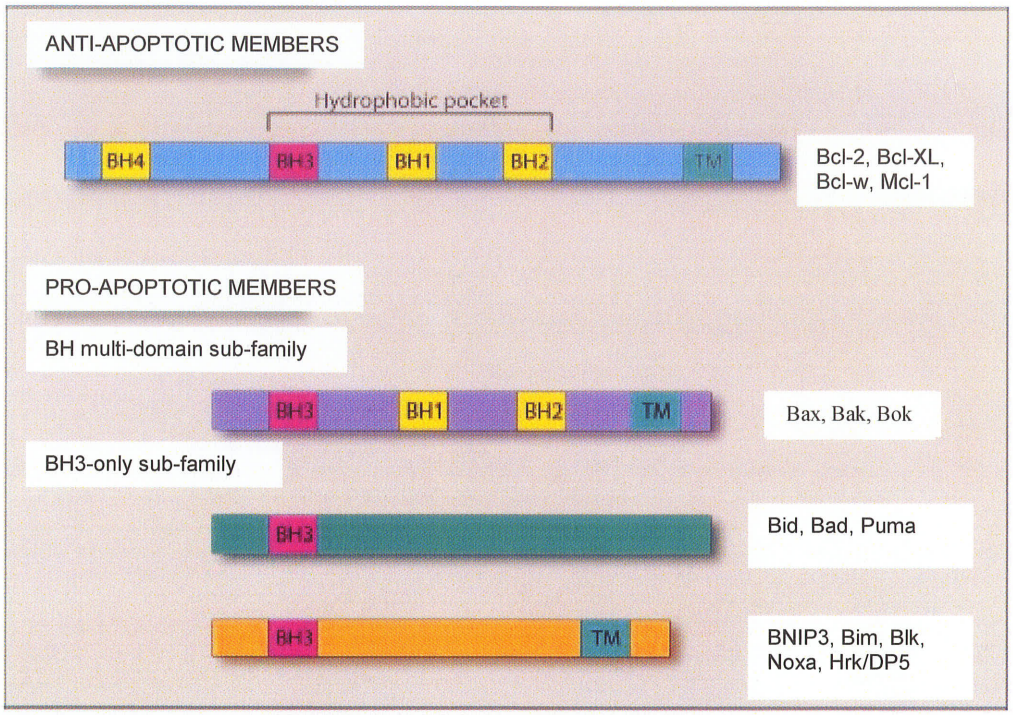
1.1.2.4.1 Anti-apoptotic Bcl-2 family members

Anti-apoptotic members contain at least BH1 and BH2, but many contain all 4 BH domains. This sub-family includes Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Boo, and Bcl-B. The function of anti-apoptotic family members is to maintain the integrity of $\Delta\psi m$ and prevent the release of apoptotic proteins from the mitochondria. Bcl-2 is able to inhibit the release of cytochrome c and SMAC thereby blocking caspase activation and apoptosis (8, 17, 18).

1.1.2.4.2 Pro-apoptotic Bcl-2 family members

The pro-apoptotic family members can be further divided into those that contain BH1-BH3 domains (Bax family) and those that contain only the BH3 domain (BH3-only family). The pro-apoptotic Bax family members are Bax, Bak, Bok, and Bcl-X_S, while

the BH3-only pro-apoptotic members include **BNIP3**, Nix, Bad, Bik, Blk, Hrk/DP5, Bid, Bim, Noxa, and Puma.

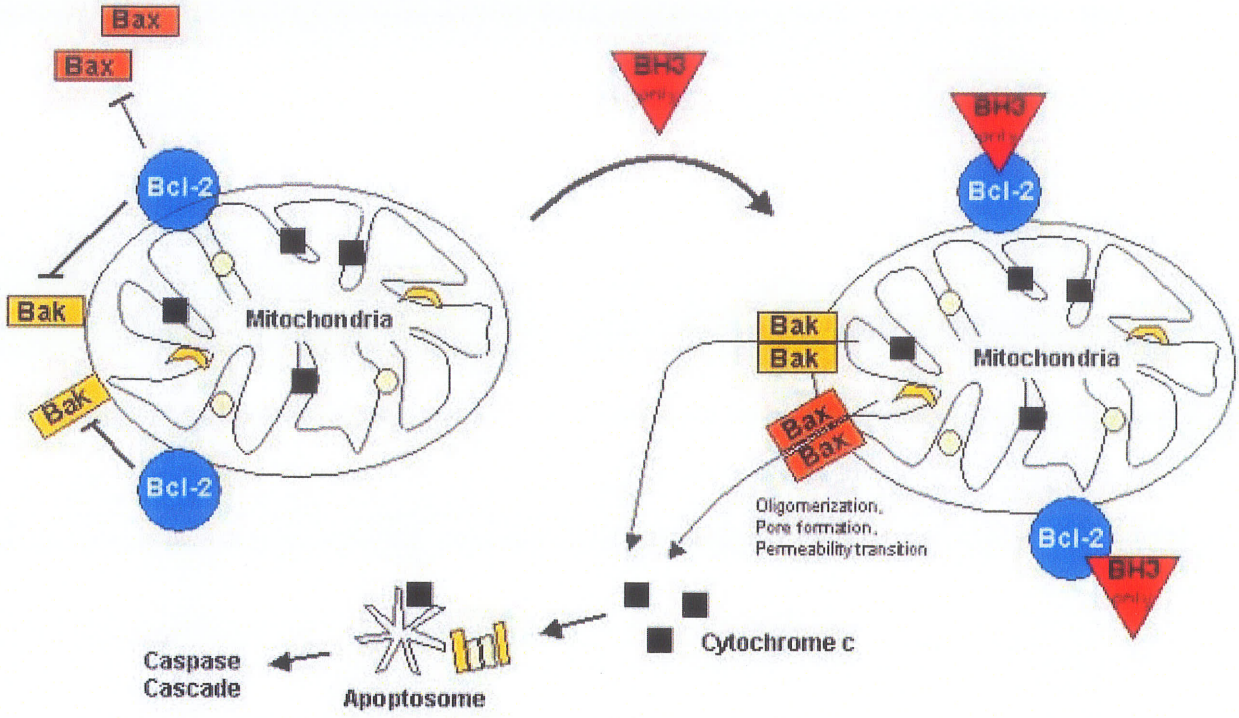


(http:// http://protein.bio.msu.ru/biokhimiya/contents/v70/full/70020284Fig3.jpg)

Figure 3. Schematic of the Bcl-2 family proteins divided into sub-families based on the presence of or absence of the BH domains 1-4.

Both types of pro-apoptotic proteins are required to initiate apoptosis. The BH-3 only proteins are thought to act as a rheostat for apoptosis and are direct antagonists of anti-apoptotic members, while the Bax family members are directly involved in mitochondrial disruption (Figure 4)(9, 15). Most BH3-only proteins neutralize the function of anti-apoptotic family members by interacting via their BH3 domain. This indirectly allows Bax and Bak to induce outer mitochondrial membrane permeabilization and efflux of apoptotic proteins by removing the inhibition of Bax and Bak imposed by their binding of

anti-apoptotic proteins (15). In fact, a synthetic BH3 peptide is sufficient to antagonize anti-apoptotic Bcl-2 family members and can promote Bax-dependent apoptosis by removing the inhibition imposed on Bax by Bcl-X_L (19). An exception to the general function of BH3-only proteins is Bid. The BH3 domain of Bid is not exposed in its native conformation (20) and only becomes active after proteolytic cleavage into a truncated Bid protein (tBid), which can directly activate Bax or Bak to induce apoptosis (20, 21). Bax is a cytosolic monomer in healthy cells, but changes conformation during apoptosis and integrates and oligomerizes in the mitochondrial outer member (15). Bak on the other hand, exists as an oligomeric integral mitochondrial member protein even in healthy cells, but also undergoes a conformational change during apoptosis (15, 20). One model, based on the resemblance of Bcl-2 family members to the channel-forming diphtheria toxin, is that Bax and Bak contribute to mitochondrial membrane permeabilization by the formation of channels in the membrane (15). An alternate hypothesis is that Bax may interact with components of the existing permeability transition (PT) pore, such as the voltage-dependent anion channel (VDAC) to create a larger channel allowing the release on proteins from the intermembrane space (15).



(http://www.celldeath.de/encyclo/aporev/revfigs/rev_7b.jpg)

Figure 4. The role of Bcl-2 family proteins at the mitochondria.

1.1.2.4.3 BNIP3

The human BNIP (Bcl-2 E1B Nineteen kDa interacting protein) family of proteins contains 4 members, BNIP1, 2 and 3 and Nix (BNIP3-like protein X), which are all pro-apoptotic BH3-only members of the Bcl-2 family of proteins (22). BNIP3 is the most well characterized member of the family and was first identified by its ability to bind the adenovirus E1B 19kDa, which has an anti-apoptotic function to preserve host cell viability during viral infection (23). The 194 amino acid BNIP3 protein contains several defined regions including a C-terminal transmembrane domain (TM), a 19 amino acid conserved domain (CD), a Bcl-2 homology (BH) 3 domain, and a PEST domain (high

frequency of Pro, Glu, Ser, Thr amino acids which are associated with having high rates of proteasomal turnover) (Figure 11) (22, 24, 25). BNIP3 is expressed in brain and skeletal muscle under normal conditions (26), but its function is unknown. In most other tissues BNIP3 expression is induced by Hypoxia-inducible factor 1 (HIF-1; discussed below) under hypoxic conditions. HIF-1α binds directly to a HIF-1α responsive element (HRE) in the BNIP3 promoter, and this binding is required for activation of the promoter (27). The BNIP3 promoter is responsive to both hypoxia-induced HIF-1 expression and over-expression of HIF-1 (28). In addition to hypoxia, BNIP3 expression can also be induced by HIF-1 activation stimulated by nitric oxide (NO) and mediated by the Ras/MEK/ERK (discussed below) signaling pathway (29).

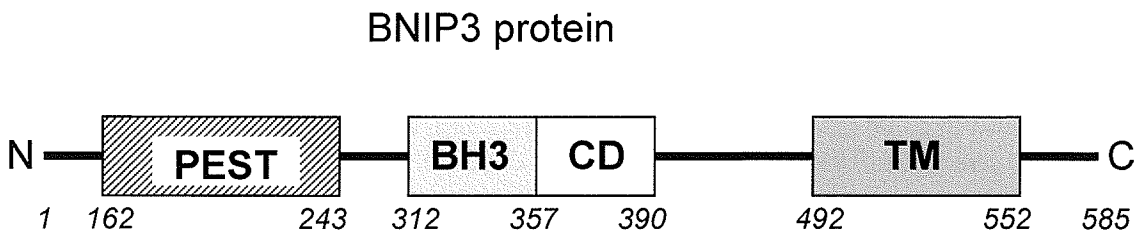


Figure 5. Schematic of BNIP3 protein structure.
BNIP3 protein contains an N-terminal PEST domain that targets it for degradation via the ubiquitin-proteasome pathway. The Bcl-2 homology 3 (BH3) domain of BNIP3 makes a member of the BH3-only sub-family of the Bcl-2 family proteins. It also contains a conserved domain (CD) and a transmembrane (TM) essential for its ability to induce cell death. The amino acid positions are listed below the schematic in italics.

BNIP3 integrates into the mitochondrial membrane where it induces necrosis-like cell death characterized by opening of the mitochondrial permeability pore, proton electrochemical gradient ($\Delta\psi m$) suppression and increased reactive oxygen species

production, which is independent of caspase activation, Apaf-1 and cytochrome c release (30). The transmembrane (TM) domain of BNIP3 is critical for homodimerization, pro-apoptotic function and integration into the mitochondrial membrane (31). The removal of the TM domain alters BNIP3's localization, ability to homodimerize and ability to induce cell death (24, 32). However, it has also been shown that a mutation in the TM domain that prevents homodimerization does not inhibit the cell death function of BNIP3 in cardiac myocytes (33)indicating that BNIP3 can induce cell death without dimerization or that its function may be affected by cell type specificity. BNIP3 is a unique member of the BH3-only proteins as it does not require its BH3 domain for its function and can directly cause changes in mitochondrial membrane potential without the involvement of other Bcl-2 family members (34). Removal of the BH3 domain of BNIP3 does not affects its ability to induce cell death (31) and therefore BNIP3 may promote apoptosis by both BH3-dependent and independent mechanisms (25). Moreover, BH3-only proteins are generally thought to act as sensors of intracellular or stress-induced damage. BNIP3 is the first BH-3 only protein shown to activate cell death via interaction of its TM domain with that of the cell surface receptor CD47, known to activate caspase and cytochrome c independent-apoptosis in lymphocytes (35).

It is clear that BNIP3 exerts its action at the mitochondria, however the mechanism of BNIP3-mediated cell death remains poorly defined. Structure analysis suggests that the BNIP3 TM domain alone can form ion conducting pathways in the membrane and helps to explain its mechanism of action in hypoxia-induced cell death (34). Alternatively, in isolated mitochondria BNIP3 induces mitochondrial permeability transition via its

carboxy terminal tail which may directly interact with one of the components of the permeability transition pore or with other mitochondrial proteins (36). It was recently shown that BNIP3 mediates mitochondrial dysfunction through activation of Bax or Bak, which is independent of mitochondrial permeability pore opening (37). Taken together these results suggest that BNIP3 may induce mitochondrial dysfunction by several different mechanisms yet to be fully elucidated.

BNIP3 appears to play a role in several pathological conditions related to hypoxia including stroke and cardiac ischemia/reperfusion injury. Under normoxic conditions, most tissues including the heart have undetectable levels of BNIP3, but activate BNIP3 transcription during hypoxia (38). BNIP3 is an inducible factor that provokes mitochondrial defects and cell death of ventricular myocytes during hypoxia (39). Cardiomyoctyes undergo apoptosis upon BNIP3 over-expression unlike epithelial cells, which undergo a necrosis-like cell death. The BNIP3-mediated apoptosis of cardiomyocytes can be suppressed by the binding of the transcription factor nuclear factor κB (NF-κB) to the BNIP3 promoter and silencing its expression (40). The cytoprotective role of NF-κB silencing of BNIP3 requires the cooperation of histone deacetylase 1 (HDAC1) (41). Kubasiak *et al.* have shown that hypoxia alone is not sufficient to induce apoptosis in ischemia-mediated death of cardiac myocytes, but requires the combination of hypoxia and acidosis. They found that hypoxia/acidosis cell death is mediated by BNIP3 (42). Acidosis stabilizes BNIP3 proteins and protects them from proteasomal degradation allowing them to induce cell death in cardiac myocytes (33). Neural cell death in stroke occurs via a caspase-independent pathway. BNIP3

has been shown to induce cell death in stroke and hypoxia by mediating the release of EndoG from the mitochondria (43). EndoG is a mitochondrial protein that is able to translocate to the nucleus and cleave DNA independent of caspases. BNIP3 leads to cell death in both cardiac myocytes and neurons in heart attack and stroke, respectively and therefore may be an important factor in the cellular damage caused by hypoxia in both cases.

1.1.3 Autophagy

Eukaryotic cells use two distinct mechanisms for large-scale degradation, the ubiquitin-proteasome-pathway and autophagy. Autophagy involves lysosomal-mediated degradation of proteins and cellular organelles and literally translates to "eat oneself". The molecular mechanisms governing autophagic vesicles were discovered using *Saccharomyces cerevisiae* as a model organism and the involved genes are called Atg (autophagy-related) genes (5). The process involves the formation of a double membrane vesicle (autophagosome) in the cytosol that encapsulates whole organelles and bulk cytoplasm. This autophagosome fuses with a lysosome where the contents are subsequently degraded (1). Autophagy is an evolutionarily conserved, normal physiological process that is active in homeostasis in routine organelle turnover. In addition to turning over cellular components, autophagy is involved in development, differentiation and tissue remodeling in various organisms (44). Autophagy can be protective and is used for the recycling of cellular materials during starvation and for the elimination of abnormal proteins. During times of stress, such as nutrient deprivation,

cells switch to a catabolic metabolism program in which cellular constituents are degraded and recycled for energy production (1). The fact that autophagy is induced to promote cell survival is generally accepted in the literature; however, there is some debate about whether autophagy is a true form of cell death. On one hand, autophagic cell death could simply describe death with autophagy that occurs when the cell is no longer able to survive under stress, and does not necessarily mean that death is occurring through an autophagic pathway (3). Alternatively, autophagy could represent a primary attempt to reestablish homeostasis but can lead to apoptosis or autophagic cell death if the autophagic capacity is overwhelmed (45). Autophagic cell death may be interpreted as the process of autophagy that does not terminate before the cell collapses (6). Autophagic cell death can be defined morphologically as death that occurs without chromatin condensation accompanied by massive autophagic vacuolization of the cytoplasm (3) and appears to be distinct from apoptosis (6). The contribution of autophagy to cell death and its role is many diseases in not clearly established (46). It appears that is has opposing roles, and can function to promote both survival and death and is likely dependent on the status of the individual cell.

1.1.4 Necrosis

Necrosis is usually considered a type of cell death with no signs of apoptosis. It is often referred to as passive or accidental, occurring due to severe cellular injury such as hypoxia (47). Regions of hypoxia in solid tumors are often found surrounding areas of necrosis (48) supporting the fact that hypoxia is lethal as a direct stress trigger that

induces necrotic cell death (49). The morphology of necrosis is defined by cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles and is associated with some moderate chromatin condensation (3). The cytoplasmic contents of the cell are released upon rupture of the plasma membrane, causing an inflammatory response (47). Until recently, necrotic cell death was considered to be a disordered mode of cell death. However, recent studies suggest that necrotic cell death occurs during normal cell physiology and development (50, 51). In addition, apoptosis and necrosis can be triggered by the same stimulus with the outcome dependent on the balance of intracellular anti-apoptotic Bcl-2 family members or endogenous caspase inhibitors (47). In the absence of activated caspases, cell death is accompanied by cytoplasmic vacuolization, suggesting that the cell dies from a "necrotic-like" autophagic programmed cell death (50-52).

1.2 Breast Cancer

1.2.1 Clinical Features

Breast cancer is the most common cancer diagnosed in Canadian women. The Canadian Cancer Society estimates that in 2007, 22 300 women will be diagnosed with breast cancer, and 5300 will die from the disease. These numbers correlate into 1 in 9 women expected to be affected, and 1 in 27 are expected to die from the disease. Despite the fact that the incidence of breast cancer has been rising in industrial countries, the mortality associated with the disease has been stable or even decreased in the last 10-15 years due to screening programs that detect cancer earlier combined with improvements in therapy (53). Breast cancer is a clinically heterogeneous disease with approximately 10%-15% of patients having aggressive disease that develops distant metastases within 3 years of the initial detection of the primary tumor. It is not uncommon, however, for other patients to develop distant metastases 10 years or longer after the initial diagnosis (54). It is not the primary tumor, but its metastases at distant sites, mainly to the bone, lung and liver (55), that are the main cause of death.

Tumorigenesis in humans is a multi-step process involving genetic alterations that drive the transformation of normal cells into malignant cells. For many years, breast carcinogenesis has been considered to be a linear progression from normal epithelium, to ductal carcinoma *in situ* (DCIS), to invasive ductal carcinoma and finally to metastatic carcinoma. This model has been challenged, and more recent evidence suggests there may be several distinct and parallel pathways that exist for both low-grade and high-grade carcinomas (56). DCIS is a group of heterogeneous lesions which are

characterized by a clonal proliferation of epithelial cells confined within the lumens of mammary ducts without evidence of invasion beyond the basement membrane into adjacent breast stroma. Therefore DCIS has no metastatic potential. Approximately 30% to 50% of patients with DCIS will develop invasive ductal carcinoma over a 10 year period (57). Margin width following resection has been shown to be the most important factor affecting the rate of local recurrence, and high-grade DCIS is associated with a high risk of distant metastases after invasive local recurrence (56). Invasive ductal carcinoma is the most commonly diagnosed histologic type of breast cancer, accounting for 70% to 80% of cases (57). The prognosis of breast cancer has become relatively good, with current 10 year relative survival approximately 70% in most Western populations. The most useful prognostic indicators of long-term survival include tumor size, lymph node involvement and margin width. Patients with lymph node involvement have 4-8 times higher mortality than those with node negative disease; and those who present with metastasis have very poor 10 year survivals (53).

The genes associated with hereditary breast cancer include BRCA1 (Breast Cancer 1, early onset), BRCA2 (Breast Cancer Type 2 susceptibility protein), p53, CHK2 (checkpoint homolog 2) and ATM (ataxia telangiectasia mutated), which are all involved in the maintenance of genomic integrity and DNA repair (58). Both BRCA1 (17q21) and BRCA2 (13q12-13) are implicated in the repair of DNA by homologous recombination and have characteristics of tumor suppressor genes that follow an autosomal-dominant pattern of inheritance (58, 59). BRCA mutations occur at a frequency of approximately 1 in 250 women. Carriers may undergo a high rate of mutation due to the deficiencies in

this damage repair pathway following oxidative stress (60). Loss of heterozygosity (LOH) is seen in carriers, with a retention of the disease-predisposing allele. LOH is seen in sporadic breast cancers, however the retained allele is usually wild type (61). BRCA1-related breast cancers are usually high-grade infiltrating ductal carcinomas and have a worse outcome compared with sporadic breast cancer. BRCA2 is less well understood and BRCA2-associated tumors cannot be readily distinguished from sporadic cancers morphologically (58). Women at highest risk for developing breast cancer are those who are gene mutation positive on testing for BRCA genes, or those with a very strong family history which includes either multiple first- and second-degree relatives who have had breast or ovarian cancer, a first-degree relative who has had breast cancer before the age of 50, male relatives who have had breast cancer and Ashkenazi Jewish women who have had a family history of breast or ovarian cancer. Other women at high risk include those who have a personal history of breast cancer or prior biopsy diagnosis of atypical ductal hyperplasia or lobular carcinoma *in situ*. Lastly, women treated for Hodgkin's disease with mantle radiation are at risk for developing radiation-induced breast cancer (57).

1.2.2 Staging

The TNM Staging system is used to stage breast cancer. The criteria used to classify tumors include the size of the tumor (T), its spread to axillary nodes (N) and its spread to distant sites (M) (Table 1A). The combined values of T, N and M are used to determine the stage of the tumor (Table 1B) with stages designated from 0-IV. Survival is inversely

correlated to the stage of the tumor with stages 0 and I have 100% survival at 5 years, but only 20% 5 year survival is seen in stage IV disease (Table 1C).

Table 1A. Definitions of the T, N and M designations for cancer staging.
Table 1B. Breast cancer stages based on the combined T, N and M designations.
Table 1C. 5-year survival rates for the different breast cancer stages.
(American Joint Committee on Cancer (AJCC) TNM System;
AJCC Cancer Staging Manual 6th Ed 2002;
http://www.cancer.org/docroot/CRI/content/CRI_2_4_3x_How_is_breast_cancer_staged_5.asp)

Table 1A)

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	≤2 cm
T2	>2 cm to <5cm
T3	>5cm
T4	Tumor of any size growing in chest wall or skin
Nx	Regional lymph nodes cannot be assessed
N0	Cancer has not spread to regional lymph nodes
N1	1-3 axillary lymph nodes affected
N2	4-9 axillary lymph nodes affected
N3	>10 axillary lymph nodes or lymph nodes in other areas around the breast affected
Mx	Presence of distant metastases cannot be assessed
M0	No distant metastases
M1	Metastasis to distant organs

Table 1B)

Stage	Tumor	Node	Metastasis
0	Tis	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Table 1C)

Stage	5-year survival
0	100%
I	100%
IIA	92%
IIB	81%
IIIA	67%
IIIB	54%
IV	20%

1.2.3 Role of estrogens

Estrogens play a major role in promoting the proliferation of both normal and neoplastic breast epithelium. Variables that modify the exposure to either endogenous or exogenous estrogens, such as early menarche, late first full-term pregnancy, or late menopause, are known to influence a woman's lifetime risk of developing breast cancer. A tumor's estrogen sensitivity has a major role in determining the chance of cure of breast cancer (62). The relationship between breast cancer and estrogen has been established for over 100 years, with George Beatson reporting in 1896 that removing the ovaries from a pre-menopausal woman with advanced cancer resulted in a significant decrease in tumor size and improved the patients' prognosis. However there is still no clear understanding of the exact mechanisms through which estrogens cause cancer. The three mechanisms that

are postulated to be involved in carcinogenic effects of estrogen are: (1) stimulation of cellular proliferation through receptor-mediated hormonal activity, which is the most widely acknowledged mechanism; (2) direct genotoxic effects by increasing mutation rates through cytochrome P450-mediated metabolic activation; and (3) induction of aneuploidy (63).

In the classical activation pathway, estrogen binds to its receptor, which alters the receptor's conformation inducing dimerization to promote binding to specific DNA sequences (usually an estrogen-response element (ERE), or an AP-1 or SP-1 site) in the promoter region of estrogen-responsive genes. This recruits co-regulators and initiates or represses transcription affecting cell proliferation, apoptosis and angiogenesis. The ER is the primary target of hormonal therapy of breast cancer and the expression of ER is a reliable marker to predict response to endocrine therapy (64). Estrogen has two receptors, estrogen receptor alpha (ERα) and beta (ERβ). ER levels are low in normal breast tissue and high levels have been directly correlated with an increased risk of breast cancer (65). Breast tumors can express both estrogen receptors, only one of the receptors or neither. ERα is expressed in 74% to 75% of breast tumors while ERβ is expressed in 76% to 79% of tumors. Most tumors are ERα+/ERβ+ (62%), followed by ERα-/ERβ+ (15%-18%), ERα+/ERβ- (13%-14%) and ERα-/ERβ- (8%-9%) (66). Until the recent discovery of ERβ, ERα was the main estrogen receptor and hence the most well characterized of the two. ERα and ERβ are products of different genes, coded on chromosomes 6 and 14, respectively. Their overall homology is only 30%, though they are 95% homologous in the DNA-binding domains and 53% homologous in hormone-

binding domains (64). ERα and ERβ appear to have opposing effects, with ERα exerting proliferative action whereas ERβ has tumor suppressor properties (64). There are several reports in which a loss of ERβ is observed during carcinogenesis, providing support for its potential role as a tumor suppressor (67). The progesterone receptor is a nuclear hormone receptor that is under the transcriptional control of ER. The combined ER/PR status of a tumor is important as a lack of either ER or PR is associated with poor prognosis and a decrease in disease free survival. ER+/PR+ tumors have the best survival rates with worsening survival seen from ER+/PR+ to ER+/PR- to ER-/PR+ to ER-/PR- (68). ER and PR status can change over the course of the disease or as a result of endocrine therapy (69). The loss of PR is associated with disease progression and more aggressive tumor phenotype (70).

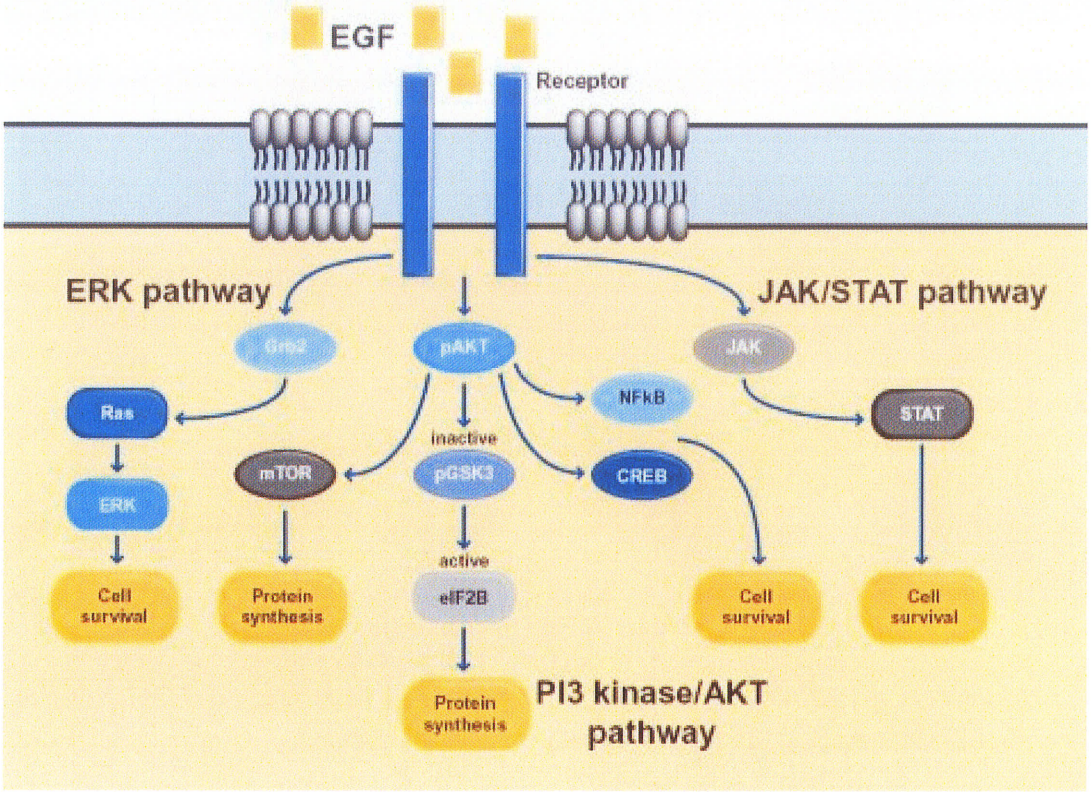
1.2.4 Epidermal growth factor (EGF) and its receptor ErbB2.

Epidermal growth factor (EGF) is an extracellular protein ligand that binds to tyrosine kinase receptors on the cell surface. Binding of EGF to its receptors induces receptor dimerization and activation which can initiate multiple signal transduction cascades including mitogen-activated protein kinases (MAPK), Akt and Jnk, leading to cell survival, proliferation and migration (Figure 5). The general MAPK and Akt signaling pathways are shown in Figures 6 and 7, respectively. One example of how EGF signaling can lead to survival is by up-regulating anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-x_L and Mcl-1 (71).

The ErbB (HER) family of EGF receptors (ErbB1-ErbB4) are well characterized tyrosine kinase receptors that activate MAPK and Akt signaling pathways involved in the control

of multiple cellular processes including apoptosis, migration, growth, adhesion and differentiation. The ErbB2 family of proteins are type I transmembrane growth factor receptors that consist of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (72). ErbB2 signaling is essential for normal cell growth; however deregulated ErbB2 signaling is found in a variety of human cancers. Unlike other family members, the extracellular domain of ErbB2 does not alternate between an active and inactive conformation; rather it exists in a constitutively active form independent of ligand binding and therefore has the strongest catalytic kinase activity. In fact ErbB2-containing heterodimers have the strongest signaling functions (72). The over-expression of ErbB2, usually as a result of gene amplification or through transcriptional deregulation, is important in 15%-30% of breast cancers where it confers aggressive biological behavior through increased kinase activity and augmented auto-phosphorylation of itself and over-phosphorylation of its cellular substrates (72, 73). Over-expression of ErbB2 can lead to an increase in Bcl-2 family member expression. The anti-apoptotic Bcl-2 family member, Mcl-1, is up-regulated by ErbB2 over-expression in estrogen receptor negative breast tumors (74). ErbB2 over-expression also leads to increased expression of HIF-1α (discussed below) which in turn can lead to an increase in HIF-1 target genes (72). The over-expression of ErbB2 leading to increased HIF-1α expression, combined with the presence of hypoxic regions within breast tumors may help to explain why BNIP3 expression is increased in breast ductal carcinoma *in situ* (DCIS) and invasive carcinoma compared with normal breast tissue. BNIP3 expression was observed in peri-necrotic regions of breast tumors (75) where it correlates with a

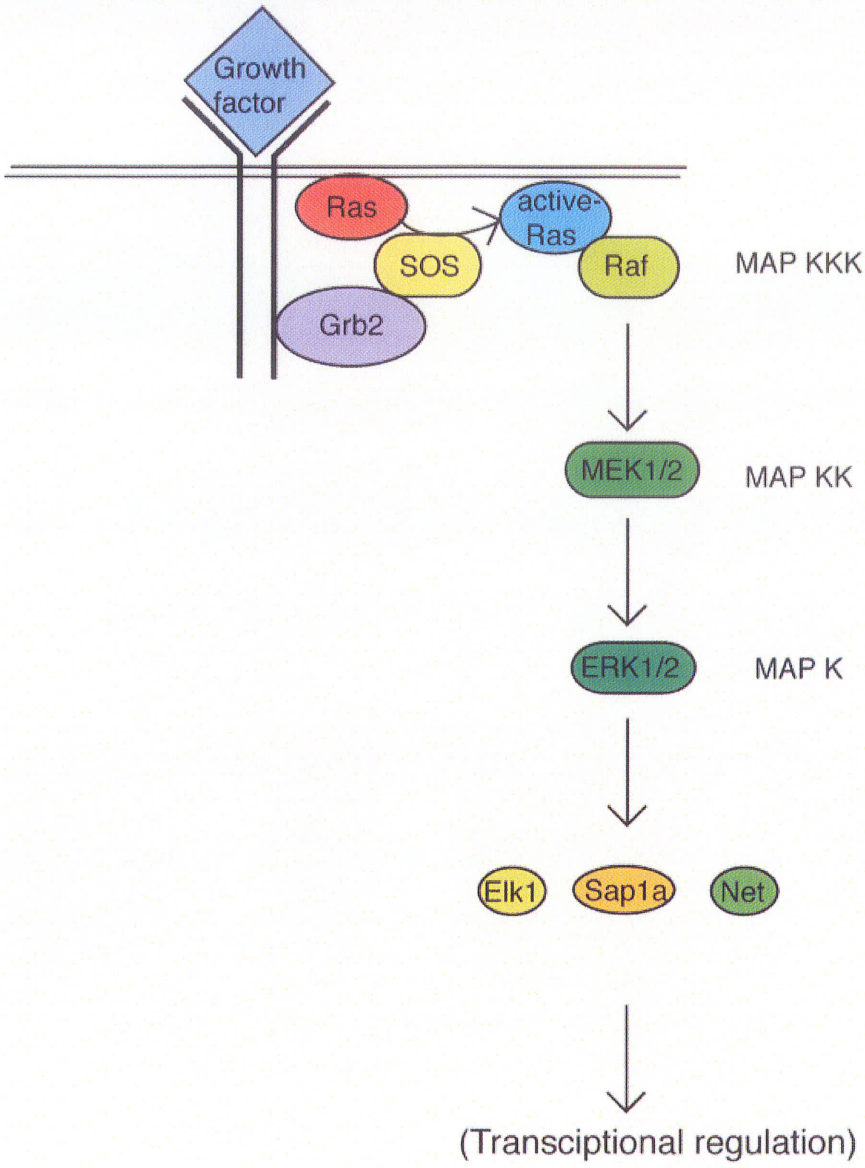
high-grade, necrotic lesion that is likely to be associated with invasive tumor (76). This data suggests that BNIP3 may play an important role in breast tumor progression.



(http://www.abcam.com/cms/displayImage.cfm?intImageID=18888)

Figure 6. EGF binding to its cell surface receptors stimulates MAPK, Akt and Jnk signal transduction pathways.

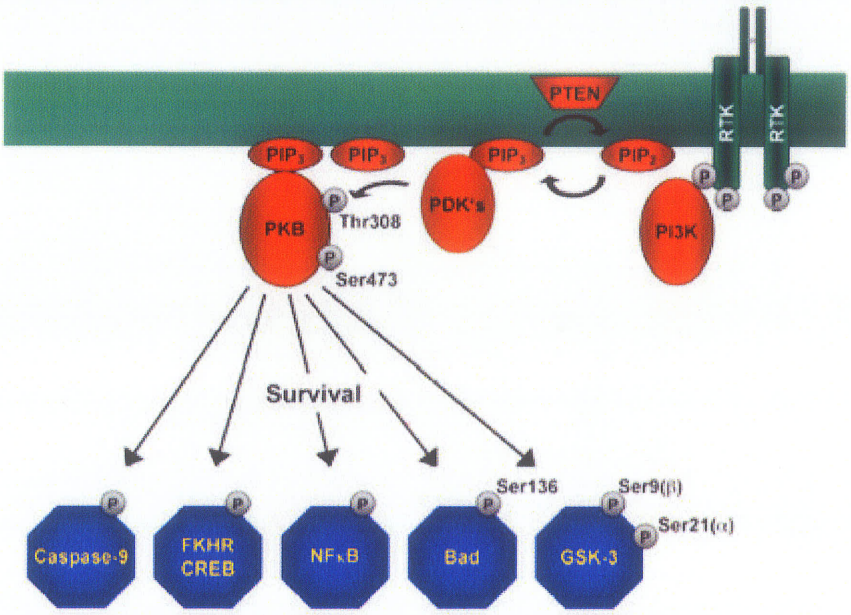
Epidermal growth factor (EGF) binds to its receptor on the cell surface leading to the activation of several downstream signaling pathways. EGF receptors can activate Ras leading to the induction of MAPK signaling pathways and cell survival. Akt signaling can be induced by EGF receptors resulting in the activation of several signaling pathways including NFκB and CREB which both stimulate cell survival. EGF receptor activation can also lead to JAK/STAT signaling pathways leading to cell survival.



Summary of Map kinase pathway

(http://www.brc.riken.jp/lab/dna/en/GENESETBANK/mapk_ras.png)

Figure7. Overview of MAPK signal transduction pathway.
Extracellular stimuli of growth factor receptors lead to the activation of Mitogen-activated protein kinase (MAPK) through a signaling cascade. Stimulation of a receptor leads to activation of Ras, which in turn activates Raf, a MAP kinase kinase kinase (MAP KKK). Raf activates a MAP kinase kinase (MEK 1/2) leading to the activation of a MAPK (ERK 1/2) which can regulate various cellular activities such as transcription, differentiation, survival and apoptosis.



(http://www.chemie.uni-kl.de/forschung/lmctox/eisenbra/forschung/pi3k.jpg)

Figure 8. Overview of Akt signal transduction pathway.
Stimulation of membrane bound receptor tyrosine kinases leads to the activation of phosphoinositide 3-kinase (PI3K), which in turn activates phosphoinositide dependent kinases (PDKs). PHDs activate Akt, also known as PKB (protein kinase B). Akt can regulate metabolism and cell survival by binding and regulating many downstream targets.

1.2.5 Diagnosis and Treatment

The management of breast cancer has been evolving towards minimally invasive approaches for both diagnosis and tumor ablation. The evolution towards minimally invasive approaches is possibly due to the fact the breast tumors are detected earlier and are smaller at the time of diagnosis (77). Mammography is the first-line in the detection and diagnosis of breast cancer; however about 10% of cancers are mammographically occult even after they are palpable, and in women with dense breasts, the sensitivity can be as low as 68% (57). As such, several other types of imaging and biopsy techniques are being investigated. Novel imaging techniques include sonography, magnetic

resonance imaging (MRI), positron emission tomography (PET) and contrast-enhanced mammography. These modalities may be important for providing increased anatomic detail and further defining residual disease, and for distinguishing between benign and malignant lesions (57).

Breast conservation therapy has become the treatment standard for early-stage disease and aims to remove the entire tumor, achieve negative surgical margins, and preserve the breast and patient's body self-image. The presence of undetected residual disease that is not removed during surgery is the rationale for performing post-operative radiation therapy in patients who are treated with breast conservation surgery (57). Chemotherapy is often used in the treatment of metastatic breast cancer. Traditional chemotherapy agents often achieve desired effects by disrupting cellular functions that are common to both malignant and normal cells. Greater understanding of the molecular biology of cancer has lead to the identification of pathways that are unique or predominantly altered in cancer cells that can be specifically targeted for therapy.
For example Trastuzumab (Herceptin), an anti-ErbB2 monoclonal antibody that binds to extracellular domain of ErbB2 and prevents its dimerization, is being used in the treatment of breast cancer (78).

Antihormone therapy, which blocks the activity of estrogen, remains the first line of defense against ER positive breast tumors. Tamoxifen is a selective estrogen receptor modulator (SERM) that was approved by the FDA in 1977 for the treatment of women with advanced breast cancer. The antagonistic effects of tamoxifen in breast tissue are

thought to result from its ability to bind to the ligand-binding domain of the ER, effectively blocking the potential for estrogen stimulation (67). Approximately 30% of ERα positive tumors do not respond to tamoxifen treatment (*de novo* resistance) and the majority of tumors that initially respond to treatment develop resistance over time (acquired resistance) despite continued expression of ERα (67). There are multiple mechanisms through which breast cancer cells can become resistant to antihormonal therapy including ER activation instead of inhibition by SERMs, ER activation in the absence of estrogen and hypersensitivity of ER to low levels of circulating estrogens (64). Altered growth factor pathways can also promote anti-hormone resistance by: (1) allowing activation of ER as a nuclear transcription factor in the presence of anti-hormones; (2) the coupling of ER with growth factor receptors resulting in the activation of downstream MAPK and Akt pathways promoting cell proliferation and survival; and (3) promoting a significant decline in the level of ER protein or ultimately ER gene silencing (79). Tamoxifen therapy has been the most advantageous targeted therapy in breast cancer over last 30 years; however, it is also associated with adverse effects such as an increased risk of endometrial cancer, osteoporosis and coronary heart disease (62, 80). The existence of tamoxifen resistance combined with its adverse side effects has led to the development of novel strategies that target how breast cancer cells interact with estrogen. Aromatase is an enzyme necessary for the conversion of androgens to estrogens (67) and aromatase inhibitors (AIs) have been developed to prevent this conversion, thereby reducing estrogen levels. Compounds that bind to ER and promote its degradation have also been developed (64).

Despite the advances in screening and treatment, women still die from breast cancer. A better understanding of the molecular mechanisms that contribute to treatment failure is required in order to develop more effective and specific therapies. Molecular characterization and greater understanding of the processes involved in breast tumorigenesis will enable more individualized treatment, be it radiotherapy, hormonal, chemotherapy, biological, or multimodality therapy (73).

1.3 Ovarian Cancer

1.3.1 Clinical features

There are three types of ovarian cancer based on the tumor's cellular origin. Tumors can originate from germ cells and stromal cells, but the most common type of ovarian cancer is derived from the outer epithelial cells of the ovary and is the leading cause of gynecological cancer death (81, 82). Most women do not experience symptoms of ovarian cancer until the disease has already metastasized, and therefore approximately 70% of patients present with advanced disease (Stage III/IV, discussed below) (82). Approximately 75% of patients relapse within two years of primary therapy and have a 5-year survival of 23% and 14% for stage III and IV disease, respectively (82). Tumor cells can grow directly into surrounding sites including the contralateral ovary, fallopian tubes, uterus, bladder, rectum and pelvic peritoneum. The tumor can also spread hematologically most commonly to the lungs, liver and brain or through the lymphatic system. The most commonly affected lymph nodes are the pelvic and aortic lymph nodes. The poor prognosis of ovarian cancer is mostly related to the ability of the cancer cells to disseminate into the abdomen where they are circulated through the abdominal fluid and can implant into the liver, omentum, large intestine, small intestine, bladder and diaphragm (81). Cure is unlikely for advanced ovarian cancer, therefore the goals of treatment are to control tumor-related symptoms and to improve or maintain quality of life (82).

1.3.2 Staging

Similar to breast cancer staging, ovarian tumors are assessed for tumor thickness (T), the number and location of lymph nodes (N) and the presence of metastasis (M). The TNM definitions are summarized in Table 2A. The stage of the tumor is determined according to the combined TNM criteria (Table 2B).

Table 2A. TNM designations in ovarian cancer.
(http://info.cancer.ca/E/CCE/cceexplorer.asp?tocid=35)

TX:	Primary tumour cannot be assessed
T0:	No evidence of primary tumour
T1:	Tumour limited to the ovaries
T1a:	• • tumour limited to one ovary • • ovary (capsule) intact • • no tumour on ovarian surface • • no cancer cells found in fluid taken from inside the abdomen
T1b:	• • tumour involves both ovaries • • ovary (capsule) intact • • no tumour on ovarian surface • • no cancer cells found in fluid taken from inside the abdomen
T1c:	• • tumour limited to one or both ovaries with any of the following: ◆ ◆ ovary (capsule) ruptured ◆ ◆ tumour on ovarian surface ◆ ◆ cancer cells found in fluid taken from inside the abdomen
T2:	Tumour involves one or both ovaries with pelvic extension
T2a:	• • extension and/or implants on uterus and/or fallopian tube(s) • • no cancer cells found in fluid taken from inside the abdomen
T2b:	• • extension to other pelvic tissues • • no cancer cells found in fluid taken from inside the abdomen
T2c:	• • pelvic extension (2a or 2b) with malignant cells in ascites or peritoneal washings
T3:	Tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis
T3a:	• • microscopic peritoneal metastasis beyond pelvis
T3b:	• • macroscopic peritoneal metastasis beyond pelvis 2 cm or less in greatest dimension
T3c:	• • peritoneal metastasis beyond pelvis >2 cm in greatest dimension
T4:	Tumour more than 4 mm in thickness • • T4a: without ulceration • • T4b: with ulceration
NX:	Regional lymph nodes cannot be assessed
N0:	No regional lymph node metastasis
N1:	Regional lymph node metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2B. Staging of ovarian cancer.

Stage I	T1	N0	M0	• tumour limited to the ovaries • no lymph node involvement • no distant metastases
Stage IA	T1a	N0	M0	• tumour limited to one ovary • ovary intact • no lymph node involvement • no distant metastases
Stage IB	T1b	N0	M0	• tumour involves both ovaries • ovary intact • no lymph node involvement • no distant metastases
Stage IC	T1c	N0	M0	• tumour involves one or both ovaries with any of the following: ◆ ovary (capsule) ruptured ◆ tumour on ovarian surface ◆ cancer cells found in abdominal fluid • no lymph node involvement • no distant metastases
Stage II	T2	N0	M0	• pelvic extension • no lymph node involvement • no distant metastases
Stage IIA	T2a	N0	M0	• involves uterus and/or fallopian tubes • no lymph node involvement • no distant metastases
Stage IIB	T2b	N0	M0	• involves other pelvic tissues • no lymph node involvement • no distant metastases
Stage IIC	T2c	N0	M0	• cancer cells found in abdominal fluid • no lymph node involvement • no distant metastases
Stage III	Any T	N0, N1	M0	• involves peritoneum beyond pelvis and/or regional lymph node involvement • no distant metastases
Stage IIIA	T3a	N0	M0	• microscopic peritoneal metastasis • no lymph node involvement • no distant metastases
Stage IIIB	T3b	N0	M0	• macroscopic peritoneal metastasis ≥2cm • no lymph node involvement • no distant metastases
Stage IIIC	T3c	N0	M0	• peritoneal metastasis > 2 cm • no lymph node involvement • no distant metastases
	Any T	N1	M0	• tumour any size • involves regional lymph nodes • no distant metastases
Stage IV	Any T	Any N	M1	• spread to distant sites, such as organs or distant lymph nodes

1.3.3 Biology

Multiple oncogenes and tumor suppressors including ErbB2, K-ras, p53, and BRCA1 appear to be involved in ovarian tumorigenesis (83). The majority of inherited ovarian cancers are caused by mutations in BRCA1 (83). Over-expression of ErbB2 occurs in approximately one third of ovarian cancers and is associated with poor prognosis (83). About 40% of ovarian cancers have abnormalities in the PI3K pathway which may lead to increased downstream signaling. Similarly to breast cancer, estrogen is considered to be a promoting factor in epithelial ovarian cancer while progesterone appears to play a protective role. Both ERα and ERβ are expressed in normal ovarian epithelial cells, however there appears to be a selective down-regulation of ERβ in ovarian cancer (82). This supports the idea that ERβ plays a protective role against ERα-mediated proliferation in both breast and ovarian cancer. In addition to its ability to stimulate ERs, estradiol has been shown to induce the expression of HIF-1α and its downstream target, vascular endothelial growth factor (VEGF), in ovarian cancer cells (82).

1.4 Hypoxia

Oxygen is essential for the development and growth of multicellular organisms, and as such mammals have developed a sophisticated network to maintain oxygen homeostasis. The ability to sense and respond to changes in oxygen tension is essential, (84) as either too little or too much oxygen can have detrimental effects (85). Changes in oxygen tension can elicit both acute responses such as altered activity of existing proteins, as well as more global changes such as altered gene expression. Hypoxia, or low oxygen (0-20 mmHg compared with 24-66 mmHg seen in normal human tissues (86)), is the result of an imbalance in oxygen delivery and consumption. In recent years, several groups have provided insight into how hypoxia regulates signal transduction pathways. However the "molecular switch" that recognizes oxygen tension remains unknown, though several candidates have been proposed (85). Giaccia *et al.* summarized the oxygen sensor candidates into several groups: the prolyl hydroxylase family of enzymes, the NAD(P)H oxidase family of enzymes, oxygen sensitive ion channels and the electron transport chain (84).

1.4.2 Hypoxia in solid tumors

Deregulation of the oxygen delivery system in multicellular organisms can lead to areas of hypoxia, which is a major component of the pathophysiology of many disease states including cancer, stroke and heart attacks. In a rapidly growing tumor, oxygen demand increases while oxygen delivery decreases, primarily due to an insufficient blood supply and increasing diffusion distances between blood vessels and cells (87). To grow beyond a diameter of approximately 1mm, tumors must form their own vasculature, either by

incorporating pre-existing host vessels or by inducing the formation of new vessels (neovascularization). Newly formed tumor vessels are often structurally and functionally abnormal, leading to poor delivery of oxygen and nutrients to the tumor. Poor delivery, combined with increasing diffusion distances and potentially, cancer-related anemia, leads to the development of hypoxic regions within the tumor (87).

Hypoxia is a stress for both normal and cancer cells; however, cancer cells often adapt and can develop the ability to survive under these stressful conditions (88). Cells that are able to survive in the hypoxic microenvironment are more likely to have traits related to invasion, metastasis, and aggressiveness which correlates with poor prognosis and treatment resistance (89, 90). Hypoxia has been shown to result in reduced cell adhesion by altering the cyotoskeleton. This may allow tumor cells to detach and begin to migrate producing a more invasive phenotype, thereby resulting in metastasis (88, 91). The tumor microenvironment may itself be mutagenic and an important cause of tumor progression. Increased frequency of mutations are observed in tumors and cells exposed to hypoxia compared to identical cells grown in culture (92). Furthermore, hypoxia can effect malignant progression by inducing proteomic and genomic changes which induce angiogenesis, anaerobic metabolism and other processes that select for increasingly aggressive clones with decreased capacity for cell cycle arrest, differentiation, or apoptosis, or increased angiogenic potential. This selection of cells with diminished apoptotic potential could explain the resistance of many solid tumors with regions of hypoxia to cancer therapy (93).

1.4.3 Hypoxia-Inducible Factor 1 (HIF-1)

1.4.3.1 Structure

HIF-1 is a transcription factor that recognizes an 8-base pair DNA sequence 5'-TACGTGCT-3' referred to as the HIF-1 responsive element (HRE)(85)where it contacts both DNA strands in the major groove(94). It is composed of 2 subunits, HIF-1α (120 kDa) and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator; 91-94 kDa) (95). Both HIF-1 subunits are basic-helix-loop-helix proteins containing a PAS (PER-ARNT-SIM) domain (96). They are ubiquitously expressed in the cytoplasm and nucleus respectively. The expression of HIF-1 is regulated at the HIF-1α protein level, which under normoxic conditions is rapidly degraded preventing dimerization and activation. HIFs represent the link between oxygen sensors and effectors at the cellular, local and systemic level (97).

1.4.3.2 Activation

Hypoxia is a common feature of solid tumors and many genes whose expression is increased by HIF activation are expressed at a higher level in cancer than in corresponding normal tissues. In addition to microenvironmental hypoxia, multiple genetic changes that promote cancer development can contribute to HIF activation (97, 98). Activation of HIF may underlie the patterns of tumor-associated gene expression (98). The full HIF-1 activation cascade includes post-translational protein phosphorylation, nuclear translocation, ARNT heterodimerization, DNA binding, recruitment of general and tissue-specific transcriptional cofactors, and target gene transactivation (97). Many groups agree that a deacetylation event is necessary for a

HIF-1-mediated response, however the target of the deacetylation is a point of disagreement (99).

Activation of HIF-1 involves the stabilization and translocation of the HIF-1α subunit to the nucleus, where it is able to bind the HIF-1β subunit (Figure 8). This results in the recruitment of CBP/p300 coactivator (100). CBP/p300 are homologous and ubiquitously expressed proteins which posses histone acetyl-transferase activity. CBP/p300 binds to the C-terminal transactivation domain (C-TAD) of HIF-1α through its cysteine-histidine rich domain (CH1), increasing transactivation likely through local chromatin remodeling (101). Factor inhibiting HIF-1 (FIH-1) is a Fe(II)- and 2-oxogluterate dependent dioxygenase enzyme that hydroxylates asparagine-803 under normoxic conditions, preventing the C-TAD of HIF-1 from interacting with its coactivators such as CBP/p300 (102, 103). FIH-1 acts as a second oxygen sensor due to its utilization of oxygen as a substrate (104). Stabilization alone is not sufficient to fully activate HIFs under normoxic conditions (97). Protein phosphorylation is required for HIF-1 activation (105, 106), but is secondary to hypoxic stabilization of HIF-1α and seems to be required to regulate the transcriptional activity of HIF-1 (97).

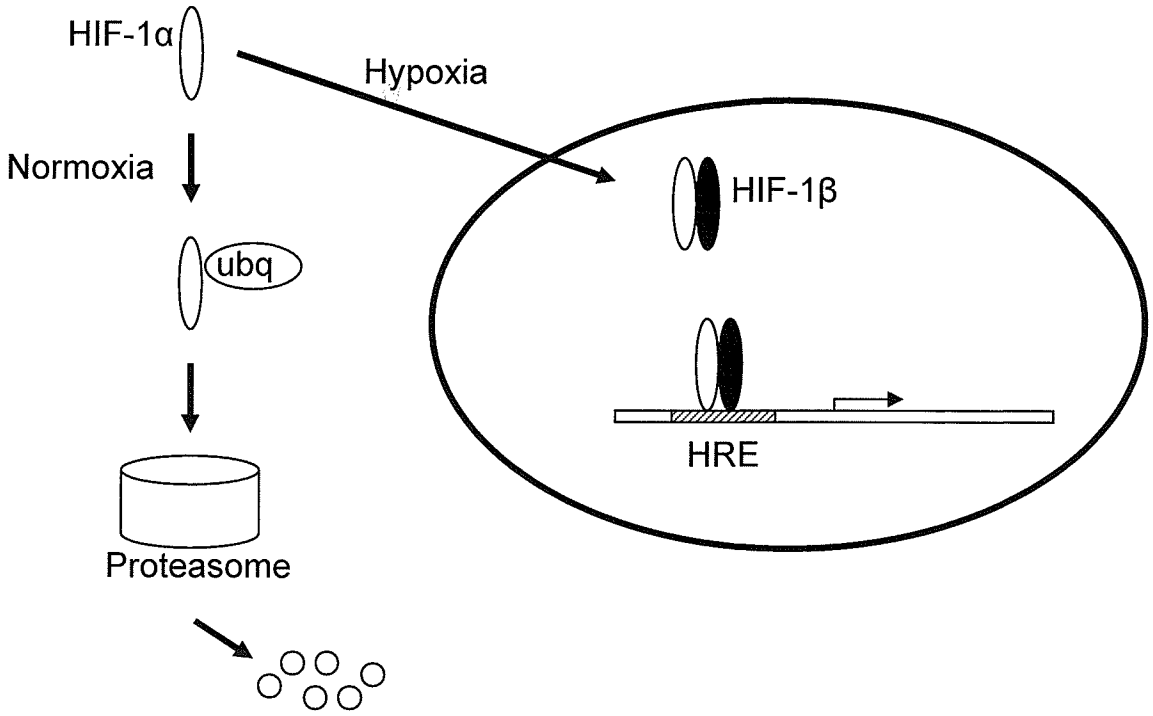


Figure 9. Model of HIF-1 activation.
Under normoxic conditions, HIF-1α is rapidly hydroxylated on proline residues 402 and 546. This marks HIF-1α for ubiquitination (ubq) and degradation by the proteasome. Under hypoxic conditions, the hydroxylation of HIF-1α is limited by the absence of oxygen. This stabilizes the HIF-1α protein and allows it to translocate to the nucleus where it binds to HIF-1β. The HIF-1α/HIF-1β dimer is then able to bind to HIF-1 responsive elements (HRE) in the promoter region of its target genes promoting gene transcription.

1.4.2.3 Regulation of HIF-1

HIF-1α is tightly regulated on multiple levels (Figure 9). The importance of the regulation of the HIF-1α subunit is supported by the fact that increased HIF-1α protein expression relative to adjacent normal tissue is observed in 13 tumor types including lung, prostate, breast, colon, gastric, skin, ovarian, pancreatic and renal carcinomas(107). Over-expression of HIF-1α is associated with treatment failure and increased mortality (108). Cells with constitutive expression of HIF-1α protein are more resistant to

apoptosis induced by hypoxia and glucose deprivation than those without constitutive HIF-1α protein expression (109). HIF-1α expression in human breast cancer is a predictive marker of chemotherapy failure (90) and increased HIF-1α expression in ovarian carcinoma correlates with increased aggressiveness and invasive potential (110).

1.4.2.3.1 Oxygen-dependent regulation: PHDs and pVHL

HIF-1α is very unstable under normoxic conditions, with a half life of less than 5 minutes (111). The oxygen-dependent degradation domain (ODD) within HIF-1α controls its degradation via the ubiquitin-proteasome pathway and may provide a means of controlling gene expression by changes in oxygen tension (112). HIF-1α contains 2 conserved (LXXLAP) motif sites for proline hydroxylation in its ODD domain (113). Prolyl hydroxylase enzymes (PHD) are iron-dependent dioxygenases requiring oxygen and 2-oxoglutarate as cosubstrates that hydroxylate proline residues Pro 402 and Pro 564 of HIF-1α subunits (97). Three prolyl hydroxylase enzymes PHD1, PHD2, and PHD3 have been characterized. PHD2 is the key limiting oxygen sensor controlling HIF-1α in normoxia. (101). The requirement of iron as a cofactor explains how iron chelators or iron antagonists (cobalt chloride) mimic the effects of hypoxia on stabilizing HIF-1 (101). Degradation of HIF-1α is mediated by the von Hippel-Lindau protein (pVHL, discussed below) which binds to the hydroxylated but not to the non-hydroxylated HIF-1α ODD domain (114). pVHL is involved in the ubiquitination of HIF-1α which marks the protein for degradation via the proteasome.

1.4.2.3.2 Negative regulation: ARD1, FIH-1, p53

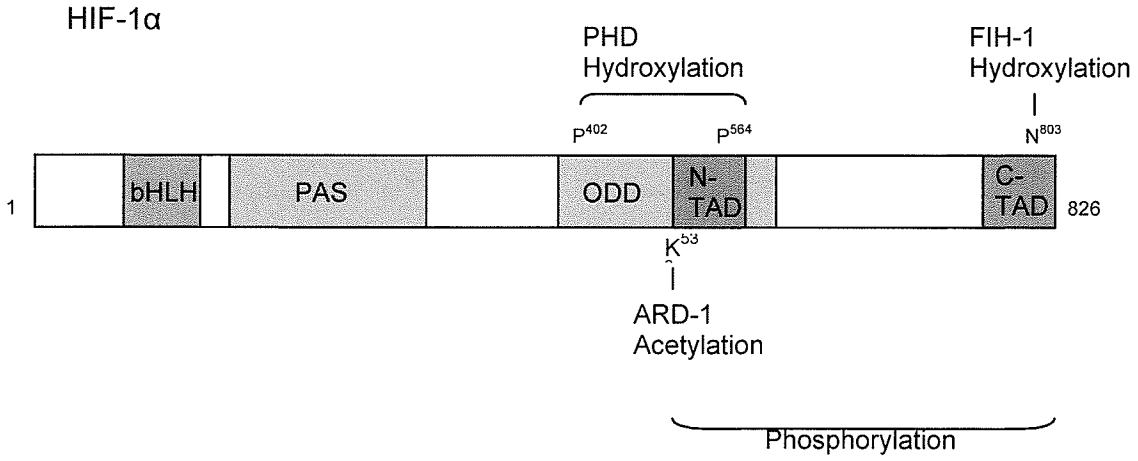
Acetylation of lysine 532 by ARD1 (arrest-defective-1 protein) is critical for the proteasomal degradation of HIF-1α (115) possibly by inducing a conformational change in HIF-1 α that increases its interaction with pVHL (104). ARD1, like PHD, acts as a negative regulator of HIF-1α by making it less stable (101). FIH-1 exerts a second level of negative control on any HIF-1α that has escaped normoxic proteasomal degradation by abolishing its interaction with its coactivator CBP/p300 (101). When Asn803 is no longer hydroxylated, p300 can bind to HIF-1α and mediate transcription (102, 103). p53 can bind HIF-1α (116) and is able to repress HIF-1 mediated transcriptional activity by binding to p300 (117).

1.4.2.3.3 Positive regulation: growth factors/PI3K/MAPK, oncogenes

Several oncogenes have been identified, including *ras* and *src*, which are thought to induce stabilization of HIF-1α through loss of hydroxylation (111).

Growth factor-dependent signaling pathways, including Ras/Raf/MAPK and PI3K/Akt cascades are involved in HIF-1 activation under normoxic conditions. ErbB2 over-expression results in constitutively active Akt, which can activate HIF-1α independent of hypoxia (72). Akt is suggested to induce HIF-1α protein translation rather than stability (118), providing a potential mechanism for how the PI3K pathway can overcome the oxygen sensor-mediated HIF-1α degradation under normoxic conditions (97). Akt can also phosphorylate HIF-1β enhancing its binding with HIF-1α, to increase HIF transcriptional activity (119). The phosphoinositide phosphatase PTEN, inhibits the PI3K pathway and therefore mutations in PTEN enhance HIF-1 activated responses

(120). Nitric oxide (NO) can also induce HIF-1 activity under normoxic conditions (121).



Adapted from Mazure *et al.*, 2004 Biochem Pharm **68**: 971-980.

Figure 10. Schematic of HIF-1α.
HIF-1α is a basic helix-loop-helix (bHLH) transcription factor with a PER-ARNT-SIM (PAS) domain. It contains two transactivation domains; (1) N-terminal transactivation domain (N-TAD) and (2) C-terminal transactivation domain (C-TAD). HIF-1 is very unstable under normoxic conditions owing to its oxygen-dependent degradation domain (ODD). Within its ODD are two conserved motifs for proline hydroxylation by prolyl hydroxylase enzymes (PHD) at proline residues 402 and 564 (P^{402} and P^{564}). Under normoxic conditions, HIF-1α is rapidly hydroxylated which targets it for degradation via the ubiquitin-proteasome pathway. ARD-1 (arrest-defective-1 protein) acetylates lysine 532 (K^{532}) which is critical for the proteasomal degradation of HIF-1α. Factor inhibiting HIF-1 (FIH-1) prevents normoxic activation of HIF-1α by hydroxylating asparagine 803 (N^{803}) under normoxic conditions, preventing its C-TAD of domain from associating with its co-activators.

1.4.2.4 Von Hippel-Lindau (VHL) tumor suppressor protein

The VHL tumor suppressor gene consists of 3 exons and encodes 2 proteins, a full length 213 amino acid protein (~28-30kDa) with no known homology to other proteins (122) and a shorter, alternatively spliced 160 amino acid protein (18-19kDa). The 4.7 kb mRNA is widely expressed in both fetal and adult tissues (123). VHL disease is a hereditary cancer syndrome associated with deregulated angiogenesis due to up-regulation of hypoxia-regulated genes by mutations in the VHL gene (122, 124). VHL disease has an incidence of ~1 in 36 000 births, and ~30% of cases arise as de novo mutations without a family history (123). It has an autosomal dominant pattern of inheritance with variable penetrance. The VHL tumor suppressor gene has features of the classical retinoblastoma (RB) tumor suppressor in that all tumor types from VHL patients have inactivation of wild-type allele by allele loss, mutation or methylation (125). Affected individuals are predisposed to develop hypervascular tumors in a number of organs (124). Most of the tumors associated with VHL disease are benign, however kidney cancer is malignant and is the principle cause of morbidity and mortality (126). Haemangioblastomas of the central nervous system are the most common tumor in VHL disease, affecting 60-80% of all patients. These tumors are benign but remain a major cause of morbidity. Retinal angiomas are seen in up to 60% of patients. Renal cell carcinomas (RCC) occur in 24%-25% of patients and are the major malignant neoplasm in VHL disease and primary cause of inherited renal cancer (127). Development of tumors in VHL disease is due, at least in part, to loss of pVHL function and consequent deregulation of hypoxia-inducible genes such as VEGF (128). Biallelic inactivation of

VHL gene is observed in the majority of sporadic RCC, establishing it as a critical "gatekeeper" of the renal epithelium (126).

The product of the VHL gene mediates ubiquitination and proteasomal degradation of HIF-1α under normoxic conditions via interaction with the core of the oxygen-dependent degradation domain of HIF-1α (129). pVHL regulates HIF-1α proteolysis by acting as the recognition component of a ubiquitin ligase complex involving Elongin C and Elongin B proteins (Figure 10) (130, 131). This complex has a central role in VHL function because most of tumor-derived mutations destabilize this complex (132). Cullin 2 (Cul2) is an E3-like ubiquitin ligase and member of the multigene cullin family. It specifically associates with the pVHL-elongin B-elongin-C complex to regulate hypoxia-inducible mRNAs (133, 134).

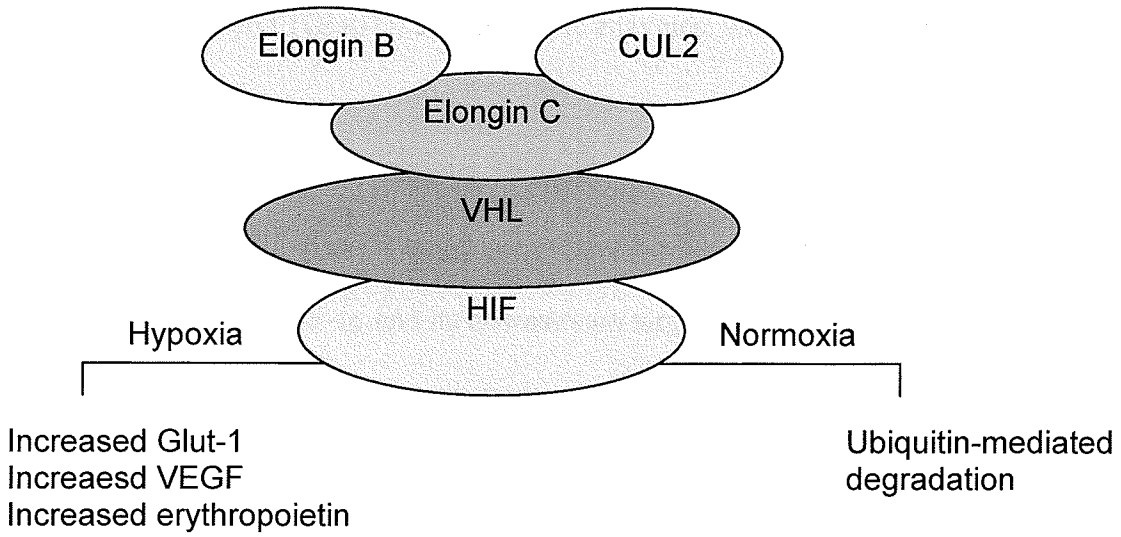


Figure 11. The pVHL complex regulates HIF-1 activity by binding HIF-1α. The Von Hippel-Lindau protein (pVHL) mediates the ubiquitination of HIF-1α under normoxic conditions leading to its degradation via the proteasome. pVHL acts as the recognition component of a ubiquitin ligase complex involving Elongin C, Elongin B and Cullin 2 (CUL2).

1.4.2.5 HIF-1 regulated genes

HIF-1 has a broad impact on cell biology which is reflected in the fact that more than 70 target genes are regulated by HIF-1 (101, 104). HIF-1 target genes are involved in three main survival strategies: energy conservation, increasing glycolysis and increasing angiogenesis (88). Target genes include angiogenic factors (vascular endothelial growth factor (VEGF) which is limited to wound healing and follicular development in adults but is a key mediator of tumor angiogenesis (73)), proliferation/survival factors (insulin-like growth factor-2 (IGF-2) and transforming growth factor-α (TGF-α)), glucose transporters

induce cell death. BNIP3 is also expressed at high levels in hypoxic regions of brain glioblastoma multiforme (GBM) tumors compared with normal brain. Mutations resulting in the loss of BNIP3's functional transmembrane domain were seen in 16% of GBM tumors screened (Dr. D.E Eisenstat, unpublished data). Since BNIP3 is over-expressed in breast tumors where it fails to induce cell death, the purpose of this project is to determine whether mutations in the BNIP3 gene play a role in breast cancer.

2. MATERIALS AND METHODS

2.1 Tissue Culture

All cell lines were maintained in a humidified 5% CO_2 environment at 37°C. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco 12800-017) supplemented with 10% (v/v) bovine calf serum (BCS; Gibco 16170-078), 1% (v/v) penicillin/streptomycin, 100units/ml and 100μg/ml respectively (Gibco 15140-122). MCF-7 breast cancer cell lines were grown in α-Modified Eagle's medium (α-MEM; Gibco 12000-022) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco 26140-079), 1% (v/v) each of 100mM sodium pyruvate (Gibco 11360-070), 1M Hepes (Gibco 15630-080), and penicillin/streptomycin. MCF-7 cells stably expressing plasmids also contained 5μg/ml Geneticin (Gibco 11811-031). SkOv3 ovarian cells were grown in RPMI (Gibco 31800-022) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100μg/ml streptomycin.

2.2 Site-directed mutagenesis

Mutant BNIP3 constructs were created by site-directed mutagenesis of 5' hemaglutinin (HA)-tagged wild type BNIP3 in a pcDNA3 (Invitrogen) vector. Primers, shown in the following table were designed to create three mutant constructs (261insC, 397insG and 409insG). The constructs were made using the QuikChange Site Directed Mutagenesis Kit (Stratagene 200519) with all of the reagents used provided in the kit unless otherwise specified. Mutant strand synthesis consisted of 30 ng of template DNA, 5μl of 10xPCR buffer, 10 pmol of both forward and reverse primer, 1μl of dNTP mix, 41μl of ddH_20 and 2.5U of *PfuTurbo* DNA polymerase. Amplification consisted of one cycle of 95°C for 30

seconds followed by 12 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 6 minutes. Parental DNA was digested by incubating the samples with 10U of *Dpn*1 enzyme at 37°C for 1 hour. Fifty microlitres of supercompetent XL1-Blue cells were transformed with 1µl of the *Dpn*1 digested DNA by incubation on ice for 30 minutes, heat-pulsed for 45 seconds at 42°C and then placed on ice for 2 minutes. Five hundred microlitres of SOC media (Invitrogen 15544-034) pre-heated to 42°C was added to each of the reactions and incubated for 1 hour at 37°C. The cells were plated on LB plates containing 50 µg/ml ampicillin (Sigma A-9518) and grown overnight at 37°C. Colonies were picked and grown in 5 ml of LB broth overnight. Plasmid DNA was extracted using Qiagen Miniprep kit (27106) per the manufacturer's directions and screened by sequencing.

261insC

F: 5'-AGATACCCATAGCACTTGGAGAGAAAAACAGCTCACAG -3'

R: 5'-CTGTGAGCTGTTTTTCTCTCCAAGTGCTATGGGTATCTG-3'

397insG

F: 5'-CCCGCCTCAGGGAGTTCACTCTTTAAACACCCG-3'

R: 5'-CGGGTGTTTAAAGAGGAACRCCCTGAGGCGGG-3'

409insG

F: 5'-CCTCTTTAAACACCGCGAAGCGCACGGCC-3'

R: 5'-GGCCGTGCGCTTCGCGGTGTTTAAAGAGG-3'

2.3 Western Blotting

Cells were pelleted at 1200 rpm for 5 minutes using an Eppendorf Centrifuge 5810R. The media was removed by aspiration and 100-300 µl lysis buffer (0.6M Hepes pH 7.25, 2M NaCl, 0.6mM ZnCl2, 0.6mM NaF, 12.5µM ethylenediaminetetraacetic acid (EDTA; Sigma D-5134), 2mM Phenylmethylsulfonyl Fluoride (PMSF; Roche 837 091) and 12.5% Igepal CA-630 (Sigma I-3021) was added to each sample. The samples were vortexed (Fisher Vortex Genie 2 12-812) for 10 seconds, followed by incubation on ice for 5 minutes. The vortexing and ice incubation steps were repeated a second time and the samples stored at -20°C. The protein concentration of each cell lysate to be assayed was determined by a Bradford assay using a 1mg/mL bovine serum albumin (BSA; Sigma A1933) solution as a standard. The absorbance values were read at 280nm on a Beckman Coulter DU 640 spectrophotometer, creating a linear standard curve with a zero-intercept and an R value of at least 0.98. Two microlitres of each sample were diluted in 2mL of Bio-Rad protein assay dye (500-0006, diluted 1:5 in dH_2O). A range of 10µg to 50µg of cell lysate protein was placed in a sterile Eppendorf microcentrifuge tube and mixed with 3-5µL of denaturing 5X sample loading buffer (0.2mL 10% sodium docedyl sulphate (SDS; Sigma L4509), 0.1mL glycerol (Sigma G8773), 0.1mL 1M Tris pH 6.8 (Fisher BP1525), 10% β-mercaptoethanol (Sigma M6250), and 1-2 grains (~60-130 mg) bromophenol blue (Sigma B-5525) for colour). The samples were placed in a boiling water bath for 10 minutes and allowed to cool before being loaded onto a 10% acrylamide SDS-gel (Stacking gel: 1.5mL ddH2O, 625µL 4X upper buffer (0.5M Tris-HCl (Invitrogen 15504-020), 0.4% SDS, pH 6.8), 338µL 30% acrylamide (Bio-Rad 161-0156), 30µL 10% ammonium persulfate (APS; Sigma A-3678) and 20µL TEMED

(N,N,N',N'-tetramethylethylenediamine; Fluka 87689); Running gel: 2.1mL ddH2O, 1.25mL 4X lower buffer (1.5M Tris base, 0.4% SDS, pH 8.8), 1.7mL 30% acrylamide, 50μL TEMED, 5μL 10% APS). 10μL of PageRuler Prestained Protein ladder (Fermentas SM0671) was loaded on each gel. Gels were run in 1X running buffer (25mM Tris base, 190mM glycine (Sigma G8898), 3mM SDS) at 100V for approximately 2 hours. Gels were transferred to nitrocellulose membranes in transfer buffer (3.03g Tris base, 14.4g glycine, 800mL ddH_2O, 200mL 100% methanol (Sigma A-412-4)) at 24V for 1-2 hours. The membranes were blocked in 1X Tris buffered saline (TBS; 20mM Tris base, 130mM NaCl (Fisher BP358-10), pH 7.4) with 1% Tween 20 (Sigma P5927-500mL) and 5% (w/v) skim milk powder (Safeway brand), for a minimum of 1 hour at room temperature to overnight at 4°C. The polyclonal BNIP3 antibody (30) was diluted 1/1000 for western blotting. The Glut-1 antibody (Medicorp RB-9052-P0) was diluted 1/250 in blocking buffer and incubated overnight at 4°C. Membranes were also probed with anti-actin antibodies (Sigma A2066) as loading control. The membranes were washed three times for 5 minutes each in 1X TBST with rocking and incubated in the secondary antibody, Horseradish peroxidase (HRP) conjugated goat α rabbit (Bio-Rad 170-6515) diluted 1/2000 in blocking buffer. The membranes were washed again three times in 1X TBST, and then visualized on autoradiography film (Kodak Scientific Imaging Film (864 6770) using chemiluminence (ECL) blotting detection reagents (Amersham RPN-2106). The blots were stripped for 30 minutes in 1x stripping buffer (Western-Re-Probe Calbiochem WB59). They were then blocked and probed with a different antibody.

2.4 Hypoxia death curves

Untreated MCF-7, MCF-7 PEST and SkOv3 cells were plated a minimum of 24 hrs before being placed in the Forma Scientific Anaerobic Sytem (hypoxic chamber), which maintains a ≤1% oxygen level. Total cell death was assayed after 0, 24, 48 and 72 hrs of hypoxia treatment by Acridine Orange – Ethidium Bromide (Sigma A-6014 and Bio-Rad 161-0433, respectively; 100μg/ml each) staining. The number of dead cells (permeable to the dye) were counted; a minimum of 200 cells were counted per sample.

2.5 Localization of BNIP3 to the mitochondria

HEK293 cells were grown on cover slips in 6-well dishes and transiently transfected with vectors expressing wild type or mutant BNIP3 constructs. All of the vectors, excluding BNIP3 Δ236, contained a 5' hemaglutinin (HA) epitope tag. Transfections were carried out with Lipofectamine 2000 reagent (Invitrogen 11668-027) using 1.5 μg of DNA per well and 12 μL of transfection reagent. The cells were incubated for 48 hrs post transfection before the addition of 0.3μg/mL of MitoTracker Red (Molecular Probes M-7512) suspended in dimethyl sulfoxide (DMSO Fisher D136-1). The cells were incubated for 1hr to allow for the uptake of the MitoTracker Red before being fixed in 3.7% formaldehyde (Fluka 47629) in 1x PBS for a minimum of 1 hr at room temperature. The cells were washed 3 times for 5 minutes each with rocking in PBS with 0.1% Igepal CA-630 (Sigma I-3021). Non-specific antibody binding was blocked by incubating the cells in 10% FBS/1xPBS/0.1% Ipegal for 1 hr at room temperature. The primary antibody used was anti-HA tag 1/100 for all constructs excluding BNIP3Δ236, which was

probed with anti-BNIP3 polyclonal antibody at a dilution of 1/750, for 2 hrs at room temperature. The cells were washed as previously described and incubated with the secondary antibody, anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate (Sigma F-0382), 1/500 for 1 hr at room temperature. The cells were washed and the slips mounted onto slides with 5µl of Slowfade Gold with DAPI (Invitrogen S-36938). The staining was visualized by confocal microscopy.

2.6 RNA isolation and Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from frozen breast cancer tissues obtained from the Manitoba Breast Tumor Bank using RNeasy Plus Mini Kit (Qiagen 74134) as per the manufacturer's instructions. The integrity of the RNA was visualized by running the isolated RNA out on a RNA Gel (42ml RNase free water, 0.5g agarose, 5ml 10x running buffer (0.2M MOPS (Sigma M-8899), 50mM sodium acetate (Fisher B9333), 10mM EDTA pH 7). The samples were quantified using the Beckman Coulter DU 640 Spectrophotometer. One microgram of each RNA sample was diluted to a final volume of 4 µl in ddH20 and then mixed with 1 µl of 0.5mg/ml ethidium bromide and 15µl of loading buffer (50% formamide, 10% 10x running buffer, 0.6% formaldehyde, 6.7% glycerol and several grain bromophenol blue). The samples were incubated at 65°C for 10 minutes and kept on ice until loaded onto the gel. The gel was run at 100V for 30-60 minutes in 1x running buffer. RT-PCR was carried out using SuperScript III One-step RT-PCR with Platinum Taq (Invitrogen 12574-026) and two sets of primers F/R and FLF/FLR (F: 5'-GCTCCGACCTCCGCTTTC-3' and R: 5'-TTGATCCCATAGGTGTAA-3'; FLF: 5'-ATGTCGCAGAACGGAGC-3' and FLR: 5'-

TCAAAAGGTGCTGGTGGAG-3'). Each reaction contained 1µg of RNA, 20 pmol of both forward and reverse primer, 1µl of Taq DNA polymerase and ddH_20 up to final volume of 50µl. The PCR consisted of 30 minutes at 55°C, 2 minutes at 94°C, followed by 40 cycles of 94°C for 15 seconds, 45°C or 49.7°C for 30 seconds and 70°C for 2 minutes, and a final extension at 72°C for 10 minutes using a Perkin Elmer GeneAmp PCR System 2400 thermocycler. Following the RT-PCR, 5µl of the resultant cDNA was purified by the addition of 2µl of ExoSAP-IT (USB 78200) to remove excess dNTPs and unused primer. The mixture was incubated at 37°C and 80°C, each for 15 minutes before the sample was used for sequencing.

2.7 Sequencing

Samples to be sequenced were subjected to an additional sequencing PCR using the Perkin Elmer GeneAmp PCR System 2400 thermocycler. A range of 0.5-2.5µl of sample was mixed with 2µl of Big Dye 3.1/buffer (Applied Biosystems 4336917) and 5pmol of primer. The samples were amplified by PCR; 96°C for 1 minute, 30 cycles of 93°C for 1 minute, 40°C for 25 seconds, 60°C for 4 minutes. The DNA was precipitated by adding 11µl of 70% ethanol for a minimum of 15 minutes and pelleted by spinning in a Labnet Spectrafuge 16M microcentrifuge at maximum speed for 15 minutes. The DNA was sequenced on the ABI Prism 310 sequencing apparatus available at the Institute.

2.8 Single-stranded conformation polymorphism

Genomic DNA was extracted from cell lines or primary breast tissue using the DNeasy Plus Mini Kit (Qiagen 69504) as per the manufacturer's directions. Primers were designed to amplify the region containing BNIP3 exon 3 (F: 5'-GGTTCACTGCCTTTGGGG-3' and R: 5'-AACCCACTCACTGTCCAAC-3') and the region spanning exons 4 and 5 together (F: 5'-FTFFFAFCTGGGTGCTTT-3' and R: 5'-RGCAACCCAGAATCGCCC-3'). The PCR reaction mixture contained 100 ng of template DNA, 5µl of 10x PCR buffer (USB 71189), 10 pmol of both forward and reverse primer, 0.4µl of dNTP mix (5mM dATP, 25mM dCTP, 25mM dGTP and 25mM dTGP), 3u of Fidelitaq (USB 71189) and ddH_20 to a final volume of 49.5µl. To each sample, 0.5µl of 9.25 mCi α-P^{32} dATP was added just before the sample was placed in the thermocycler (Perkin Elmer GeneAmp PCR System 2400). Amplification consisted of one cycle of 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute, followed by a 10 minute final extension at 72°C. Five microlitres of the labeled PCR products were mixed with 5 µl of formamide loading dye (95% formamide (Fisher AC18109), 20mM EDTA, 0.05% each xylene cyanol (Sigma X-4126) and bromophenol blue for colour) and denatured by incubating the samples at 94°C for 5 minutes. The samples were kept on ice until they were loaded onto a non-denaturing 10% acrylamide gel containing 5% glycerol (5ml glycerol (Sigma G8733)), 33.2ml 30% acrylamide (Bio-Rad 161-0156), 10ml 5xTBE (0.4M Tris base, 0.4M Boric Acid, 10mM EDTA (ethylenediaminetetraacetic acid; Sigma E-5134) and ddH_20 to a final volume of 100ml. The samples were loaded onto the gel in the Bio-Rad Sequi-Gen GT Sequencing Cell apparatus and run at 10mA at 4°C for 18hrs in 0.5x TBE (40mM

Tris, 40mM Boric Acid, 1mM EDTA). The gel was dried for 1 hour at 80°C using the Savant Slab Gel Dryer SGD5040 and then exposed to autoradiography film and/or a phosphoimaging screen overnight at room temperature.

2.9 Immunofluorescent staining (BNIP3 & Glut-1) of paraffin-embedded tissue

Paraffin-embedded breast tumor sections were obtained from the Manitoba Breast Tumor Bank. Slides were baked at 60°C for 20 minutes and treated with xylene (Fisher XS-4) for 10 minutes, plus two additional 2 minute washes in xylene to remove paraffin. The tissue was re-hydrated by treatment in each of the following for 2 minutes: 100% ethanol, 100% ethanol, 95% ethanol, 85% ethanol, 75% ethanol and 50% ethanol with a final 5 minutes in ddH_2O. Antigen presentation was achieved by placing the slides in a pressure cooker (Tender Cooker by Nordicware) filled with 1 L of 10mM citrate buffer pH 6.0 (prepared fresh) and microwaved on high power for 20 minutes. The slides were cooled to room temperature and washed 3 times with rocking in 1x PBS. To block non-specific antibody binding, the cells were incubated in 1x Roche Blocking Buffer (Roche 1096176) diluted in 1x PBS for 2 hours in a humidity chamber. The cells were washed 3 times for 5 minutes each with rocking in 1xPBST 0.1% Tween 20 (Sigma P5927 (PBST) following blocking, and after each subsequent incubation step with an antibody. All of the incubations used 100µl per slide, and were carried out in a humidity chamber. The slides were incubated with the primary anti-BNIP3 antibody (polyclonal antibody raised in rabbit; (30)) diluted 1/1000 in blocking buffer overnight at 4°C. The secondary antibody, biotinylated anti-rabbit (Vector Labs BA-1000) was diluted 1/200 in blocking buffer and applied to the slides for 1 hour at room temperature. The fluorescence

detection reagent, streptavidin Alexafluor 488 (Molecular Probes S-11223), was diluted 1/500 in blocking buffer and applied to the slides for 1 hr at room temperature in the dark. The slides were blocked with 100% bovine calf serum for 30 minutes at room temperature, followed by an one hour incubation with swine anti-rabbit IgG (DAKAcytomation Z 0196) diluted 1/25 in blocking buffer. The second primary antibody, anti-Glut-1 (Medicorp RB-9052-P0) was diluted 1/100 in blocking buffer and incubated overnight at 4°C. The secondary antibody was biotinylated anti-rabbit, followed by a 1 hour incubation with streptavidin Alexafluor 633 (Molecular Probes S-21375) diluted 1/500 in blocking buffer. Five microlitres of Slowfade Gold with DAPI (Invitrogen S-36938) was applied to each slide and mounted with cover glass. The staining was visualized by confocal microscopy using the Olympus FV500 confocal microscope.

2.10 Animal Models

Forty-eight hours before injection of tumor cells, an estrogen pellet was implanted subcutaneously in the athymic nude mice (CD-1 Nude; Charles River Laboratories, MA) to support the growth needs of the MCF-7 cells. Mice were anesthetized by intraperitoneal (I.P.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) solution in PBS. A 1.7 mg 17β-estradiol sustained-release pellet (Innovative Research of America SE-121) was implanted subcutaneously in a 5mm incision made in the dorsal neck region. The incision was sealed with Vetbond Tissue Adhesive (3M 1469SB). After a minimum of 48hrs, the mice were once again anesthetized as previously described for the injection of the tumor cells. Approximately $1x10^7$ MCF-7 breast cancer cells stably

expressing empty vector (pcDNA3) or BNIP3Δ236 (PEST) constructs were washed in 1x PBS and suspended in 250μL of plain α-MEM media. The cells were kept on ice until just before injection, at which time they were mixed with 250μL of Matrigel (BD Biosciences 354234). The cells were injected subcutaneously in the flank region of the mouse using an 18-gauge needle. Tumors cells were injected similarly into both the left and right flank regions. Tumor size was measured by monitoring the length and width of the visible tumor burden every 3-4 days. After 45 days, the mice were injected (intraperitoneal) with 150μL of 15 mg/mL hypoxyprobe 1 (Chemicon 90203). After 20 minutes the mice were sacrificed using CO_2 and the tumors excised. Half of the tumor was fixed in 10% buffered formalin (Fisher SF100) and taken to the Manitoba Breast Tumor Bank to be embedded in paraffin and sectioned for immunohistochemical analysis. The remaining tumor was stored at -80°C. All animal work was performed following protocols approved by the Central Animal Care Committee under the auspices of the Canadian Council on Animal Care (CCAC).

2.11 Immunohistochemical staining of mouse tumors

Slides containing 5μm sections of mouse tumors were prepared by the Manitoba Breast Tumor Bank. The slides were deparaffinized and rehydrated as previously described. The slides were washed once for 5 minutes in ddH_20, followed by two washes of 5 minutes each in 1x PBS. Endogenous peroxidase activity was blocked by incubating the slides in freshly prepared 0.3 % H_2O_2 (Fisher H324-500) solution in methanol for 10 minutes. The slides were washed with PBST 3 times for 5 minutes each with rocking between every incubation step. The slides were blocked in 1x Roche blocking buffer

(Roche 1096176) for 2 hours at room temperature in a humidity chamber. The primary antibody, anti-BNIP3 (1/500) or anti-hypoxyprobe 1 (Chemicon International 90204; 1/50), was incubated with the slides overnight at 4°C. The slides were incubated with secondary antibody, biotinylated anti-rabbit IgG (Vector Laboratories BA-1000) or biotinylated anti-mouse IgG (BA-9200), respectively, at a concentration of 1/1000 for 1 hour at room temperature in a humidity chamber. The slides were incubated with ABC reagent (Vector Laboratories PK-6100) for 30 minutes (prepared by adding one drop of solution A to 2.5 mL of 1x PBS, followed by the addition of one drop of solution B, mixed and incubated 30 minutes at room temperature before use). The slides were incubated with DAB (Vector Laboratories SK-4100) prepared by mixing 1 drop of buffer solution, 2 drops of DAB stock solution, 1 drop of H_20_2 solution to 2.5 mL distilled water) for 2 minutes 30 seconds. The slides were washed in water for 5 minutes to stop the reaction. Slides were stained with Hematoxylin (Sigma MSH32) for 30 seconds, followed by washing in running tap water for 5 minutes and incubated in ddH_2O for 5 minutes. The slides were dehydrated by successive washing in 50%, 75%, 85%, 95%, 100%, 100% ethanol for 2 minutes each followed by 2 washes in xylene. The slides were mounted with Permount (Fisher SP15-100).

3. HYPOTHESES

BNIP3 is mutated in a subset of breast tumors. (1) These mutations eliminate BNIP3's cell killing function. (2) BNIP3 mutant protein acts in a dominant negative fashion interfering with wild type BNIP3 function, thereby inhibiting hypoxia-induced cell death.

4. RESULTS

4.1 The ovarian cell line SkOv3 has a mutation in the BNIP3 gene.

BNIP3 is a pro-cell death member of the Bcl-2 protein family. It has previously been shown that BNIP3 mRNA is expressed at higher levels in breast tumors compared with normal breast tissue and that BNIP3 over-expression in ductal carcinoma *in situ* correlates with a high grade necrotic lesion that is likely to be associated with an invasive phenotype. This is paradoxical since BNIP3 is expressed in viable breast tumor cells where it fails to induce cell death. We wanted to determine if BNIP3 is mutated in any breast cancer cell lines rendering it unable to induce cell death. Total RNA was isolated from six different breast cancer cell lines including: MCF-7, ZR-75-1, MDA MB 231, MDA MB 468, T47D, and SkBr3. Each sample and subjected to a reverse-transcriptase polymerase chain reaction (RT-PCR) and the resultant cDNA sequenced using 2 sets of BNIP3 specific primers that allowed complete coverage of the cDNA. No mutations were detected in any of the breast cancer cell lines screened; however, all of the cell lines did have a single nucleotide polymorphism (SNP) at nucleotide 235. This SNP did not change the coded amino acid and therefore has no effect on BNIP3 protein function. We also screened an ovarian cell line, SkOv3, for mutations in BNIP3, since hypoxia is associated with a worsened prognosis in ovarian cancer. We found an insertion of an adenine at nucleotide 236 (Figure 12) which results in a shift in the open reading frame and introduction of a premature stop codon. This mutation is located within the PEST domain of the protein and will be referred to as PEST mutant BNIP3. This mutation eliminates the transmembrane domain of BNIP3, which has been shown to be essential

for BNIP3's ability to dimerize and integrate into the mitochondrial membrane where it induces cell death.

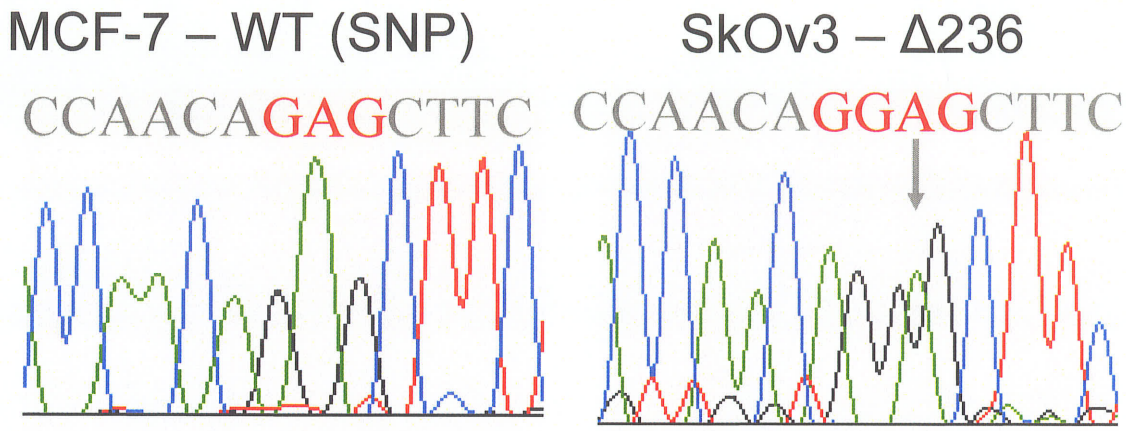


Figure 12. The ovarian cell line SkOv3 has a mutation in the BNIP3 gene.

RNA was isolated from MCF-7 and SkOv3 cell lines and reverse-transcriptase PCR was performed using 2 different primer sets to screen the entire BNIP3 cDNA for mutations. The cDNA was sequenced using these same primer sets using the ABI310 sequencing apparatus.

4.2 SkOv3 cells express truncated BNIP3 protein.

The mutation detected in the BNIP3 gene in the SkOv3 cell line results in a shift in the open reading frame of the protein and introduces a premature stop codon (nucleotides 241-243) that eliminates the C-terminal portion of the protein. To determine if we could detect this truncated protein, we evaluated BNIP3 protein expression by both western blotting and immunofluorescent staining using a polyclonal antibody raised against the N-terminal portion of BNIP3. This would allow us to detect both wild type and mutant protein using the same antibody. In both experiments we used human embryonic kidney 293 (HEK293) cells as both a negative and positive control for BNIP3. HEK293 cells do not express any detectible BNIP3 under normoxic conditions, but endogenous BNIP3 expression can be induced by incubating the cells under hypoxia. Increased expression of BNIP3 can be seen in HEK293 cells after 48 hours of hypoxia, and therefore can be used as a positive control for BNIP3 expression. BNIP3 can be visualized by western blot as a 30 kDa band or 60 kDa band representing a BNIP3 dimer. The predicted size of the PEST mutant BNIP3 is less than that of the wild type protein. In the SkOv3 cells grown under normoxic conditions, we see the appearance of an additional band at 40kDa (Figure 13A) which may be a dimer of mutant BNIP3. This 40 kDa band was shown to be specific for BNIP3 by competing out the signal by pre-incubation of the antibody with recombinant BNIP3 protein. We confirmed the expression of BNIP3 protein in SkOv3 cells grown under normoxic conditions by immunofluorescence. Under normoxic conditions mutant BNIP3 protein was expressed in SkOv3 cells but not in HEK293 cells as expected, whereas under hypoxic conditions, we observe mutant and wild type BNIP3 expression in both cell lines, respectively (Figure 13B).

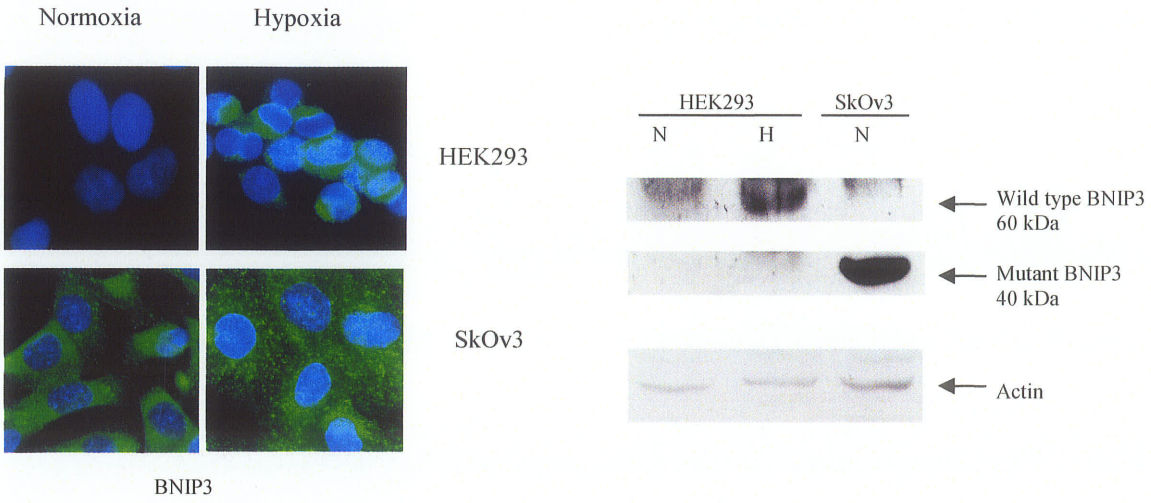


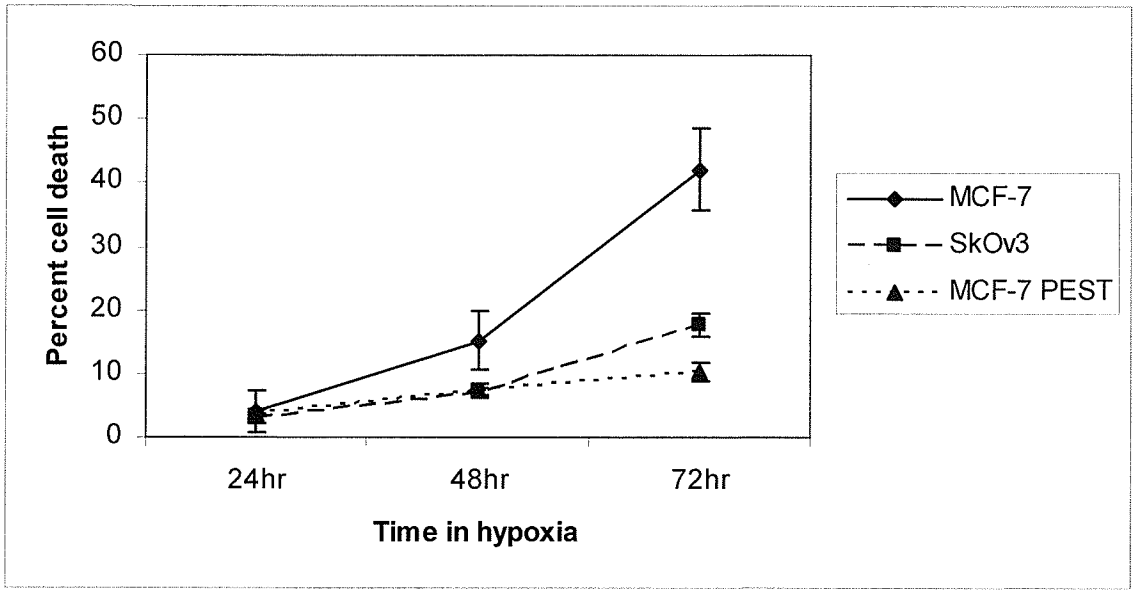
Figure 13. SkOv3 cells express truncated BNIP3 protein.

A) HEK293 and SkOv3 cells were grown on coverslips for 48 hours under normoxic or hypoxic conditions. The cells were then fixed and probed with a BNIP3 rabbit polyclonal antibody that recognizes an N-terminal portion of the protein. A FITC (green) conjugated anti-rabbit secondary antibody was used to detect the BNIP3 antibody indicating the presence of BNIP3 protein. B) HEK293 and SkOv3 were grown under either normoxic (N) or hypoxic (H) conditions for 48 hours. The cells were lysed and the proteins western blotted using a polyclonal BNIP3 antibody. The band that appears at 60 kDa in the HEK293 cells grown under hypoxia is specific and represents a wild type BNIP3 dimer. The 60 kDa band seen in the HEK93 cells is missing from the SkOv3 cells, but an additional band at 40 kDa appears. This band is specific and likely represents a dimer of mutant BNIP3. The bands seen just above 60 kDa are non-specific background bands. The blots were stripped and reprobed for β-actin expression as a loading control.

4.3 Cells expressing PEST mutant BNIP3 are resistant to hypoxia-induced cell death.

BNIP3 expression is induced by the transcription factor Hypoxia-inducible Factor-1 (HIF-1) under conditions of low oxygen. Since BNIP3 induces cell death via its transmembrane domain (TM), we evaluated what affect the PEST mutant BNIP3, which is lacking a TM domain, had on hypoxia-induced cell death in cell lines expressing the mutant protein. We used MCF-7 cells which express wild type BNIP3 under hypoxic conditions, SkOv3 cells with endogenous PEST mutant BNIP3 and an MCF-7 cell line stably over-expressing a BNIP3 PEST mutant construct. The cells were grown under hypoxic conditions (less that 1% oxygen) for a time course of 24, 48 and 72 hours. Cell death was determined by the permeability of the cells to acridine orange-ethidium bromide staining. For each sample a minimum of 200 cells were counted for dye uptake. We found that cells expressing PEST mutant BNIP3 were significantly more resistant to hypoxia-induced cell death compared to cells expressing wild type BNIP3. At 72 hrs of hypoxia, 42% of MCF-7 cells under went cell death, compared to the 18% cell death in the SkOv3 cells and 10% cell death in the MCF-7 cells expressing PEST mutant BNIP3 (Figure 14A). These results indicate a role for wild type BNIP3 in hypoxia-induced cell death. To confirm that the cells were responding to hypoxia, we also western blotted for Glut-1 expression, which is a marker of hypoxia. We show an increase in Glut-1 expression over time (Figure 14B) indicating that the SkOv3 cells are indeed responding to the hypoxic environment by up-regulating a HIF-1 responsive gene; however, the cells are undergoing less cell death when compared to the MCF-7 cells expressing wild type BNIP3.

A)



B)

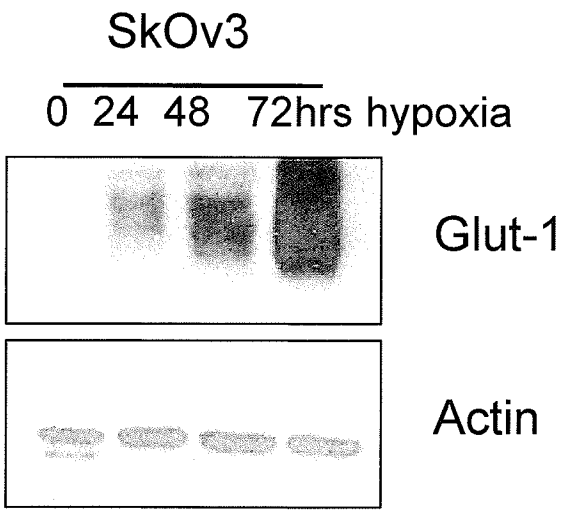


Figure 14. Cells expressing PEST mutant BNIP3 are resistant to hypoxia-induced cell death.
A) MCF-7, SkOv3 and MCF-7 cells stably expressing PEST mutant BNIP3 were cultured under hypoxic conditions (<1% oxygen) for a time course of 24, 48 and 72 hours. Following hypoxic treatment cell death was determined by membrane permeabilization using acridine orange staining. A minimum of 200 cells were counted per sample. These results represent a minimum of 3 independent experiments. B) SkOv3 cell lysates were assayed for Glut-1 expression by western blot. The blots were stripped and re-probed for β-actin as loading control.

4.4 Wild type BNIP3 induces cell death in transiently transfected SkOv3 cells.

The major function of wild type BNIP3 is to induce cell death. Since SkOv3 cells expressing PEST mutant BNIP3 are more resistant to hypoxia-induced cell death, we sought to determine whether the addition of wild type BNIP3 is able to induce cell death in the presence of mutant BNIP3. SkOv3 cells were transiently transfected with an HA-tagged wild type BNIP3 construct and pcDNA3 (empty vector) for transfection control. Twenty four hours post transfection the cells, grown on cover slips, were fixed and stained for the HA-tag indicating the positive transfection of the cell with wild type BNIP3. Positively stained cells were then assessed for viability based on nuclear morphology. Fifty five percent of SkOv3 cells transfected with wild type BNIP3 were dead 24 hours after transfection, compared to less than 10% cell death in the vector alone control cells (Figure 15). This confirms that wild type BNIP3 is able to induce cell death under normoxic conditions, and that SkOv3 cells are sensitive to BNIP3-induced cell death.

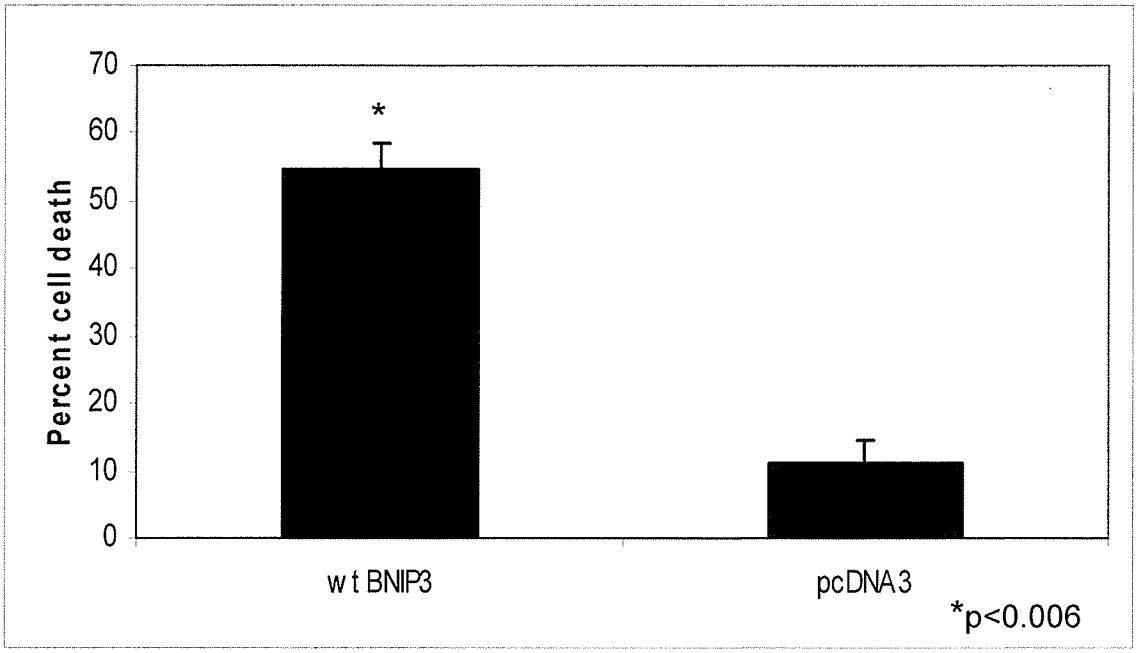


Figure 15. Wild type BNIP3 induces cell death in transiently transfected SkOv3 cells.
SkOv3 cells growing on coverslips were transiently transfected with HA-tagged wild type BNIP3 or empty vector (pcDNA3) using Lipofectamine 2000. The cells were cultured under normoxic conditions for 24 hours before being fixed and immunofluorescence detection of the HA tag. DNA was stained with DAPI and nuclear morphology was used to determine the viability of positively transfected cells. A minimum of 200 cells were counted per sample. These results represent 3 independent experiments.

4.5 HEK293 and MCF-7 cells transfected with BNIP3 mutants are more resistant to hypoxia-induced cell death.

The transmembrane domain of the BNIP3 protein is essential for its ability to dimerize and induce cell death. It has previously been shown that a BNIP3 mutant lacking its transmembrane domain (TM) is able to inhibit hypoxia-induced cell death in a dominant negative fashion. PEST mutant BNIP3 is lacking its TM domain due to the introduction of a premature stop codon resulting in protein truncation. To determine if PEST mutant BNIP3 is also able to inhibit hypoxia-induced cell death similarly to the TM mutant in a dominant negative fashion, HEK293 and MCF-7 cells were transfected with pcDNA3 (empty vector), TM mutant (positive control) and PEST mutant. The cells were cultured under hypoxic conditions (<1% oxygen) for 48 hrs, after which the cells were cytospun onto slides and fixed for immunoflourescence. Nuclear morphology was used to determine viability in positively transfected cells. HEK293 cells transfected with pcDNA, TM and PEST constructs underwent 28%, 22% and 17% cell death respectively. Similarly, MCF-7 cells exhibited 30%, 17% and 17% cell death when transfected with empty vector, TM and PEST constructs respectively. These results indicate that cells expressing PEST mutant BNIP3 were more-resistant to hypoxia-induced cell death when compared with cells expressing empty vector BNIP3 (Figure 16). This increased resistance supports the hypothesis that PEST mutant BNIP3 is able to block the function of wild type BNIP3 and may act in a dominant negative fashion.

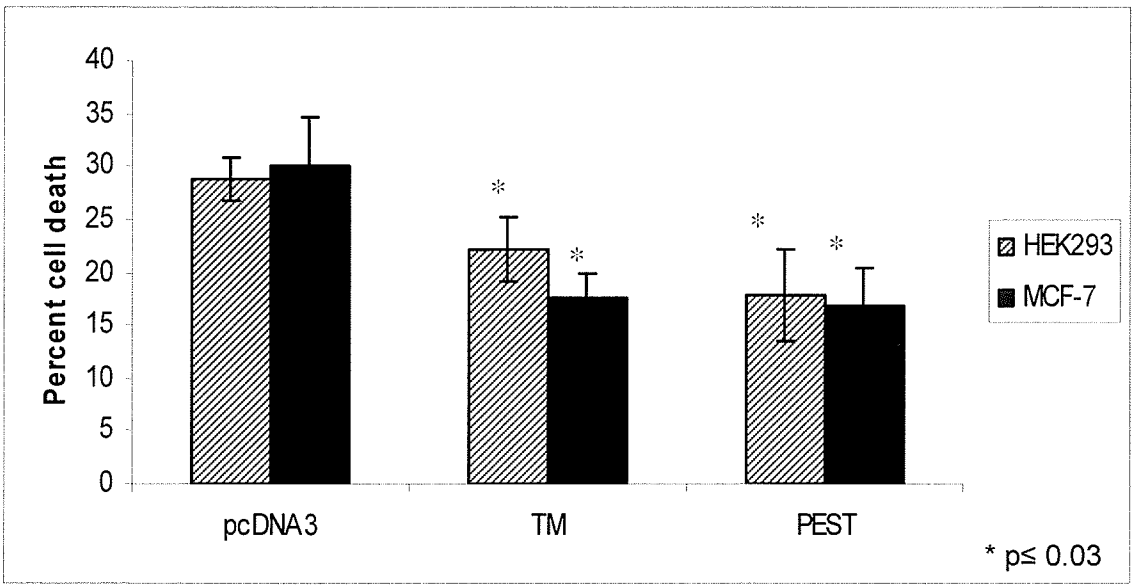


Figure 16. HEK293 and MCF-7 cells transfected with BNIP3 mutants are more resistant to hypoxia-induced cell death.
HEK293 and MCF-7 cells were transfected with pcDNA3 (empty vector), TM mutant (positive control) and PEST mutant using Lipofectamine 2000 and Gene porter, respectively. The cells were cultured under hypoxic conditions (<1% oxygen) for 48 hrs, after which the cells were cytospun onto slides and fixed for immunoflourescence. Cells immuno-positive for mutant BNIP3 were detected using polyclonal antibodies against BNIP3. Nuclear morphology was used to assess viability, with a total of 200 cells counted per sample.

4.6 23% (7/30) of sequenced breast tumors have a mutation in the BNIP3 gene.

BNIP3 is over-expressed in peri-necrotic regions of breast tumors where it paradoxically fails to induce cell death and correlates with worsened prognosis (75, 76). Inactivation of BNIP3 by hypermethylation has been seen in a variety of human tumors including; haematopoietic, colorectal, pancreatic and gastric tumors suggesting that tumors cells that do not express BNIP3 have a growth advantage related to their ability to escape cell death induced by hypoxia (41, 138-142). The presence of inactivating mutations is another mechanism by which BNIP3 may be unable to induce cell death. Since a mutation in the BNIP3 gene was found in an ovarian cancer cell line, we wanted to investigate whether BNIP3 mutations may also play a role in breast tumors. We obtained 30 high grade tumors from the Manitoba Breast Tumor Bank. Some of the tumors contained regions of necrosis indicated by the value 1 (a value of 0 indicated no areas of necrosis) which suggests that the tumor may contain hypoxic regions. The ER (value $\geq$3 ndicates positive expression) and PR (value $\geq$10 indicates positive expression) status, whether the tumor was treated with hormone therapy (HT), radiation (RT), chemotherapy (CT) or surgery (SX), as well as any known survival data was obtained from the tumor bank. The anonymized clinical data available for each sample is summarized in Table 3. Total RNA was isolated from each sample and subjected to a reverse-transcriptase polymerase chain reaction (RT-PCR) and the resultant cDNA was sequenced using two sets of BNIP3 specific primers that allowed complete coverage of the cDNA. We found a total of 7 out of 30 tumors (23%) had a mutation in the BNIP3 gene. All of the mutations detected were single nucleotide insertions (Figure 17). Four of the tumors contained the same mutation observed in the SkOv3 cell line, an insertion of an adenine at nucleotide

236. Three novel single nucleotide insertions were also observed: (1) 261insC; (2) 397insG; and (3) 409insG. All of the mutations with the exception of 409insG result in the introduction of a stop codon and predict protein truncation. The mutation at nucleotide 409 results in a frameshift mutation. In all cases, the mutations would result in a loss of the function transmembrane domain of the BNIP3 protein. The proposed protein structures are summarized in Table 4.

Table 3. Clinical data available on the breast tumors screened for BNIP3 mutations.

TUMOR	BNIP3 Mutation	Necrosis	ER	PR	HT	RT	CT	SX	Time to Recurrence	Time to new primary	Patient Status	Status Time
10158A	0	1	1.8	2.4			y	y	15 months		died of disease	22
10898A	0	0	104	11.1	y	y		y	215*/328	240	died of other cause	329
11319A	0	0	6.9	14.3			y	y			lost	50
12123A	0	0	0.2	0	y	y		y	39*/39/52		died of disease	56
12200A	0	0	1.6	6.9			y	y			lost	83
12569B	1	1	0.5	4.4				y	26		died of disease	27
12785B	0	1	28	19.2	y			y	15		died of disease	28
12831B	0	1	5.6	16.5	y		y	y	48		died of disease	60
12909B	0	1	1.7	13.4		y		y			alive & well	97
12979A	1	1	1.3	7.7				y		29	alive & well	80
13029A	0	1	2.1	6.2		y	y	y			alive & well	109
13099A	1	1	0.4	4.4	y	y	y	y			alive & well	121
13134D	0	0	1.1	10.4			y	y			lost	23
13147A	1	0	3.4	13.5		y	y	y	14	32	died of disease	45
13153A	1	1	4.9	2.4	y			y			alive & well	120
13282A	0	0	19.4	13.9			y	y			alive & well	98
13329A	1	1	1.4	3.3	y	y		y			alive & well	77
13408B	0	0	32	15.3	y	y		y			alive & well	104
13443A	0	0	40	49			y	y	90		alive with disease	119
13513A	0	0	145	118	y	y		y			alive & well	116
13546A	1	0	31	57	y			y		36	alive & well	110
13557A	0	0	6.4	26	y		y				died of other cause	56
13847A	0	1	3.5	7.7		y	y	y	16		died of disease	30
13895C	0	0	0	12.5	y			y		53	alive & well	108
14189A	0	0	15.3	178	y	y		y		100	alive with other cancer	106
14220B	0	0	3.5	8.2			y	y	15		died of disease	15
14236B	0	0	29	39		y		y			alive & well	102
14285A	0	0	4.8	16.4		y	y	y			alive & well	88
14386A	1	1	1.1	16.6		y	y	y		54	alive & well	100
14482A	0	0	1.7	14.6		y	y	y	61		died of disease	73

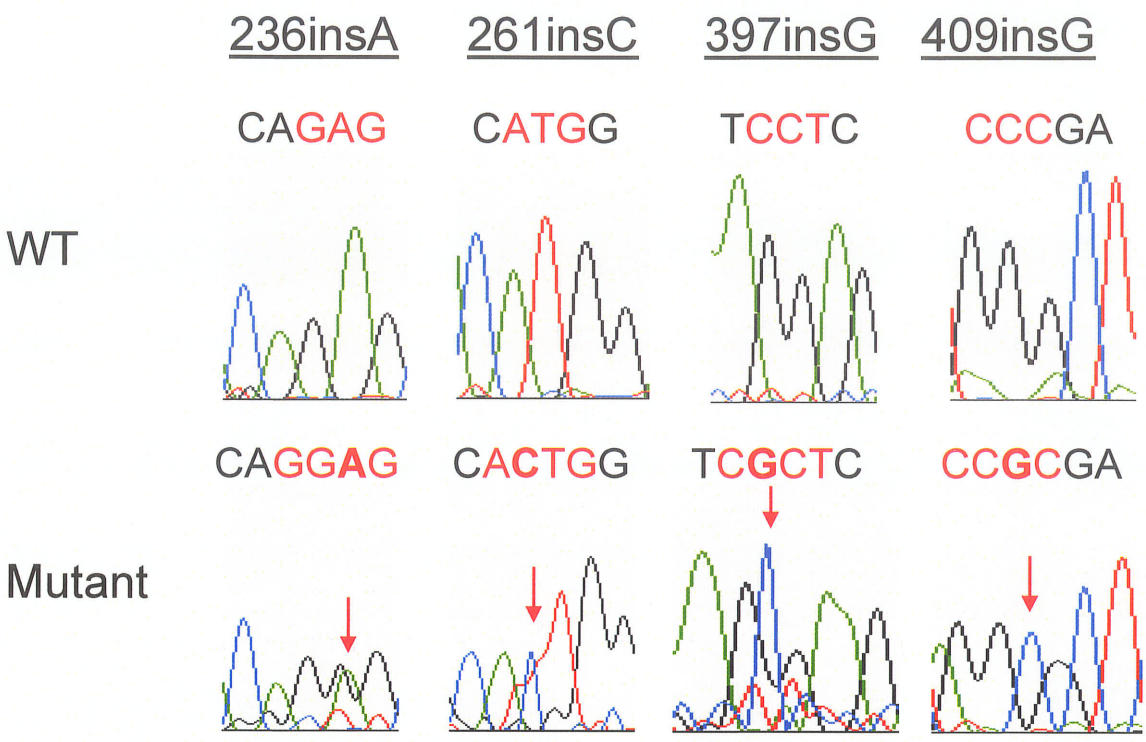


Figure 17. Single nucleotide insertions observed in 30 high grade breast tumors. RNA was isolated from 30 high grade frozen breast tumors obtained from the Manitoba Breast Tumor Bank. RT-PCR was performed and the cDNA amplified using a high fidelity Taq polymerase using 2 different primer sets to screen the entire BNIP3 cDNA for mutations. The cDNA was sequenced using these same primer sets using the ABI310 sequencing apparatus.

Table 4. Summary of the mutations detected in breast tumors and their proposed protein structure.

Tumor	Sequence Alteration	Type of Mutation	Schematic of proposed protein structure
12123	Wild type		
12979 13147 13153 13546	235_236insA	Nonsense	
13099	260_261insC	Nonsense	
14386	396_397insG	Nonsense	
13329	408_409insG	Frameshift	

4.7 Single-Stranded Conformation Polymorphism (SSCP) confirms the presence of mutations in breast tumor samples.

To confirm the results obtained by DNA sequencing, we tested for the presence of mutations using the single-stranded conformation polymorphism technique. We radiolabeled a 574 base pair region of DNA in exon 3 using α-P^{32} dATP in a PCR reaction. The primers were designed to cover the region containing both the mutations at nucleotide 236 and 261. The DNA strands were denatured by incubation at 95°C for five minutes. The denatured DNA was immediately placed on ice until being loaded onto a 45 cm non-denaturing acrylamide/glycerol gel on a sequencing apparatus. The gel was run at 10mA at 4°C for 18 hours. We observed the appearance of an additional band not seen in the wild type control sample, confirming the presence of an altered DNA sequence (Figure 18).

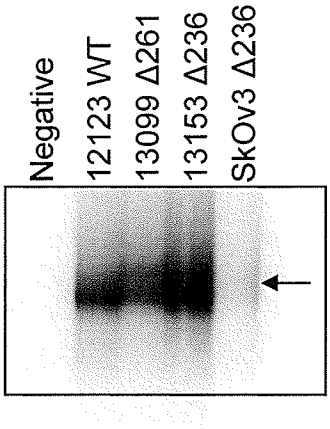


Figure 18. Single-Stranded Conformation Polymorphism confirms the presence of mutations in the BNIP3 gene of breast tumors.
DNA was isolated from breast tumors and the cell line SkOv3 using the DNeasy isolation procedure. A region of DNA containing the mutations sites at nucleotide 236 and 261 was amplified using BNIP3 Exon 3 specific primers. The DNA was radiolabled using α-P^{32} dATP and run on a glycerol gel in a Bio-Rad sequencing apparatus for 18 hours. The bands were visualized by exposure to X-ray film.

4.8 Breast tumors with BNIP3 mutations trend towards reduced overall recurrence and primary-free survival.

To determine whether there is any potential clinical significance of the BNIP3 mutations detected in breast tumors, we had the anonymized patient data analyzed for a number of variables. There was no statistical difference in overall survival or recurrence-free survival when wild type tumors were compared to tumors possessing mutations in the BNIP3 gene. However, there appears to be a trend towards reduced overall recurrence and primary-free survival in the breast tumors containing BNIP3 mutations (Figure 19). These results are not statistically significant since the cohort (n=30) is too small. More tumors must be analyzed to determine if indeed any difference in survival may be attributed to the presence of mutations in BNIP3.

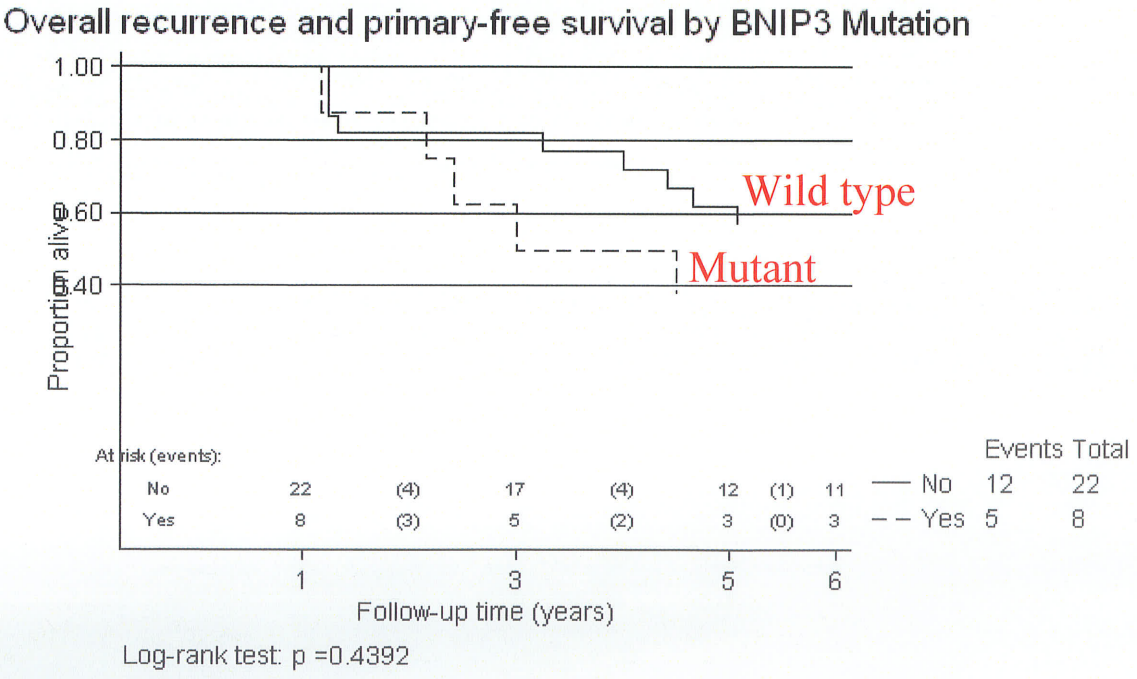


Figure 19. Statistical analysis of survival data comparing breast tumors with wild type BNIP3 and those with mutant BNIP3.

4.9 Breast tumors with mutations in the BNIP3 gene express BNIP3 protein.
Next we wanted to determine whether the breast tumors with BNIP3 mutations produced BNIP3 protein. We obtained paraffin-embedded breast tumor sections from the Manitoba Breast Tumor Bank that corresponded to ten of the tumors we screened for mutations in the BNIP3 gene. We immunostained the sections for BNIP3 protein expression using a polyclonal BNIP3 antibody that recognizes both wild type and mutant BNIP3. We observed positive staining in sections containing a wild type BNIP3 gene, while other wild type sections were negative for BNIP3 staining. Positive staining was seen in sections containing a mutation in the BNIP3 gene (Figure 20). Since the antibody we used detects both wild type and mutant BNIP3, we cannot determine whether the protein expressed in the sections containing a mutation in the BNIP3 gene is wild type protein or a truncated form of the protein.

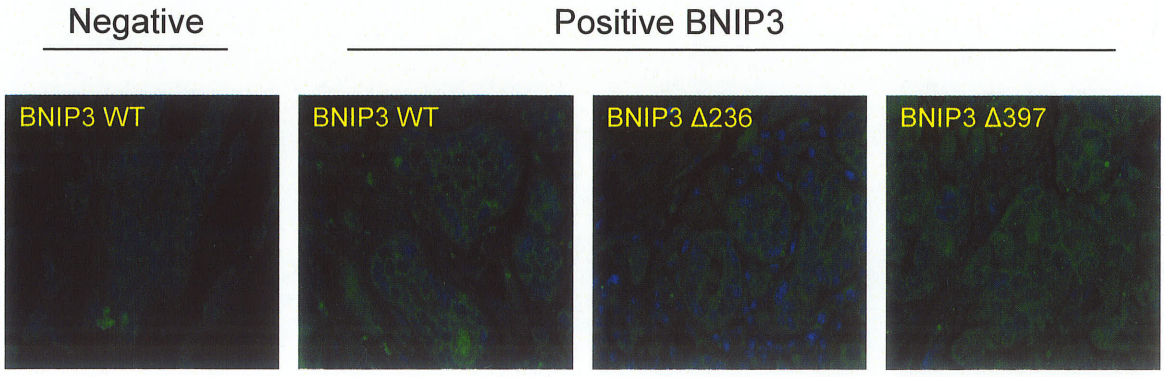


Figure 20. Breast tumors express BNIP3 protein.
Paraffin-embedded tumor sections from the Manitoba Breast Tumor Bank were immunofluorescently stained for BNIP3 protein expression using an in-house polyclonal BNIP3 antibody to an N-terminal epitope that recognizes both wild type and mutant protein. BNIP3 protein expression (green) can be seen in tumors that contain a mutation in the BNIP3 gene. DNA is stained with DAPI (blue).

4.10 BNIP3 protein expression correlates with the hypoxia marker Glut-1 in breast tumors that express wild type or mutant BNIP3.

Since BNIP3 is normally induced under hypoxia by HIF-1, we wanted to evaluate whether the protein expression observed in the breast tumors correlated with a marker of hypoxia. Paraffin-embedded sections of breast tumors obtained from the Manitoba Breast Tumor Bank were immunofluorescently stained for both BNIP3 expression and Glut-1 expression. We observed that BNIP3 expression correlated with Glut-1 expression in both breast tumors with wild type and mutant BNIP3 genes (Figure 21).

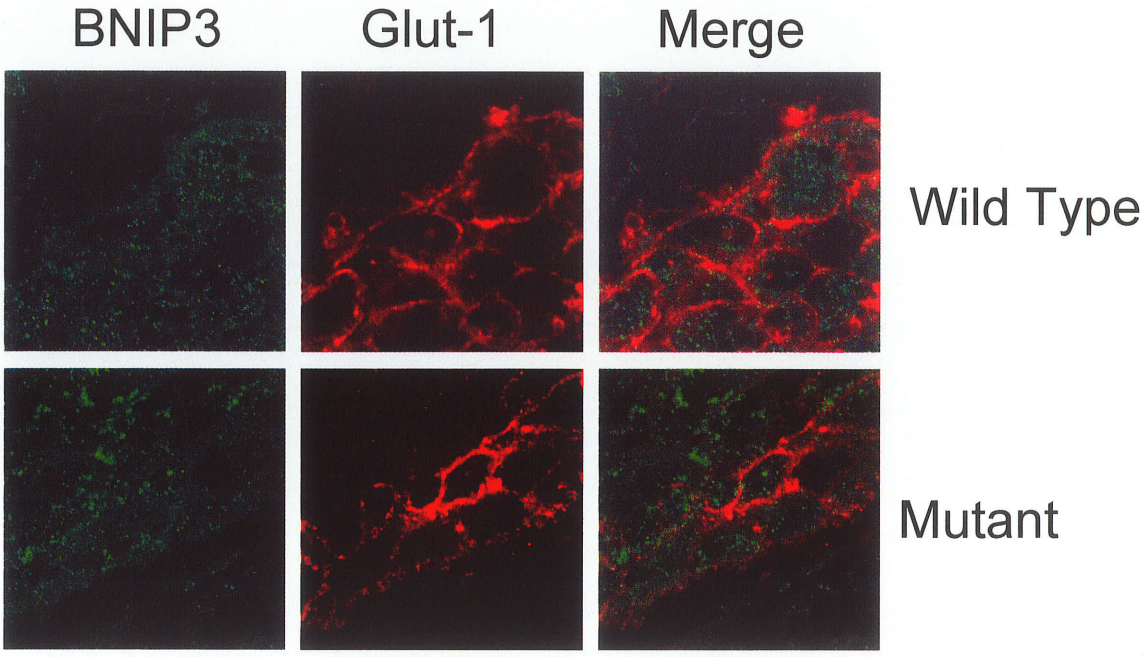
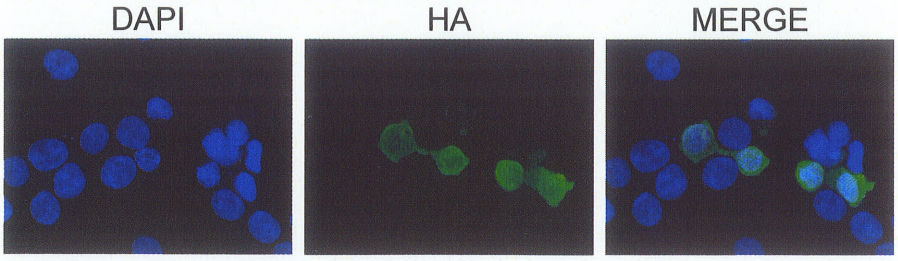


Figure 21. BNIP3 protein expression in tumor sections correlates with the hypoxia marker Glut-1.
Paraffin-embedded tumor sections from the Manitoba Breast Tumor Bank were immunofluorescently stained for BNIP3 (green) protein expression using an in-house polyclonal BNIP3 antibody that recognizes both wild type and mutant protein. The sections were also stained for a marker of hypoxia, Glut-1 (red). The sections were imaged by confocal microscopy.

4.11 All BNIP3 mutations observed in breast tumors appear to inhibit hypoxia-induced cell death in a dominant negative fashion.

Cells expressing either TM or PEST mutant BNIP3 are more resistant to hypoxia-induced cell death. Both the TM and PEST mutant are able to inhibit hypoxia-induced cell death in a dominant negative fashion. We wanted to determine if all of the mutations detected in breast tumors were more resistant to hypoxia-induced cell death. Hypoxia treatment induces the expression of endogenous wild type BNIP3 protein in HEK293 cells therefore, cells transfected with mutant constructs and exposed to hypoxia would express both wild type and mutant protein. HEK293 cells were transiently transfected with BNIP3 mutant constructs (Δ261 = 261insC, Δ397 = 397insG and Δ409 = 409 insG) or pcDNA (empty vector) and cultured under normoxic or hypoxic conditions for 48 hours. The cells were then fixed and immunofluorescently stained for positively transfected cells. Nuclear morphology was assayed to determine cell viability. Cells transfected with pcDNA3, Δ261, Δ397 and Δ409 underwent 40%, 22%, 29% and 26% cell death respectively (Figure 22) indicating the mutant BNIP3 functions to block hypoxia-induced cell death over cells expressing wild type BNIP3.

A)



B)

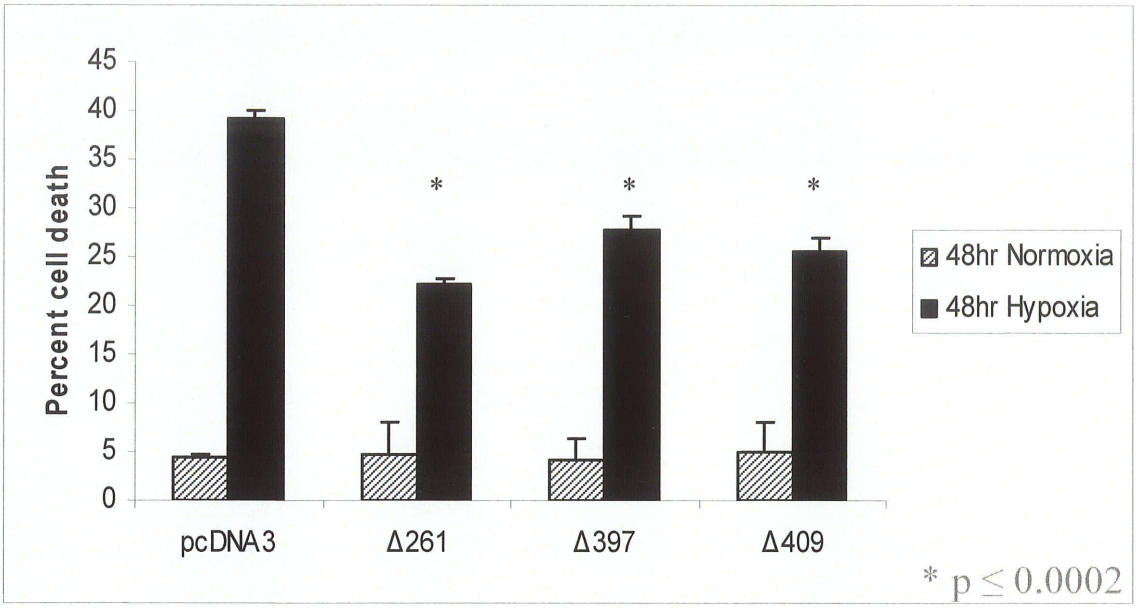


Figure 22. All BNIP3 mutations observed in breast tumors appear to be more resistant to hypoxia-induced cell death.
HEK293 cells were transfected with HA-tagged pcDNA3 (empty vector), Δ261, Δ397 and Δ409 mutant vectors using Lipofectamine 2000. The cells were cultured under hypoxic conditions (<1% oxygen) or normoxic conditions for 48hrs, after which time the cells were cytospun onto slides and fixed for immunoflourescent staining. Cells positive for mutant BNIP3 were identified using polyclonal antibodies against the HA tag. DNA was stained with DAPI and nuclear morphology was used to determine viability, with a total of 200 cells counted per sample.

4.12 Mutant BNIP3 does not localize to the mitochondrial membrane.

The transmembrane domain of BNIP3 is essential for its ability to integrate into the mitochondrial membrane. Since all of the mutations detected in the breast tumors are predicted to result in the loss of the TM domain, we wanted to confirm that the mutant BNIP3 protein is not localizing to the mitochondria. HEK293 cells were transiently transfected with BNIP3 mutant constructs (Δ261, Δ397 and Δ409) or pcDNA (empty vector) and cultured under normoxic conditions for 48 hours. Mitotracker Red, which is a dye taken up by the mitochondria, was added to the cells 30 minutes prior to fixation. Positively transfected cells were identified by immunofluorescence. Visualization of BNIP3 localization was performed using the confocal microscope. Figure 23 shows that none of the BNIP3 mutant constructs tested were able to localize to the mitochondria.

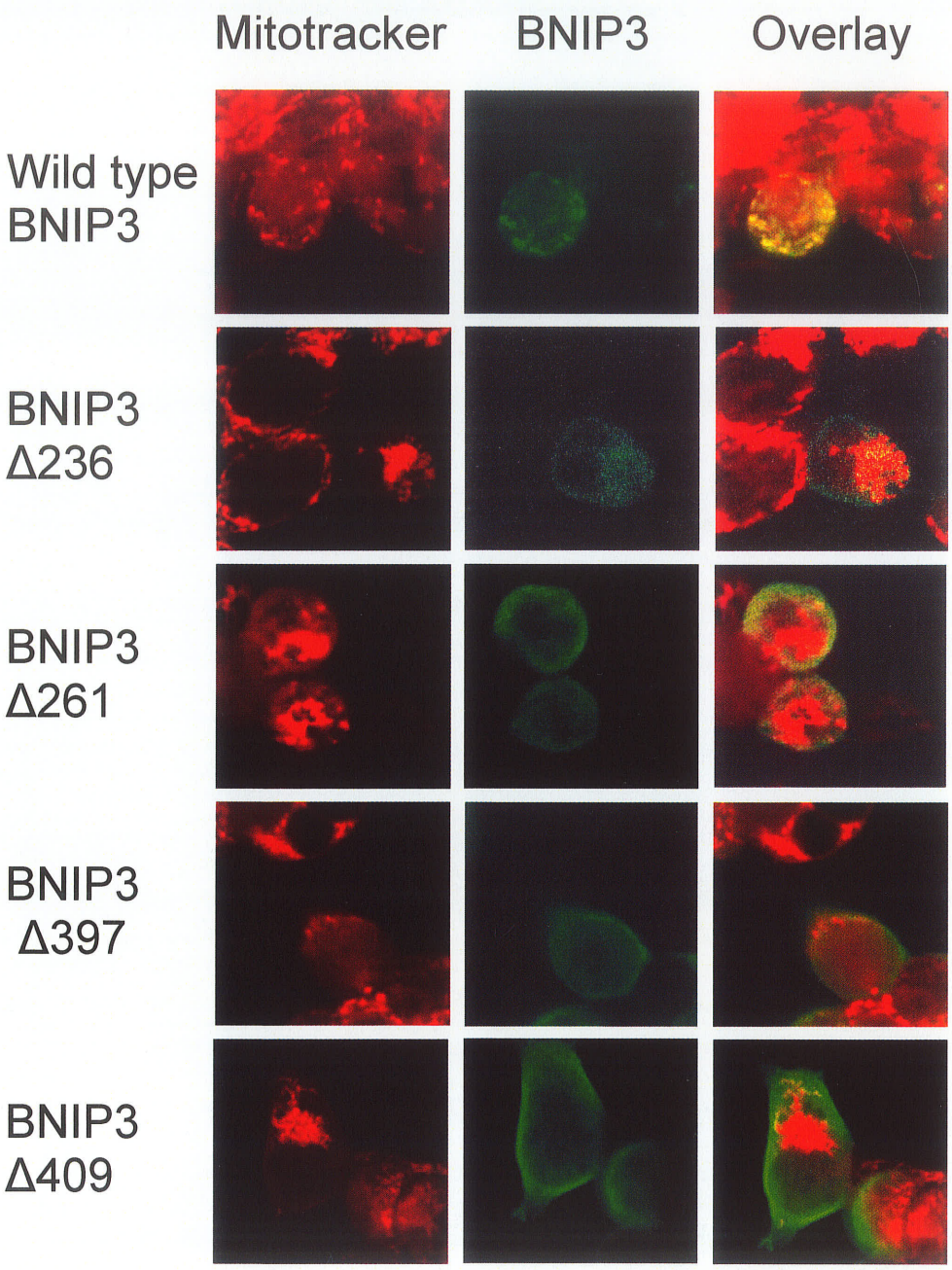
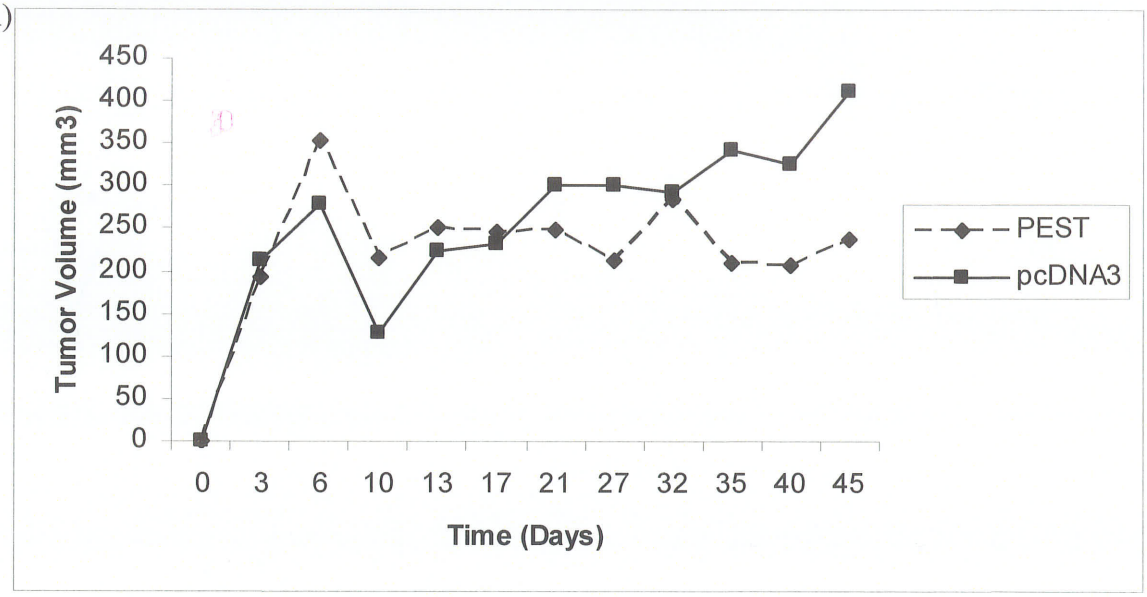


Figure 23. Mutant BNIP3 is unable to localize to the mitochondrial membrane. HEK293 cells were transfected with HA-tagged pcDNA3 (empty vector), Δ261, Δ397 and Δ409 mutant vectors using Lipofectamine 2000. The cells were cultured under normoxic conditions for 48hrs. MitoTracker Red was added to the cells 30 minutes before the cells were cytospun onto slides and fixed for immunoflourescent staining. Cells positive for mutant BNIP3 were determined using polyclonal antibodies against the HA tag.

4.13 MCF-7 cells stably expressing PEST mutant BNIP3 do not have a growth advantage over cells expressing pcDNA3 *in vivo*.

MCF-7 cells stably expressing PEST mutant BNIP3 or empty vector (pcDNA3) were implanted subcutaneously into athymic nude mice to evaluate the potential effects of BNIP3 mutations on tumorigenesis *in vivo*. Forty-five days after tumor implantation, no difference in tumor volume was observed between tumors containing MCF-7 PEST cells and MCF-7 pcDNA3 cells (Figure 24A). These results represent data obtained from 6 mice. The possibility exists that a larger number of mice and/or a greater amount of time may be required before any potential differences in tumor growth could be observed. The tumors were immunohistochemically stained for BNIP3 to confirm that the MCF-7 PEST cells continued to express BNIP3 mutant protein. As expected, no BNIP3 was observed in the MCF-7 pcDNA3 tumors (Figure 24B). Prior to sacrifice, the mice were given an intraperitoneal injection of hypoxyprobe to determine whether any hypoxic regions were present within the tumors. MCF-7 PEST tumors were positive for hypoxyprobe staining, indicating the presence of hypoxia within these tumors whereas no positive staining was observed in the MCF-7 pcDNA3 tumors (Figure 24B).

A)



B)

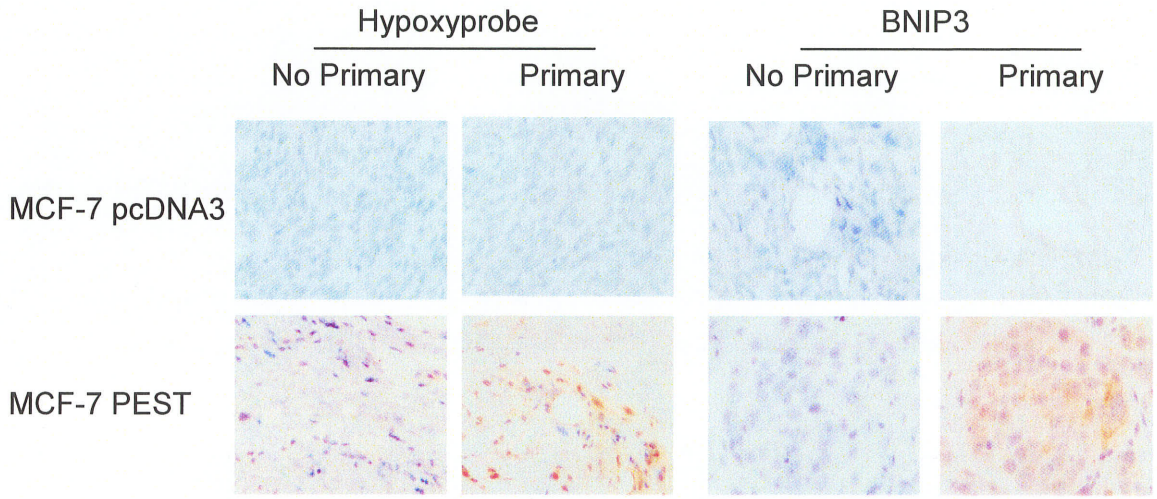


Figure 24. Mouse xenograph tumors expressing PEST mutant BNIP3 in MCF-7 cells showed no growth advantage over tumors expressing empty vector MCF-7 cells *in vivo*. A) No difference in tumor size/growth was observed between mice implanted with MCF-7 PEST mutant cells and MCF-7 pcDNA3 cells over a time course of 45 days. B) Tumors were analyzed by immunohistochemistry for BNIP3 expression using a polyclonal BNIP3 antibody. Tumors derived from MCF-7 cells over-expressing PEST mutant BNIP3 showed positive BNIP3 staining (brown) throughout the tumor as expected. There was no positive staining for BNIP3 seen in tumors derived from cells over-expressing pcDNA3. The tumors were also analyzed for regions of hypoxia using the hypoxyprobe antibody that recognizes pimonidazole adducts. Positive hypoxyprobe staining (brown) was observed in MCF-7 PEST tumors, while MCF-7 pcDNA3 tumors were negative for regions of hypoxia.

5. DISCUSSION

Uncontrolled cell proliferation is the generally accepted hallmark of cancer. Cells accumulate a series of genetic or epigenetic (i.e. methylation) changes that lead to alterations in gene activity, ultimately creating a population of cells that proliferate outside of normal regulatory controls (143). Genes can be inactivated by deletion, mutation or epigenetic events or activated/deregulated by point mutations, amplifications or translocations and gene fusions (144). Futreal *et al.* reviewed the literature and summarized the genes known to be mutated and causally implicated in oncogenesis. They found that at least 291 human genes accounting for more than 1% of the total number of genes, all of which encode proteins, are mutated in cancer. Approximately 90% of the cancer genes show somatic mutations, 20% show germline mutations, and 10% show both. The most common protein domain affected were tyrosine kinases, followed by proteins involved in transcriptional regulation and DNA maintenance and repair (145). DNA methylation maintains a large amount of non-coding DNA in cells of higher organisms in a transcriptionally inert state (146). Both loss of methylation and gain of methylation occur in cancer, resulting in activation or repression, respectively, of the affected gene.

BNIP3 is a pro-cell death BH3-only Bcl-2 family member whose expression is paradoxically increased in viable tumors cells in hypoxic regions of breast tumors where it fails to induce cell death. Several groups have published data indicating the loss of BNIP3 function occurs in various tumors where it correlates with worsened prognosis. Aberrant methylation of the BNIP3 promoter and histone deacetylation are involved in

silencing the BNIP3 gene in a subset of haematopoietic, colorectal, pancreatic and gastric tumors suggesting that tumors cells that do not express BNIP3 have a growth advantage related to their ability to escape cell death induced by hypoxia (41, 138-142). The inactivation of BNIP3 is specific and is not a general down-regulation of BH3-only family members (147). To date, no studies have been published that indicate loss of BNIP3 expression in breast tumors occurs via aberrant methylation. However, knock down of BNIP3 is sufficient to enable metastasis of primary breast cancer to multiple organs including lung, liver and bone suggesting a critical role for BNIP3 silencing in the pathogenesis of metastasis (148). In addition to gene silencing, negative regulation of BNIP3 occurs by nuclear sequestration of the protein. Nuclear localization of BNIP3 was correlated to increased survival compared to cytoplasmic BNIP3 expression in breast tumors (149) and has also been observed in the brain tumor glioblastoma multiforme (GBM) (150) and lung carcinomas (151) further supporting a role for loss of BNIP3 in tumor progression. Nuclear localization of BNIP3 may play a role in breast tumor progression, but does not explain why breast tumor cells with cytoplasmic BNIP3 expression are viable and do not undergo cell death.

The presence of mutations in the BNIP3 gene in breast tumors may help to explain why BNIP3 is expressed in breast tumors, but correlates with a worsened prognosis. Our results demonstrating BNIP3 mutations provide a novel mechanism to explain how cells escape hypoxia-induced cell death mediated by BNIP3. The transmembrane domain of BNIP3 is essential for its ability to integrate into the mitochondrial membrane and induce cell death. All of the BNIP3 mutants observed in breast tumors are predicted to lack a

functional transmembrane domain and may not localize to the mitochondrial membrane. Mutant BNIP3 appears to block the function of wild type BNIP3, shifting the balance towards survival. The fact that all of the detected mutations are able to inhibit hypoxia-induced cell death in a dominant-negative fashion provides insight into how the presence of BNIP3 mutations could provide a survival advantage. Mutant BNIP3 may inhibit wild type BNIP3 by competing with regulatory factor(s) required for mitochondrial targeting, or by directly preventing wild type BNIP3 from inducing cell death, perhaps by forming (inactive) heterodimers that cannot integrate within the mitochondrial outer membrane.

BNIP3 functions to induce cell death; however, the type of cell death it induces is still under some debate, as BNIP3 may contribute to both apoptosis and autophagy in a cell type and/or stimulus specific manner. In the heart, a BNIP3-mediated increase in autophagy is observed in ischemia and reperfusion injury, where it acts in a protective response to remove damaged mitochondria (152). There is a correlation between reduced autophagy and cancer, which suggests that a failure in autophagy signaling may be important in cancer pathogenesis (5). The situation is complex though, and autophagy appears to act to both promote and prevent cancer. In the early stages of tumor development, autophagy functions as a tumor suppressor (153). As a tumor grows, autophagy may promote tumor progression by allowing cells to survive in nutrient- and oxygen-limiting conditions and may also protect against ionizing radiation by removing damaged macromolecules or organelles (46). Furthermore, autophagy may protect some cancer cells against anticancer treatments by blocking apoptosis, while other cancer cells undergo autophagic cell death after cancer therapies (5, 153). Beclin-1 is a mammalian

autophagy gene that maps to a tumor susceptibility locus on human chromosome 17q21 and is mono-allelically deleted in up to 40%-75% of cases of sporadic breast and ovarian carcinomas (154-156). It can inhibit tumorigenesis and is expressed at decreased levels in breast carcinomas compared with normal tissue, where it may contribute to the development or progression of the disease (156). It has also been shown that tamoxifen may activate autophagy by up-regulating beclin-1 in a process mediated by ceramide (46). Ceramide is an important sphingolipid that acts as second messenger in apoptotic signaling pathways, and also induces autophagic cell death in malignant glioma cells via the activation of BNIP3 (157). Arsenic trioxide, which is used to induce apoptosis in hematological malignancies, also induces autophagy but not apoptosis in malignant glioma cells (158). The autophagy induced by arsenic trioxide is mediated by BNIP3 and its over-expression is sufficient to induce autophagic cell death in the absence of arsenic trioxide (158). Induction of autophagy in ischemia and reperfusion injury is mediated by BNIP3. There is evidence to suggest that apoptosis and autophagy may be interconnected in some settings, likely at the level of the mitochondria. Since BNIP3 appears to induce both apoptosis and autophagy in a cell type/system specific manner, it may be a protein involved in connecting the two types of cell death (5). BNIP3's role in both apoptosis and autophagy provides insight into how BNIP3 may function unlike any other Bcl-2 family member. It may explain why mutant BNIP3 is able to block cell death without the presence of either its BH3 or TM domain.

Hypoxia in tumors is associated with metastasis and poor prognosis, and is an important predictive indicator of therapeutic response. Cells within hypoxic regions often become

resistant to chemotherapeutic agents and radiation therapy (86, 159, 160). Hypoxic cells are considered to be resistant to most anticancer drugs for several reasons: (1) hypoxic cells are distant from blood vessels, and as a result are not adequately exposed to the drug; (2) hypoxia can induce a decrease in cellular proliferation; (3) hypoxia often selects cells with reduced apoptotic potential; (4) the cytotoxicity of DNA lesions caused by some anticancer drugs is enhanced by oxygen, and (5) hypoxia has been shown to upregulate genes involved in drug resistance (48). HIF-1 is central to most hypoxia responses, and as such inhibition of HIF-1 activity may be important in combinatory cancer therapy (161). Four general strategies are being developed to target tumor hypoxia including pro-drugs activated by hypoxia (toxic metabolite present in higher concentration of hypoxic regions, selectively kills hypoxic cells), hypoxia-selective gene therapy (use of hypoxia-specific promoters providing selective transcription of enzymes that can convert pro-drugs to toxic metabolite), targeting the HIF-1 transcription factor (HIF-1 promotes tumor cell survival under hypoxia, target its activity), and the use of obligate anaerobe bacteria (colonizes necrotic regions of tumor after systemic administration) (48, 104, 162). In addition, BNIP3, as a downstream target of HIF-1 may a potential therapeutic target. BNIP3 has been identified as a mediator of cell death during ischemia in both cardiac and neuronal cells. Blocking its function may be important in reducing the amount of tissue loss during both stroke and ischemic heart disease or congestive heart failure (38, 43). In cancer, activating BNIP3-induced cell death may be important in tumors with loss of BNIP3 due to mutation, methylation or nuclear localization.

6. CONCLUSIONS

Hypoxia and its related genomic instability can result in the selection of tumor cells with reduced apoptotic potential. The selection of tumor cells with mutations in the BNIP3 gene provides a mechanism for tumors cells to escape hypoxia-induced cell death leading to disease progression. Our results demonstrate that BNIP3 is mutated in a subset of primary breast tumors, and that these mutations inhibit hypoxia-induced cell death in cell lines. These results provide a potential explanation as to why BNIP3 is expressed in viable breast tumors cells where its expression counter-intuitively correlates with a worsened prognosis. In order to further investigate the role of mutations in breast tumors, more tumors need to be screened for BNIP3 mutations. A greater number of tumors will allow us to correlate the presence of mutations with patient outcome and determine if the presence of mutations has any statistically, as well as clinically significant implications for patients with breast cancer. In addition, the molecular mechanisms involved in mutant BNIP3's ability to inhibit hypoxia-induced cell death need to be elucidated to fully understand the role of BNIP3 mutations in breast tumor progression. The presence of a BNIP3 mutation in the ovarian cell line implies that mutations in BNIP3 likely occur in other tumor types, specifically ovarian tumors and needs to be further investigated.

REFERENCES

1. Edinger AL, Thompson CB. Death by design: Apoptosis, necrosis and autophagy. Curr Opin Cell Biol 2004;16(6):663-9.

2. Lockshin RA, Zakeri Z. Caspase-independent cell death? Oncogene 2004;23(16):2766-73.

3. Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, et al. Classification of cell death: Recommendations of the nomenclature committee on cell death. Cell Death Differ 2005;12 Suppl 2:1463-7.

4. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. Annu Rev Biochem 2000;69:217-45.

5. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. Oncogene 2004;23(16):2891-906.

6. Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. Int J Biochem Cell Biol 2004;36(12):2405-19.

7. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407(6805):770-6.

8. Adams JM, Cory S. The bcl-2 protein family: Arbiters of cell survival. Science 1998;281(5381):1322-6.

9. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med 2005;11(7):725-30.

10. Kakkar P, Singh BK. Mitochondria: A hub of redox activities and cellular distress control. Mol Cell Biochem 2007.

11. Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 2003;22(17):4385-99.

12. Grimm S, Brdiczka D. The permeability transition pore in cell death. Apoptosis 2007;12(5):841-55.

13. Finkel E. The mitochondrion: Is it central to apoptosis? Science 2001;292(5517):624-6.

14. Madesh M, Hajnoczky G. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. J Cell Biol 2001;155(6):1003-15.

15. Cory S, Adams JM. The Bcl2 family: Regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2(9):647-56.

16. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13(15):1899-911.

17. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by bcl-2. EMBO J 2001;20(23):6627-36.

18. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: A primary site for bcl-2 regulation of apoptosis. Science 1997;275(5303):1132-6.

19. Moreau C, Cartron PF, Hunt A, Meflah K, Green DR, Evan G, et al. Minimal BH3 peptides promote cell death by antagonizing anti-apoptotic proteins. J Biol Chem 2003;278(21):19426-35.

20. Lalier L, Cartron PF, Juin P, Nedelkina S, Manon S, Bechinger B, et al. Bax activation and mitochondrial insertion during apoptosis. Apoptosis 2007;12(5):887-96.

21. Burlacu A. Regulation of apoptosis by bcl-2 family proteins. J Cell Mol Med 2003;7(3):249-57.

22. Zhang HM, Cheung P, Yanagawa B, McManus BM, Yang DC. BNips: A group of pro-apoptotic proteins in the bcl-2 family. Apoptosis 2003;8(3):229-36.

23. Subramanian T, Kuppuswamy M, Gysbers J, Mak S, Chinnadurai G. 19-kDa tumor antigen coded by early region E1b of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. J Biol Chem 1984;259(19):11777-83.

24. Chen G, Cizeau J, Vande Velde C, Park JH, Bozek G, Bolton J, et al. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. J Biol Chem 1999;274(1):7-10.

25. Yasuda M, Han JW, Dionne CA, Boyd JM, Chinnadurai G. BNIP3alpha: A human homolog of mitochondrial proapoptotic protein BNIP3. Cancer Res 1999;59(3):533-7.

26. Lee H, Paik SG. Regulation of BNIP3 in normal and cancer cells. Mol Cells 2006;21(1):1-6.

27. Kothari S, Cizeau J, McMillan-Ward E, Israels SJ, Bailes M, Ens K, et al. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene 2003;22(30):4734-44.

28. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A 2000;97(16):9082-7.

29. An HJ, Maeng O, Kang KH, Lee JO, Kim YS, Paik SG, et al. Activation of ras up-regulates pro-apoptotic BNIP3 in nitric oxide-induced cell death. J Biol Chem 2006;281(45):33939-48.

30. Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, et al. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol 2000;20(15):5454-68.

31. Ray R, Chen G, Vande Velde C, Cizeau J, Park JH, Reed JC, et al. BNIP3 heterodimerizes with bcl-2/Bcl-X(L) and induces cell death independent of a bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. J Biol Chem 2000;275(2):1439-48.

32. Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, et al. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. J Exp Med 1997;186(12):1975-83.

33. Frazier DP, Wilson A, Graham RM, Thompson JW, Bishopric NH, Webster KA. Acidosis regulates the stability, hydrophobicity, and activity of the BH3-only protein Bnip3. Antioxid Redox Signal 2006;8(9-10):1625-34.

34. Bocharov EV, Pustovalova YE, Pavlov KV, Volynsky PE, Goncharuk MV, Ermolyuk YS, et al. Unique dimeric structure of BNip3 transmembrane domain suggests membrane permeabilization as a cell death trigger. J Biol Chem 2007.

35. Lamy L, Ticchioni M, Rouquette-Jazdanian AK, Samson M, Deckert M, Greenberg AH, et al. CD47 and the 19 kDa interacting protein-3 (BNIP3) in T cell apoptosis. J Biol Chem 2003;278(26):23915-21.

36. Kim JY, Cho JJ, Ha J, Park JH. The carboxy terminal C-tail of BNip3 is crucial in induction of mitochondrial permeability transition in isolated mitochondria. Arch Biochem Biophys 2002;398(2):147-52.

37. Kubli DA, Ycaza JE, Gustafsson AB. Bnip3 mediates mitochondrial dysfunction and cell death through bax and bak. Biochem J 2007.

38. Webster KA, Graham RM, Bishopric NH. BNip3 and signal-specific programmed death in the heart. J Mol Cell Cardiol 2005;38(1):35-45.

39. Regula KM, Ens K, Kirshenbaum LA. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. Circ Res 2002;91(3):226-31.

40. Baetz D, Regula KM, Ens K, Shaw J, Kothari S, Yurkova N, et al. Nuclear factor-kappaB-mediated cell survival involves transcriptional silencing of the mitochondrial death gene BNIP3 in ventricular myocytes. Circulation 2005;112(24):3777-85.

41. Shaw J, Zhang T, Rzeszutek M, Yurkova N, Baetz D, Davie JR, et al. Transcriptional silencing of the death gene BNIP3 by cooperative action of NF-kappaB and histone deacetylase 1 in ventricular myocytes. Circ Res 2006;99(12):1347-54.

42. Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the bcl-2 family protein BNIP3. Proc Natl Acad Sci U S A 2002;99(20):12825-30.

43. Zhang Z, Yang X, Zhang S, Ma X, Kong J. BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia. Stroke 2007;38(5):1606-13.

44. Levine B, Klionsky DJ. Development by self-digestion: Molecular mechanisms and biological functions of autophagy. Dev Cell 2004;6(4):463-77.

45. Rodriguez-Enriquez S, He L, Lemasters JJ. Role of mitochondrial permeability transition pores in mitochondrial autophagy. Int J Biochem Cell Biol 2004;36(12):2463-72.

46. Shintani T, Klionsky DJ. Autophagy in health and disease: A double-edged sword. Science 2004;306(5698):990-5.

47. Denecker G, Vercammen D, Declercq W, Vandenabeele P. Apoptotic and necrotic cell death induced by death domain receptors. Cell Mol Life Sci 2001;58(3):356-70.

48. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 2004;4(6):437-47.

49. Schmaltz C, Hardenbergh PH, Wells A, Fisher DE. Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Biol 1998;18(5):2845-54.

50. Chautan M, Chazal G, Cecconi F, Gruss P, Golstein P. Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr Biol 1999;9(17):967-70.

51. Kitanaka C, Kuchino Y. Caspase-independent programmed cell death with necrotic morphology. Cell Death Differ 1999;6(6):508-15.

52. Schweichel JU, Merker HJ. The morphology of various types of cell death in prenatal tissues. Teratology 1973;7(3):253-66.

53. Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW. An overview of prognostic factors for long-term survivors of breast cancer. Breast Cancer Res Treat 2007.

54. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: Markers and models. Nat Rev Cancer 2005;5(8):591-602.

55. Lee YT. Breast carcinoma: Pattern of metastasis at autopsy. J Surg Oncol 1983;23(3):175-80.

56. Tang P, Hajdu SI, Lyman GH. Ductal carcinoma in situ: A review of recent advances. Curr Opin Obstet Gynecol 2007;19(1):63-7.

57. Bartella L, Smith CS, Dershaw DD, Liberman L. Imaging breast cancer. Radiol Clin North Am 2007;45(1):45-67.

58. Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer 2004;4(9):665-76.

59. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell 1999;4(4):511-8.

60. Le Page F, Randrianarison V, Marot D, Cabannes J, Perricaudet M, Feunteun J, et al. BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8-oxoguanine lesion in human cells. Cancer Res 2000;60(19):5548-52.

61. Scully R. Role of BRCA gene dysfunction in breast and ovarian cancer predisposition. Breast Cancer Res 2000;2(5):324-30.

62. Ponzone R, Biglia N, Jacomuzzi ME, Mariani L, Dominguez A, Sismondi P. Antihormones in prevention and treatment of breast cancer. Ann N Y Acad Sci 2006;1089:143-58.

63. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol 2006;102(1-5):89-96.

64. Cordera F, Jordan VC. Steroid receptors and their role in the biology and control of breast cancer growth. Semin Oncol 2006;33(6):631-41.

65. Khan SA, Rogers MA, Obando JA, Tamsen A. Estrogen receptor expression of benign breast epithelium and its association with breast cancer. Cancer Res 1994;54(4):993-7.

66. Skliris GP, Parkes AT, Limer JL, Burdall SE, Carder PJ, Speirs V. Evaluation of seven oestrogen receptor beta antibodies for immunohistochemistry, western blotting, and flow cytometry in human breast tissue. J Pathol 2002;197(2):155-62.

67. Riggins RB, Schrecengost RS, Guerrero MS, Bouton AH. Pathways to tamoxifen resistance. Cancer Lett 2007.

68. Anderson WF, Chu KC, Chatterjee N, Brawley O, Brinton LA. Tumor variants by hormone receptor expression in white patients with node-negative breast cancer from the surveillance, epidemiology, and end results database. J Clin Oncol 2001;19(1):18-27.

69. Hull DF,3rd, Clark GM, Osborne CK, Chamness GC, Knight WA,3rd, McGuire WL. Multiple estrogen receptor assays in human breast cancer. Cancer Res 1983;43(1):413-6.

70. Balleine RL, Earl MJ, Greenberg ML, Clarke CL. Absence of progesterone receptor associated with secondary breast cancer in postmenopausal women. Br J Cancer 1999;79(9-10):1564-71.

71. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: Implications for cancer therapy. Cell Signal 2006;18(12):2089-97.

72. Moasser MM. The oncogene HER2: Its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007.

73. Yarden Y, Baselga J, Miles D. Molecular approach to breast cancer treatment. Semin Oncol 2004;31(5 Suppl 10):6-13.

74. Henson ES, Hu X, Gibson SB. Herceptin sensitizes ErbB2-overexpressing cells to apoptosis by reducing antiapoptotic mcl-1 expression. Clin Cancer Res 2006;12(3 Pt 1):845-53.

75. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res 2001;61(18):6669-73.

76. Sowter HM, Ferguson M, Pym C, Watson P, Fox SB, Han C, et al. Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol 2003;201(4):573-80.

77. Vlastos G, Verkooijen HM. Minimally invasive approaches for diagnosis and treatment of early-stage breast cancer. Oncologist 2007;12(1):1-10.

78. Roy V, Perez EA. New therapies in the treatment of breast cancer. Semin Oncol 2006;33(3 Suppl 9):S3-8.

79. Nicholson RI, Hutcheson IR, Jones HE, Hiscox SE, Giles M, Taylor KM, et al. Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. Rev Endocr Metab Disord 2007.

80. Jensen EV, Jordan VC. The estrogen receptor: A model for molecular medicine. Clin Cancer Res 2003;9(6):1980-9.

81. Jiang H, Feng Y. Hypoxia-inducible factor 1alpha (HIF-1alpha) correlated with tumor growth and apoptosis in ovarian cancer. Int J Gynecol Cancer 2006;16 Suppl 1:405-12.

82. Zheng H, Kavanagh JJ, Hu W, Liao Q, Fu S. Hormonal therapy in ovarian cancer. Int J Gynecol Cancer 2007;17(2):325-38.

83. Gallion HH, Pieretti M, DePriest PD, van Nagell JR,Jr. The molecular basis of ovarian cancer. Cancer 1995;76(10 Suppl):1992-7.

84. Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: The role of oxygen sensing in development, normal function, and disease. Genes Dev 2004;18(18):2183-94.

85. D'Angio CT, Finkelstein JN. Oxygen regulation of gene expression: A study in opposites. Mol Genet Metab 2000;71(1-2):371-80.

86. Knowles HJ, Harris AL. Hypoxia and oxidative stress in breast cancer. hypoxia and tumourigenesis. Breast Cancer Res 2001;3(5):318-22.

87. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. Cancer Res 1989;49(23):6449-65.

88. Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, et al. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol 2005;206(3):291-304.

89. Vaupel P. The role of hypoxia-induced factors in tumor progression. Oncologist 2004;9 Suppl 5:10-7.

90. Generali D, Berruti A, Brizzi MP, Campo L, Bonardi S, Wigfield S, et al. Hypoxia-inducible factor-1alpha expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer. Clin Cancer Res 2006;12(15):4562-8.

91. Hasan NM, Adams GE, Joiner MC, Marshall JF, Hart IR. Hypoxia facilitates tumour cell detachment by reducing expression of surface adhesion molecules and adhesion to extracellular matrices without loss of cell viability. Br J Cancer 1998;77(11):1799-805.

92. Reynolds TY, Rockwell S, Glazer PM. Genetic instability induced by the tumor microenvironment. Cancer Res 1996;56(24):5754-7.

93. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379(6560):88-91.

94. Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem 1993;268(29):21513-8.

95. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;270(3):1230-7.

96. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92(12):5510-4.

97. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J 2002;16(10):1151-62.

98. Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. Curr Opin Genet Dev 2001;11(3):293-9.

99. Johnson AB, Barton MC. Hypoxia-induced and stress-specific changes in chromatin structure and function. Mutat Res 2007.

100. Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, et al. Signal transduction in hypoxic cells: Inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J 1998;17(22):6573-86.

101. Mazure NM, Brahimi-Horn MC, Berta MA, Benizri E, Bilton RL, Dayan F, et al. HIF-1: Master and commander of the hypoxic world. A pharmacological approach to its regulation by siRNAs. Biochem Pharmacol 2004;68(6):971-80.

102. Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, et al. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem 2002;277(29):26351-5.

103. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science 2002;295(5556):858-61.

104. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. Hypoxia-inducible factor (HIF-1)alpha: Its protein stability and biological functions. Exp Mol Med 2004;36(1):1-12.

105. Salceda S, Beck I, Srinivas V, Caro J. Complex role of protein phosphorylation in gene activation by hypoxia. Kidney Int 1997;51(2):556-9.

106. Wang GL, Jiang BH, Semenza GL. Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. Biochem Biophys Res Commun 1995;216(2):669-75.

107. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59(22):5830-5.

108. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3(10):721-32.

109. Akakura N, Kobayashi M, Horiuchi I, Suzuki A, Wang J, Chen J, et al. Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res 2001;61(17):6548-54.

110. Osada R, Horiuchi A, Kikuchi N, Yoshida J, Hayashi A, Ota M, et al. Expressions of hypoxia-inducible factor 1alpha, hypoxia-inducible factor 2alpha, and von hippel-

lindau protein in epithelial ovarian neoplasms and allelic loss of von hippel-lindau gene: Nuclear expression of hypoxia-inducible factor 1alpha is an independent prognostic factor in ovarian carcinoma. Hum Pathol 2007.

111. Bardos JI, Ashcroft M. Negative and positive regulation of HIF-1: A complex network. Biochim Biophys Acta 2005;1755(2):107-20.

112. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 1998;95(14):7987-92.

113. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J 2001;20(18):5197-206.

114. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399(6733):271-5.

115. Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, et al. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 2002;111(5):709-20.

116. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 1998;392(6674):405-8.

117. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem 1998;273(20):11995-8.

118. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21(12):3995-4004.

119. Li YM, Zhou BP, Deng J, Pan Y, Hay N, Hung MC. A hypoxia-independent hypoxia-inducible factor-1 activation pathway induced by phosphatidylinositol-3 kinase/Akt in HER2 overexpressing cells. Cancer Res 2005;65(8):3257-63.

120. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, et al. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14(4):391-6.

121. Hirota K, Semenza GL. Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. Biochem Biophys Res Commun 2005;338(1):610-6.

122. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, et al. Identification of the von hippel-lindau disease tumor suppressor gene. Science 1993;260(5112):1317-20.

123. Woodward ER, Maher ER. Von hippel-lindau disease and endocrine tumour susceptibility. Endocr Relat Cancer 2006;13(2):415-25.

124. Linehan WM, Lerman MI, Zbar B. Identification of the von hippel-lindau (VHL) gene. its role in renal cancer. JAMA 1995;273(7):564-70.

125. Prowse AH, Webster AR, Richards FM, Richard S, Olschwang S, Resche F, et al. Somatic inactivation of the VHL gene in von hippel-lindau disease tumors. Am J Hum Genet 1997;60(4):765-71.

126. Ohh M. Ubiquitin pathway in VHL cancer syndrome. Neoplasia 2006;8(8):623-9.

127. Lonser RR, Glenn GM, Walther M, Chew EY, Libutti SK, Linehan WM, et al. Von hippel-lindau disease. Lancet 2003;361(9374):2059-67.

128. Iliopoulos O, Levy AP, Jiang C, Kaelin WG,Jr, Goldberg MA. Negative regulation of hypoxia-inducible genes by the von hippel-lindau protein. Proc Natl Acad Sci U S A 1996;93(20):10595-9.

129. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von hippel-lindau tumor suppressor protein. EMBO J 2000;19(16):4298-309.

130. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, et al. Hypoxia inducible factor-alpha binding and ubiquitylation by the von hippel-lindau tumor suppressor protein. J Biol Chem 2000;275(33):25733-41.

131. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von hippel-lindau protein. Nat Cell Biol 2000;2(7):423-7.

132. Stebbins CE, Kaelin WG,Jr, Pavletich NP. Structure of the VHL-ElonginC-ElonginB complex: Implications for VHL tumor suppressor function. Science 1999;284(5413):455-61.

133. Lonergan KM, Iliopoulos O, Ohh M, Kamura T, Conaway RC, Conaway JW, et al. Regulation of hypoxia-inducible mRNAs by the von hippel-lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol 1998;18(2):732-41.

134. Pause A, Lee S, Worrell RA, Chen DY, Burgess WH, Linehan WM, et al. The von hippel-lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci U S A 1997;94(6):2156-61.

135. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. interaction between H-ras and hypoxia. J Biol Chem 2001;276(12):9519-25.

136. Wiesener MS, Munchenhagen PM, Berger I, Morgan NV, Roigas J, Schwiertz A, et al. Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas. Cancer Res 2001;61(13):5215-22.

137. Sobhanifar S, Aquino-Parsons C, Stanbridge EJ, Olive P. Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. Cancer Res 2005;65(16):7259-66.

138. Akada M, Crnogorac-Jurcevic T, Lattimore S, Mahon P, Lopes R, Sunamura M, et al. Intrinsic chemoresistance to gemcitabine is associated with decreased expression of BNIP3 in pancreatic cancer. Clin Cancer Res 2005;11(8):3094-101.

139. Erkan M, Kleeff J, Esposito I, Giese T, Ketterer K, Buchler MW, et al. Loss of BNIP3 expression is a late event in pancreatic cancer contributing to chemoresistance and worsened prognosis. Oncogene 2005;24(27):4421-32.

140. Murai M, Toyota M, Satoh A, Suzuki H, Akino K, Mita H, et al. Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. Br J Cancer 2005;92(6):1165-72.

141. Murai M, Toyota M, Suzuki H, Satoh A, Sasaki Y, Akino K, et al. Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer. Clin Cancer Res 2005;11(3):1021-7.

142. Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res 2004;64(15):5338-46.

143. Ponder BA. Cancer genetics. Nature 2001;411(6835):336-41.

144. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 2007;7(4):233-45.

145. Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, et al. A census of human cancer genes. Nat Rev Cancer 2004;4(3):177-83.

146. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349(21):2042-54.

147. Bacon AL, Fox S, Turley H, Harris AL. Selective silencing of the hypoxia-inducible factor 1 target gene BNIP3 by histone deacetylation and methylation in colorectal cancer. Oncogene 2007;26(1):132-41.

148. Manka D, Spicer Z, Millhorn DE. Bcl-2/adenovirus E1B 19 kDa interacting protein-3 knockdown enables growth of breast cancer metastases in the lung, liver, and bone. Cancer Res 2005;65(24):11689-93.

149. Tan EY, Campo L, Han C, Turley H, Pezzella F, Gatter KC, et al. BNIP3 as a progression marker in primary human breast cancer; opposing functions in in situ versus invasive cancer. Clin Cancer Res 2007;13(2 Pt 1):467-74.

150. Burton TR, Henson ES, Baijal P, Eisenstat DD, Gibson SB. The pro-cell death bcl-2 family member, BNIP3, is localized to the nucleus of human glial cells: Implications for glioblastoma multiforme tumor cell survival under hypoxia. Int J Cancer 2006;118(7):1660-9.

151. Giatromanolaki A, Koukourakis MI, Sowter HM, Sivridis E, Gibson S, Gatter KC, et al. BNIP3 expression is linked with hypoxia-regulated protein expression and with poor prognosis in non-small cell lung cancer. Clin Cancer Res 2004;10(16):5566-71.

152. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, et al. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ 2007;14(1):146-57.

153. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 2005;5(9):726-34.

154. Friedman LS, Ostermeyer EA, Lynch ED, Szabo CI, Anderson LA, Dowd P, et al. The search for BRCA1. Cancer Res 1994;54(24):6374-82.

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

156. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999;402(6762):672-6.

157. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, Kondo S. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. Cancer Res 2004;64(12):4286-93.

158. Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene 2005;24(6):980-91.

159. Caro J. Hypoxia regulation of gene transcription. High Alt Med Biol 2001;2(2):145-54.

160. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2(1):38-47.

161. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. Nat Rev Drug Discov 2003;2(10):803-11.

162. Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. Cancer 2004;100(12):2491-9.