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Anaerobic Nucleolar Proteome Dynamics

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Anaerobic Nucleolar Proteome Dynamics

By

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This thesis is submitted as a partial fulfillment of

the M.Sc. program in Cellular and Molecular Medicine

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Abstract

Anaerobic metabolism as a consequence of low oxygen tension (hypoxia) is observed in various physiological and pathological conditions. Through the study of adaptive mechanisms to anaerobic metabolism, it was recently shown that an increase in the extracellular [H^+] causes the relocalization and sequestration of the von Hippel-Lindau (VHL) tumor suppressor to the nucleolus. This results in an indirect increase in energy production through HIF transcription factor stabilization and a decrease in energy demand through silencing of ribosomal biogenesis. Mutagenesis of VHL revealed a pH-dependent nucleolar targeting sequence, $NoDS^{H+}$. A bioinformatic search of this sequence identified proteins involved in major metabolic activities including: POLD1, the catalytic subunit of DNA polymerase delta; TAF1, subunit 1 of the general transcription factor TFIID; APC2, subunit of cell cycle protein APC/C; and UAP56, an mRNA splicing and export factor. Here we demonstrate that in response to acidic conditions, these proteins accumulate and become detained within the nucleolus. We also show that disruption of pH-dependent nucleolar sequestration of $NoDS^{H+}$-containing proteins reduces cell viability through increasing cellular energy consumption. This data suggests that during anaerobic metabolism, cells utilize the nucleolus to sequester key proteins of basal metabolic processes, preventing their function, in order to maintain energy equilibrium by reducing metabolic demand.

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ADP	Adenosine Diphosphate
AP	Acidosis Permissive
APC/C	Anaphase Promoting Complex
APC2	Anaphase Promoting Complex Subunit 2
ATP	Adenosine Triphosphate
B23	rRNA Processing Factor Nucleophosmin
BrdU	Bromodeoxyuridine
CAIX	Carbonic Anhydrase IX
CBE	Chloride/Bicarbonate Exchanger
Cdc14	Cell Division Cycle 14 Phosphatase
cIAP2	Inhibitor of Apoptosis 1
Δ157	VHL Truncation N-terminal Mutant
ΔE	Change in Energy, $Energy_{supply}$-$Energy_{demand}$
E3	Ubiquitin Ligase
eIF2B	Eukaryotic Initiation Factor 2B
ETC	Electron Transport Chain
FAD^+	Flavin Adenine Dinucleotide
FLIP	Fluorescence Loss in Photobleaching
FRAP	Fluorescence Recovery after Photobleaching
GTP	Guanosine Triphosphate
HIF	Hypoxia-Inducible Factor
HRE	Hypoxia Response Element
HSC70	Heat Shock Cognate 71kDa Protein
HSP110	110kDa Heat Shock Protein
LDH	Lactate Dehydrogenase
MCT	Monocarboxyate Transporter
Mdm2	Murine Double Minute Protein
NAD^+	Nicontinamide Adenine Dinucleotide
NHE	Na^+-H^+ exchanger
$NoDS^{H+}$	Nucleolar Detention Signal Regulated by H^+

PHD	Prolyl Hydroxylase
PI	Propidium Iodide
POLD1	DNA Polymerase Delta Catalytic Subunit 125kDa
PP1	Protein Phosphatase 1
RNF8	RING Finger Protein 8 Ubiquitin Ligase
SD	Standard (media)
STAD	Subnuclear Targeting Arginine Domain
STHD	Subnuclear Targeting Hydrophobic Domain
SUMO	Small Ubiquitin-Like Modifier
TAF1	TATA-Box Binding Protein Associated Factor 1, 250kDa
TBP	TATA-Box Binding Protein
TCA	Tricarboxylic Acid Cycle
TFII	General Transcription Factor of DNA Polymerase II
UAP56	HLA-B associated transcript 1
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau

Acknoledgements

I would first like to recognize and thank my supervisor Dr. Lee, whose passion, direction and support have made my post-graduate research experience invaluable and rewarding. I am very grateful for the knowledge and skills I have learned from working with him and being part of his team. I would particularly like to thank one of my colleagues, Mireille Khacho, for her assistance and expertise in the lab. Her teaching and direction has helped shape me as a researcher. I would additionally like to recognize Isabelle Robert for her assistance and friendship on both personal and scientific matters. I would also like to thank the other members of Dr. Lee's lab – Aleksandra Franovic, Chet Holterman, Tim Audas and Josianne Payette- for their help and support. A special thanks to the members of my advisory committee, Dr. David Lohnes and Dr. David Picketts for their criticism, advice and insight. Last but not least, I would like to thank my mother, father and brother for all their support and encouragement these past two years, and without them I would not be where I am today.

INTRODUCTION

Chapter 1: Introduction

1.1 Energy Production in the Cell

1.1.1 Accumulation of Oxygen in the Atmosphere

Approximately 2.7 billion years ago, atmospheric oxygen began to be produced as a result of early photosynthetic prokaryotes. The oxygen liberated from these reactions was likely dissolved in surrounding water bodies, which then precipitated as iron oxide through reactions with dissolved iron. Once all the iron had precipitated, excess oxygen began to be released from seas and lakes and gradually accumulated in the atmosphere. This change in atmospheric content had a significant impact on existing life as the corrosive oxygen attacked chemical bonds, destroying most prokaryotic groups save those in anaerobic habitats. This resulted in an evolution of adaptations to the changing environment, notably cellular respiration. Through this process, cells were able to create increased energy in a more efficient manner permitting more complex and demanding pathways to be created (Falkowski, 2006).

1.1.2 Aerobic Metabolism

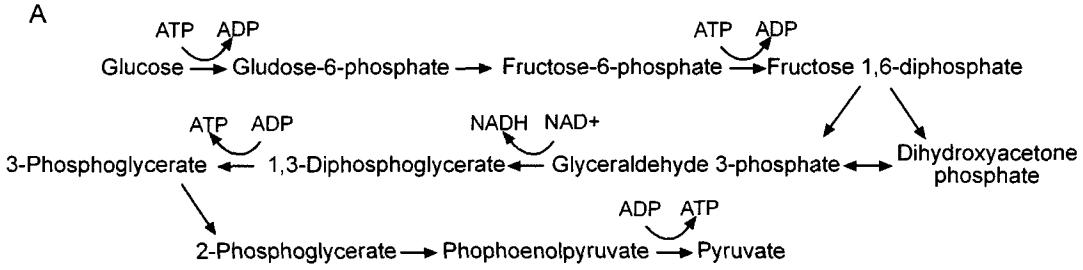
The immediate source of energy that fuels the majority of processes within the cell is adenosine triphosphate (ATP). Through the use of specific enzymes, the cell is able to harness the energy released through the hydrolysis of ATP, coupling it to endergonic reactions. Nearly all cellular work is dependent on the ability of ATP to energize molecules through the transfer of phosphate groups. In order to produce required energy, cells catabolize complex organic molecules, rich in energy potential, into simpler products. Although fats and proteins can be processed and used as fuel, the simple carbohydrate glucose is most widely used due to its ease of catabolism and its

ability to be used in conditions of low oxygen levels. Under normal oxygen tension, normoxia, oxygen constitutes 21% of the total gas content. The steps of glucose catabolism can be divided into two main stages: glycolysis and cellular respiration.

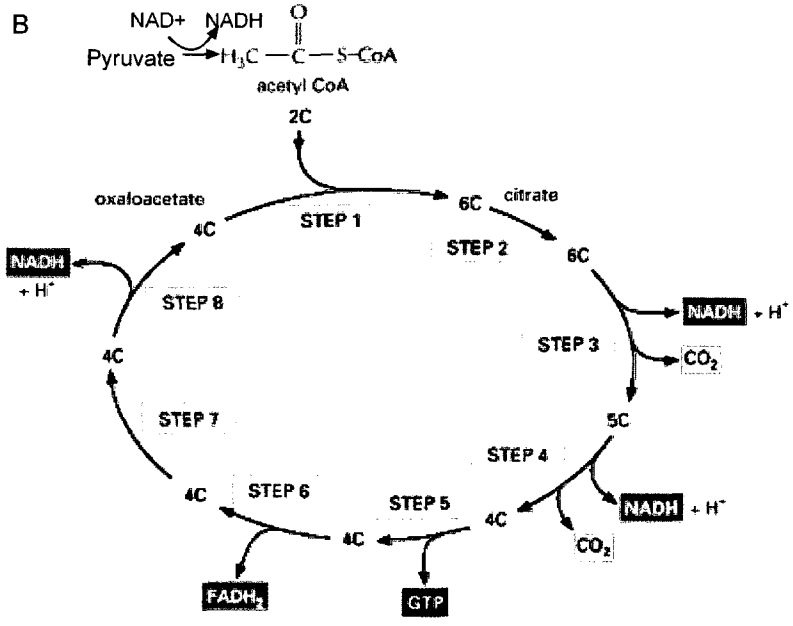
Glycolysis begins with the breakdown of glucose through a series of biochemical reactions occurring in the cytosol. This results in the creation of two molecules of the compound pyruvate, the production of two molecules of ATP, and the reduction of the coenzyme NAD^+ (nicotinamide adenine dinucleotide) into NADH (Figure 1A). Cellular respiration can be divided into three steps: pyruvate decarboxylation, Krebs cycle (also known as the citric acid cycle; tricarboxylic acid cycle, TCA) and the electron transport chain (ETC) (Figure 1B and C). Pyruvate molecules from glycolysis are transported from the cytosol to the mitochondrial matrix, where decarboxylation and association with coenzyme A occur, resulting in the formation of acetyl CoA. This product is now prepared to feed into the Krebs cycle for further oxidation. For each turn of the Krebs cycle, two carbons enter in the form of acetate and two different carbons exit as the completely oxidized form of CO_2. The process begins with enzymatic addition of acetate to oxaloacetate to form citrate, which subsequently gets broken back down to oxaloacetate producing CO_2 as a byproduct. Most of the energy gathered is stored in the form of NADH, and in one reaction through the reduction of FAD^+ (flavin adenine dinucleotide) to $FADH_2$. There is also a step within the Krebs cycle which forms an ATP molecule directly through substrate-level phosphorylation, similar to the process by which ATP is generated in glycolysis. While glycolysis and Krebs cycle produce a net four ATP per molecule of glucose, the majority of ATP produced in aerobic metabolism occurs through the ETC.

Figure 1. Aerobic metabolism of glucose. Under conditions of normal oxygen tension, glucose catabolism occurs through three major steps: (A) glycolysis; (B) the Krebs cycle; (C) the electron transport chain adapted from (Berg et al., 2002).

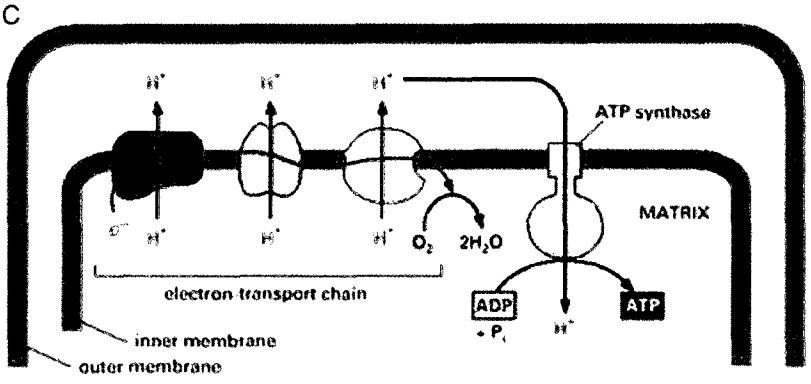
A


ATP ADP
Glucose
Gludose-6-phosphate
Fructose-6-phosphate
ATP ADP
Fructose 1,6-diphosphate
ATP ADP
NADH NAD+
3-Phosphoglycerate
1,3-Diphosphoglycerate
Glyceraldehyde 3-phosphate
Dihydroxyacetone phosphate
ADP ATP
2-Phosphoglycerate
Phophoenolpyruvate
Pyruvate

B


NAD+ NADH
Pyruvate
acetyl CoA
2C
oxaloacetate
4C
STEP 1
6C
citrate
STEP 2
6C
NADH + H+
CO2
STEP 3
5C
STEP 4
NADH + H+
CO2
4C
STEP 5
GTP
4C
STEP 6
FADH2
4C
STEP 7
4C
STEP 8
NADH + H+

C


ATP synthase
MATRIX
O2
2H2O
electron transport chain
ADP + Pi
ATP
inner membrane
outer membrane

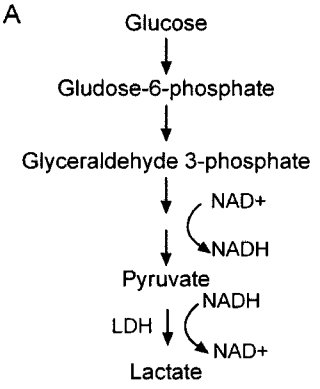
Each component of the ETC fluctuates between a reduced and oxidized state, as it gains electrons from neighboring less-electronegative proteins and transfers them to more-electronegative ones (Figure 1C). At the end of the chain, the final electron acceptor is the highly electronegative oxygen which becomes reduced to form H_2O. At certain regions along the chain, electron-carrying protein complexes move H^+ from the matrix to the intermembrane space. The storing of energy as a proton-motive force results in the release of H^+ back into the mitochondrial matrix through ATP synthase. This exergonic passage of protons drives the phosphorylation of ADP into ATP. Aerobic metabolism of glucose results in the net production of 36 ATP molecules.

1.1.3 Anaerobic Metabolism

In a variety of conditions, the cellular environment becomes hypoxic: when oxygen tension is decreased by 50-80%. Since cellular respiration is dependent on oxygen as a final electron receptor, this process is inhibited during these conditions. Glycolysis, though, is not oxygen dependent and can still function regardless of oxygen tension so long as there is a sufficient supply of the coenzyme NAD^+ available to accept the electrons during the oxidation reactions. Therefore in hypoxia, cells rely on fermentation to regenerate NAD^+ by transferring electrons from NADH to pyruvate or derivatives of pyruvate. In mammalian cells, this occurs through reduction of pyruvate to lactate by the enzyme lactate dehydrogenase (LDH) (Figure 2A). Though lactic acid fermentation has the advantage of remaining functional in hypoxia, it is far less efficient in terms of energy production compared to respiration (Figure 2B). As a means of maximizing energy production, the cell undergoes what is known as the Pasteur Effect whereby the rate at which glycolysis occurs is increased, though it is only approximately

Figure 2. Anaerobic metabolism of glucose. Under conditions of low oxygen tension, oxidative phosphorylation is inhibited and pyruvate from glycolysis is converted into lactate in order to regenerate the coenzyme NAD^+ (A); (B) ATP yield from the different steps of glucose metabolism. Net production from the glycolytic metabolism of glucose is 2 molecules while maximal net ATP production is achieved through oxidative phosphorylation, 36 ATP per molecule of glucose.

A



B

Reaction Sequence	**ATP Yield**
Glycolysis:	
Phosphorylation of glucose	- 1
Phosphorylation of fructose 6-phosphate	- 1
Dephosphorylation of 2 molecules of 1,3-BPG	+ 2
Dephosphorylation of 2 molecules of phosphoenolpyruvate	+ 2
2 molecules of NADH are formed	
Pyruvate Decarboylation	
2 molecules of NADH are formed	
Krebs Cycle	
2 molecules of GTP are formed	+ 2
6 molecules of NADH are formed	
2 molecules of FADH2 are formed	
Oxidative phosphorylation (inside mitochondria)	
2 molecules of NADH (glycolysis), each yields 2 ATP	+ 4
2 molecules of NADH (pyruvate decarboxylation), each yields 3 ATP	+ 6
2 molecules of FADH2 (Krebs), each yields 2 molecules of ATP	+ 4
6 molecules of NADH (Krebs), each yields 3 molecules of ATP	+18
Net Yield Per Glucose Molecule	36

1.5 times faster (Krebs, 1972). Though lactic acid fermentation is the sole method of producing energy under hypoxia, it is also a process that can occur under normal oxygen tension as well. Scientist Otto Warburg observed that human cancer cells also undergo lactic acid fermentation in normoxia, despite the lower net production in energy (Warburg, 1956), now known as the Warburg effect. Lactic acid fermentation results in the production of lactic acid within the cell which dissociates into a lactate and hydrogen ion. To prevent their intracellular accumulation, these components are exported from the cell causing the acidification of the extracellular milieu (See Section 1.3.1).

1.2 Fluctuation in Oxygen Tension

1.2.1 Hypoxia in Physiological Settings

Oxygen is required by cells for consumption by the mitochondria during the production of ATP through oxidative phosphorylation. The energy produced through this process is required to satisfy the energy requirements of the majority of cellular processes. When oxygen supply is compromised or the demand is significantly increased, insufficient ATP levels prevent normal cellular functioning (Taylor and Pouyssegur, 2007). Since gene activation by the transcription factor HIF (hypoxia inducible factor) is critical in the cellular response to low oxygen tension, it will be used as the basis for the definition of hypoxia. Hypoxia occurs when oxygen tension is below 5%, resulting in the transcription of genes involved in the hypoxic response through activity of the HIF protein (Walshe and D'Amore, 2008) (See Section 1.4).

Hypoxia occurs within human tissues in a variety of physiological and pathological conditions. Low oxygen concentrations have been demonstrated to be

integral for proper embryonic development (Li and Foote, 1993; Quinn and Harlow, 1978). It has also been suggested that hypoxia is required for proper closure of the neural tube, mediation of apoptosis and proper morphological development during gestation (Chen et al., 1999; Genbacev et al., 1997; Iyer et al., 1998). Consequently, embryos must rely on anaerobic metabolism to fulfill their energy requirements.

During strenuous exercise, energy reserves within the muscle cannot sustain continuous activity. As a result of the limiting availability of oxygen, cells undergo lactic acid fermentation as a means to produce energy under these conditions (Richardson et al., 2002; Richardson et al., 1995).

1.2.2 Hypoxia in Pathological Conditions

Hypoxia is also observed in a variety of pathological conditions. Ischemia occurs when blood flow to a tissue or organ is reduced due to obstruction of blood vessels, resulting in impairment in oxygen and metabolic substrate delivery. In an ischemic stroke, blood supply to a region within the brain is decreased causing damage in that region (Donnan et al., 2008; Shuaib and Hachinski, 1991; Stam, 2005). Ischemic heart disease is characterized by reduced myocardial blood flow and is typically caused by atherosclerosis of the coronary arteries (Loria et al., 2008; Sen et al., 2006).

A well studied example of low oxygen tension is the tumor microenvironment. Pre-invasive cancers are spatially and temporally diverse. Mutant cells on an epithelial surface are separated from underlying blood vessels by an intact basement membrane, leading to pre-malignant tumors which are typically avascular. Selection pressures under these conditions are thought to be crucial for the development into more invasive phenotypes (Fang et al., 2008; Gatenby and Gillies, 2008; Gillies and Gatenby, 2007).

Solid tumors account for the vast majority of human tumors (Kallinowski et al., 1989). An important aspect to proliferation is the development of a tumor's own vacularization. The vessels that develop though, are typically abnormal, disorganized, highly permeable, and suffer from low concentrations of oxygen. The result is the development of chronic and acute hypoxic cells (Cairns et al., 2001; Fang et al., 2008; Gimbrone et al., 1972) (Figure 3).

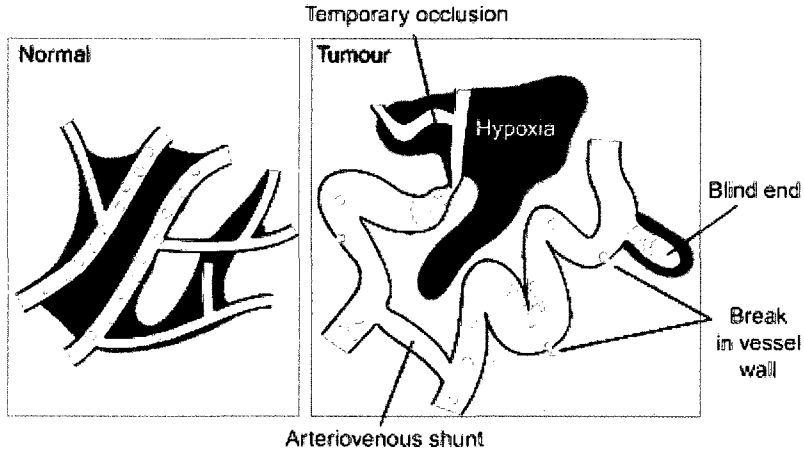
1.3 Acidosis

1.3.1 Regulation of pH by the Cell

Many processes within the cell such as enzyme activity, cell division, membrane transport, protein synthesis, cell-cell communication and differentiation are dependent on a stable, regulated intracellular pH. Variation in pH not only disrupts these processes, but may also result in cell death (Boron, 1986). It is therefore important that when lactic acid is produced during anaerobic metabolism, that its dissociated components, lactate (La^-) and a hydrogen ion (H^+), do not accumulate within the cell. The maintenance of a stable pH within the cell depends on the use of both short and long term mechanisms. In response to an acute increase in acidity, the cell rapidly uses several mechanisms to consume the excess H^+, followed by a return to normal pH through extrusion of H^+ from the cell. Short term mechanisms include physiochemical buffering, cellular consumption of non-volatile acids, and transfer of acid between the cytosol and organelles (Roos and Boron, 1981).

Short term buffering mechanisms (physiochemical, biochemical and organellar) have limited capacity to neutralizing the intracellular environment. Return to normal pH

Figure 3. Illustration of the difference in vasculature of normal and malignant tissues. In normal tissues, there is sufficient vasculature to oxygenate all of the tissue. In malignant tissues, vasculature is abnormal and disorganized contributing to slow and irregular blood flow. As a result, tumors have regions affected by acute and chronic hypoxia. Adapted from (Brown, 2000).


Temporary occlusion
Normal
Tumour
Hypoxia
Blind end
Break
in vessel
wall
Arteriovenous shunt

values is dependent on transport mechanisms that remove acid from the cell. To prevent accumulation of lactate and H^+ within the cell, these molecules are removed through the monocarboxyate transporter (MCT) in a symport manner. One of the most studied membrane transporters involved in H^+ removal from the cell is the amiloride-sensitive Na^+-H^+ exchanger (NHE) (Bianchini and Poussegur 1994; Bobulescu and Moe 2006). This transporter exchanges sodium ions from the extracellular environment for hydrogen ions within the cell. Another type of transporter which extrudes H^+ from the cell is the vacuolar-type (V-type) H^+-ATPase. This pump uses the energy from ATP hydrolysis to transport H^+ across various biological membranes when the free energy of ATP hydrolysis is greater than the ionic electrochemical potential (Beyenbach and Wieczorek, 2006; De Milito and Fais, 2005). Extrusion of H^+ from the cell is not always direct and can also occur indirectly such as through the chloride/bicarbonate exchanger (CBE). This cotransporter exports intracellular chloride ions while simultaneously importing HCO_3^-, which is then used to neutralize H^+ within the cell, generating carbon dioxide as a product. The CO_2 is then exported from the cell through carbonic anhydrase IX (CAIX) which can then be broken back down into HCO_3^- and H^+ (Tannock and Rotin 1989; Potter and Harris 2004). These examples demonstrate the various mechanisms cells employ in order to maintain proper pH regulation within the cell.

1.3.2 Role of Lactic Acidosis

Lactate is continuously produced through the enzyme lactate dehydrogenase during normal metabolism and at increased rates during anaerobic metabolism. Many studies have been performed investigating the role and effect of this molecule on the cell, with a growing consensus leaning toward its beneficial properties.

A recent study looking at the function of lactic acid in muscle tissue has shown that H^+ can reduce the decline in muscle force caused by muscle fatigue and aid in recovery of action potential generation (Nielsen et al., 2001). This knowledge has been advanced in another study showing lactic acid to be involved in influencing the activity of chloride ion (Cl^-) channels. Cl^- channels act to increase the amount of sodium ion (Na^+) needed to cause an action potential, which results in calcium ion (Ca^{2+}) release. During muscle fatigue, accumulated potassium ions depress the ability of action potentials to release Ca^{2+} and acidosis helps counteract this effect by decreasing the contribution of Cl^- channels (Pedersen et al., 2004). These and other studies (Bangsbo et al., 1996; Westerblad et al., 2002) have challenged previous notions suggesting lactic acidosis to be responsible for depressing muscle function (Fabiato and Fabiato, 1978; Hill and Kupalov, 1929). These earlier studies have been shown to be flawed in that they used large amounts of lactic acid and were performed at or below room temperature (Bruton et al., 1998; Pate et al., 1995).

Other studies show lactic acidosis to play a protective role during hypoxic and even anoxic conditions. It has been shown that acidosis protects against anoxic cell killing in rat liver cells and that injury is avoided if the pH is increased incrementally, as opposed to abruptly (Currin et al., 1991). Additional studies have shown that hypoxic cell killing of hepatocytes and renal tubule cells is dramatically reduced by decreasing the pH (Bonventre and Cheung, 1985; Gores et al., 1988).

An area of current interest is the suggestion that lactate can act as a fuel for the neural tissue of the brain. Early research suggested that lactic acidosis protects neural tissue in hypoxia (Schurr, West et al. 1987; Schurr, Dong et al. 1988). A later study

supported this concept through demonstration that lactate is used as an aerobic energy substrate by neural tissue immediately following hypoxia *in vitro* (Schurr and Rigor, 1998). This was further supported *in vivo* through evidence indicating lactate to be critical for oxidative phosphorylation post-ischemia in the rat brain (Schurr, Payne et al. 2001). A recent study even suggested that lactate may partially replace glucose as a substrate for oxidation during various forms of brain activation (Quistorff et al., 2008). Together, these studies oppose the concepts of lactate being a dead-end product of glycolysis and acidosis being detrimental to cells.

1.4 The Hypoxia Inducible Factor (HIF)

1.4.1 HIF Functions

To compensate for the effects of hypoxia, organisms undergo a variety of systemic and local changes to restore oxygen homeostasis and limit negative effects (Wenger, 2002). At the cellular level, the most notable response is a reduction in oxidation-phosphorylation, along with an increase in the glycolytic rate to compensate for lowered energy production (Hochachka and Lutz, 2001). Though hypoxia generally inhibits transcription, mRNA synthesis of specific genes does increase (Kaluz et al., 2008; Kiang and Tsen, 2006). At the molecular level, this is controlled through the activation of the hypoxia inducible factor (HIF) (Harris, 2002; Semenza, 2003). Its importance in adaptation to hypoxia is exemplified by the fact that it can be found in almost all higher eukaryotes (Huang and Bunn, 2003).

HIF is a heterodimeric transcription factor involved in the hypoxic response. It consists of a highly regulated alpha subunit and constitutively expressed beta subunit

(Ema, Taya et al. 1997; Hogenesch, Chan et al. 1997; Tian, McKnight et al. 1997; Semenza 1999). HIF binds to the hypoxia response elements (HRE) within the regulatory regions of target genes and recruits transcriptional coactivators, forming an intact initiation complex (Chapman-Smith and Whitelaw, 2006). More than 100 genes have been identified as HIF targets in a variety of cellular pathways (Denko et al., 2003; Semenza, 2003) (Table 1).

Among some of the HIF targets are genes involved in the shift from respiration to fermentation including glycolytic enzymes and glucose transporters such as aldolase A and GLUT-1 (Carmeliet, Dor et al. 1998; Vaupel 2004). HIF also decreases respiration pathways through activation of pyruvate dehydrogenase kinase 1 and halting the Krebs cycle (Papandreou et al., 2006). This transcription factor also upregulates genes involved with local and systemic responses to hypoxia such as the angiogenic factor VEGF (vascular endothelial growth factor) and erythropoietin, a central regulator of red blood cell maturation (Gordan and Simon, 2007; Semenza, 1999).

1.4.2 Oxygen and pH Dependent Regulation of HIF

Regulation of the HIF transcription factor occurs through modification of the stability of its alpha subunit (HIF-α). In normoxia, the half-life of HIF-α has been shown to be less than a few minutes (Huang and Bunn, 2003; Yu et al., 1998a). In these conditions, HIF-α is modified at specific proline residues within its oxygen-dependent degradation (ODD) domain by prolyl hydroxylases (PHDs) (Bruick and McKnight, 2001; Wang et al., 1995). This allows for its recognition by the VBC/Cul-2 ubiquitin ligase complex; formed by the association of the von Hippel-Lindau (VHL) tumor suppressor with four other partners: elongin B, elongin C, cullin-2, and Rbx1. The VBC/Cul-2

Table 1. Partial list of target genes of the Hypoxia Inducible Factor Transcription Complex

Oxygen Supply	
Transferrin	Erythropoietin
Transferrin receptor	Plaminogen activator inhibitor 1
VEGF	Heme oxygenase 1 (HO-1)
VEGF recepto	
Cellular Metabolism	
Enolase 1	Lactate dehydrogenase A
Phosphoglycerate kinase 1	GAPDH
Phosphofructokinase L	Carbonic anhydrase 9
MRD1	
Transcription	
DEC 1 and 2	ETS-1
ID2	CITED2/p35srj
Apoptosis	
NIP3	BNIP3
Noxa	Mcl-1
Other	
PHD2	PHD3

For references, see (Kaluz et al. 2008, Semenza, 2003)

complex then ubiquitinates HIF-α in the nucleus and translocates it to the cytoplasm for degradation via the 26S proteasome (Cockman et al., 2000; Ivan et al., 2001; Tanimoto et al., 2000) (Figure 4A).

In hypoxia, HIF-α is not recognized by VHL due to inhibition of hydroxylation by the PHDs, an oxygen dependent process (Lando et al., 2003; Schofield and Ratcliffe, 2004). This enables HIF-α to dimerize with HIF-β forming the active HIF transcription factor, resulting in transcription of hypoxia inducible genes (Maxwell et al., 1999; Yu et al., 2001) (Figure 4B).

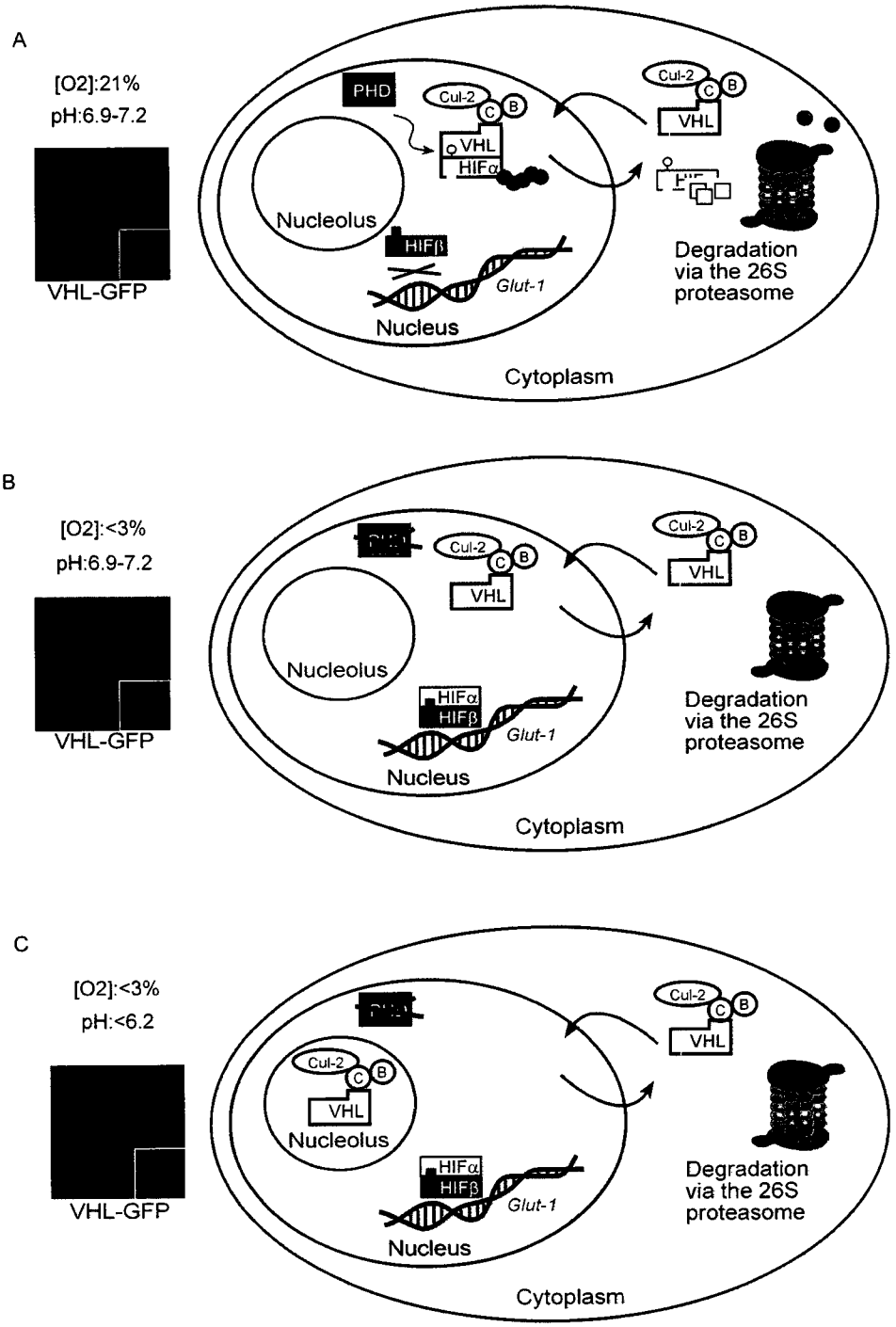
Hypoxia causes cells to undergo fermentation resulting in an accumulation of H^+ in the extracellular milieu. Once cell-type specific thresholds are attained, VHL becomes relocalized and statically detained in the nucleolus (Figure 4C). Reoxygenation of acidic cells does not release nucleolar VHL and HIF-α stability is maintained, even though the PHDs are active. Subsequent neutralization of the extracellular pH causes the release of VHL from nucleoli resulting in the resumption of HIF-α degradation (Mekhail et al., 2004a). This shows the importance of VHL subcellular dynamics in the regulation of HIF-α.

1.5 Nucleolar Detention of VHL

1.5.1 Photobleaching

Proteins are dynamic molecules whose mobility profiles are affected by interactions with other elements within the cell. They engage in random movement which allows for increased probability of interaction with its target participants (Misteli, 2001). Though nuclear compartments appear to be stable entities, they are in fact

Figure 4. Oxygen-dependent regulation of HIF-α by the VBC/Cul2 complex. (A) At normal oxygen tension (21% O_2) and neutral pH, prolyl hydroxylase (PHD) enzymes use molecular oxygen to modify HIF-α on its oxygen-dependent degradation (ODD) domain. This allows for recognition by the VBC/Cul2 complex and subsequent proteasomal degradation in the cytoplasm. Under these conditions, VHL undergoes nuclear-cytoplasmic shuttling (as observed through VHL-GFP immunofluorescence) necessary for the degradation of HIFα and thus the inhibition of HIF activation. (B) When oxygen tension is low (<3% O2), VHL maintains its dynamic shuttling, but HIF-α is stabilized because of inhibition of PHD activity, preventing recognition by VHL. Stabilized HIF-α then dimerizes with the constitutively expressed β subunit forming the active HIF transcription factor complex. HIF then binds to hypoxia response elements (HRE) within target genes, such as GLUT-1, activating their transcription. (C) Hypoxia induction causes the cells to undergo fermentation resulting in a decreased extracellular pH. Once cell-type specific thresholds are attained, VHL becomes detained within nucleoli making it unable to bind HIF-α and target it for degradation. Acidosis is also observed at normoxia resulting from the Warburg effect. In this case, HIF-α remains stabilized even though the PHDs are active due to detention of VHL. B and C refer to elongins B and C respectively.

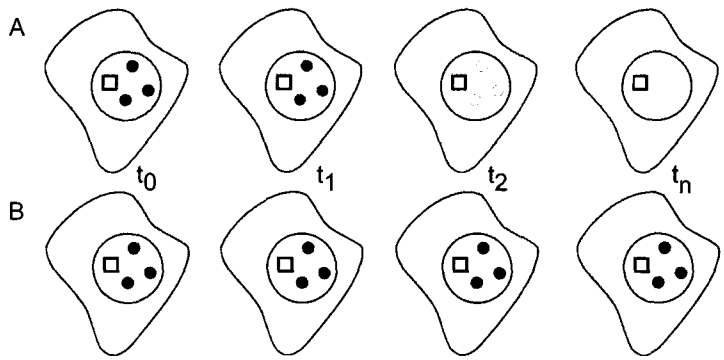

A
[O2]:21%
pH:6.9-7.2
VHL-GFP
PHD
Cul-2
C
B
VHL
HIFα
Nucleolus
HIFβ
Glut-1
Nucleus
Degradation
via the 26S
proteasome
Cytoplasm
B
[O2]:<3%
pH:6.9-7.2
VHL-GFP
Cul-2
C
B
VHL
Nucleolus
HIFα
HIFβ
Glut-1
Nucleus
Degradation
via the 26S
proteasome
Cytoplasm
C
[O2]:<3%
pH:<6.2
VHL-GFP
Cul-2
C
B
VHL
Nucleolus
HIFα
HIFβ
Glut-1
Nucleus
Degradation
via the 26S
proteasome
Cytoplasm

involved in rapid, continuous flux. While the nucleus contains large amounts of DNA, RNA and proteins, protein movement within this compartment is far from restricted, and it has been shown that non-physiological solutes within the nucleus have only four-times less diffusional mobility compared to an aqueous solution (Fushimi and Verkman, 1991; Seksek et al., 1997). It has been demonstrated that even resident nuclear proteins shuttle between compartments and the nucleoplasm (Phair and Misteli, 2000; Snaar et al., 2000). Most protein movement occurs in a diffusion-based, energy-independent fashion, facilitated by the lack of delineating membranes of nuclear compartments. This facilitates the cell to respond rapidly to various signaling cues.

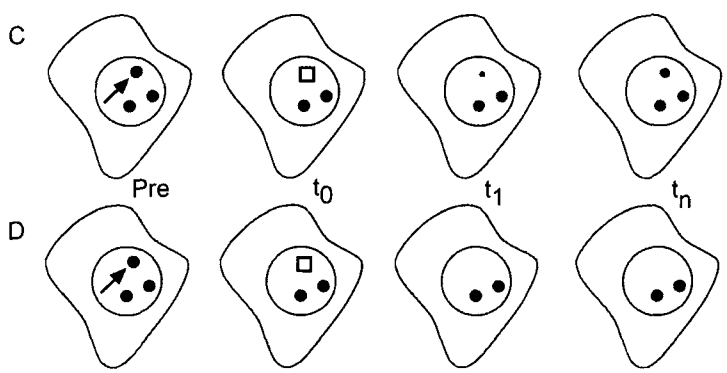
While conventional microscopy is unable to show the constant movement of proteins within the cell, an especially useful tool for studying protein kinetics is photobleaching. In this method, a fluorophore within a small region is made non-fluorescent through the use of a high intensity illumination. The exchange of bleached and unbleached populations is then monitored. The fluorophore is not destroyed, but instead irreversibly altered, making it incapable of fluorescence. There are two main types: fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP). FLIP involves the repeated bleaching of a small area within the cell, resulting in a decrease in total fluorescence for dynamic proteins through eventual passing of the bleaching area (Figure 5A and B). FRAP consists of quenching a small region of fluorescence within the cell, followed by recovery of the fluorescence if the protein is dynamic through eventual exchange of bleached and unbleached proteins (Figure 5C and D) (White and Stelzer, 1999). When a protein loses its dynamic mobility, it is said to be statically detained. This inhibits the proteins movement to other areas of

Figure 5. Types of Photobleaching Used to Study Protein Dynamics. There are two main types of photobleaching: fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP). (A, B) FLIP involves the repeated bleaching of a small area within the cell with a laser, and the total cellular fluorescence is measured after each bleaching. (A) GFP-tagged protein maintains mobility and overall loss of fluorescence is observed. (B) GFP-tagged protein is statically detained and no overall loss of fluorescence is observed. (C, D) FRAP involves quenching a small region of fluorescence within the cell with a laser, and measuring recovery of fluorescence in the bleached area over time. (C) GFP-tagged protein maintains mobility and recovery of fluorescence is observed within specified region of bleaching. (D) GFP-tagged protein is statically detained and no recovery of fluorescence is observed within specified region of bleaching.

Fluorescence Loss In Photobleaching (FLIP)



Fluorescence Recovery After Photobleaching (FRAP)



the cell. For example, nucleolar detention of a protein involved in translation would prevent it from interacting with its partners within the cytoplasm, thus inhibiting its ability to carry out its function. In this manner, static detainment serves as a mechanism to regulate protein activity.

1.5.2 Effects of Acidosis on the Dynamic Properties of VHL

In neutral conditions, VHL is constantly shuttling between the nucleus and the cytoplasm. This trafficking is necessary for HIF-α binding, ubiquitination and subsequent degradation (Khacho et al., 2008; Lee et al., 1999). The accumulation of lactic acid in response to anaerobic metabolism causes a decrease in the pH of the extracellular milieu. Once it has reduced to a cell-specific threshold, VHL becomes relocalized to the nucleolus (Mekhail et al., 2004a). To confirm that this shift in localization was not just a change in the steady-state distribution but actual detainment, photobleaching experiments were performed. Results revealed nucleolar detainment of VHL in acidosis (Mekhail et al., 2005). This showed that an accumulation of extracellular H^+ is sufficient to shift VHL from a dynamic nuclear-cytoplasmic distribution to static detainment within the nucleolus of the cell.

1.5.3 Activation of HIF-α through Detention of VHL

A dynamic nuclear-cytoplasmic distribution of VHL is required for its ability to target HIF-α for degradation in normoxia (Groulx and Lee, 2002; Khacho et al., 2008). As stated previously, upon acidification of the extracellular milieu, as observed in anaerobic metabolism, VHL becomes statically detained within the nucleolus (Mekhail et al., 2004a; Mekhail et al., 2005). This prevents its ability to regulate HIF-α since the VBC/Cul2 complex must leave the nucleus to target HIF-α for degradation. Nucleolar

sequestration of VHL has been demonstrated to be a reversible, oxygen-independent process and this indicates that even upon reoxygenation, HIF-α remains stable within the cell. A possible explanation for these dual mechanisms in HIF-α regulation, the two being oxygen-dependent prolyl hydroxylation and pH-dependent VHL dynamics, has been termed "hypoxic cell memory". This hypothesis states that having VHL detained within the nucleolus upon reoxygenation serves as a protective measure against sudden changes in oxygen levels until the environment stabilizes itself and the extracellular milieu returns to its normal pH. These dual mechanisms would also allow cells to respond differentially to acute vs. chronic exposures to hypoxia (Mekhail et al., 2004b).

1.6 The Nucleolus

1.6.1 Nucleolar Structure and Function in Ribosomal Biogenesis

The nucleolus represents the largest sub-nuclear domain and is the location of ribosomal biogenesis. Within the interphase nucleolus, ribosomal RNA (rRNA) genes are organized within ribosomal DNA (rDNA) as tandem repeats, which cluster at chromosomal loci called nucleolar organizer regions (NORs) (McKeown and Shaw, 2009). Through electron microscopy, three main nucleolar components are visible. Clear areas represent fibrillar centers (FCs) which are partially or completely surrounded by high contrasted regions representing dense fibrillar components (DFCs). The remaining areas of the nucleolus are filled with granules (called granular component, GC). The different sections represent pre-ribosomal particles in various stages of maturation (Goessens et al., 1987). Through the use of fluorescent-tagged proteins and photobleaching, investigation into intranuclear dynamics has been made possible. This

area of research has shown that nucleolar proteins are involved in rapid association/dissociation reactions with nucleolar components and the nucleoplasm, demonstrating the dynamic mobility of nucleolar proteins (Phair and Misteli, 2000).

1.6.2 Diverse Functions of the Nucleolus

With the advances in large scale nucleoli purification and protein identification through mass spectrometry, investigation of the nucleolar proteome has been made possible. Several proteomic studies on the nucleolus have been performed identifying more than 700 proteins. Bioinformatic analysis of these results have aided in suggesting possible functions for some previously undescribed proteins affirming that the nucleolus is not just involved in ribosomal biogenesis (Coute et al., 2006; Hinsby et al., 2006; Leung et al., 2003).

In the cell cycle for example, the nucleolus has been suggested in various studies to play a role in regulating protein activity. Conjugation of small protein chains, such as the SUMO (small ubiquitin-like modifier) protein, is one mechanism of protein modification to regulate cellular activities. It has been shown that a SUMO-specific protease, SENP5, is predominantly localized in the nucleolus and that its knockdown disrupts cell division as well as morphology (Di Bacco et al., 2006; Gong and Yeh, 2006). This suggests that the nucleolus may be involved in regulating SUMO conjugation of proteins involved in cell division. Another example further implicating a role for the nucleolus in cell-cycle regulation is that of protein phosphatase 1 (PP1), a serine-threonine phosphatase that has been shown to regulate cell division. One of its isoforms, PPI-γ, is localized in the nucleolus in interphase but upon entry into mitosis, accumulates in the cytoplasm where it plays a role in chromosome condensation

(Vagnarelli et al., 2006). These examples support the concept of the nucleolus being involved in cell division. In addition to cell cycle involvement, other non-traditional roles have also been identified including modification of small RNAs, control of aging, and signal recognition particle (SNP) assembly (Olson, 2004; Olson et al., 2002).

Besides modification and degradation of specific components of various cellular pathways, many studies have been published showing an alternate mode of regulation, protein detention within the nucleolus. One example is Cdc14, a protein phosphatase crucial for mitotic exit, which acts by dephosphorylating an activator of mitotic cyclin degradation (Visintin et al., 1998). Cdc14 is inactive and detained in the nucleolus during interphase; however upon initiation of anaphase, active Cdc14 is released from the nucleolus allowing it to perform its role in the cell cycle (D'Amours et al., 2004). Additionally, it has been suggested that while in the nucleolus, Cdc14 plays an additional role in maintaining a favorable kinase/phosphatase balance regulating DNA replication (Bloom and Cross, 2007). Another example is telomerase reverse transcriptase, a ribonucleoprotein polymerase which is involved in maintaining telomere ends through the addition of the telomerase repeat sequence (Kirkpatrick and Mokbel, 2001). This RNP enzyme has been shown to remain sequestered in nucleoli until telomeres are replicated near the end of S-phase, demonstrating a role for nucleolar localization in regulating the cell cycle (Wong et al., 2002). The murine double minute protein (Mdm2, HDM2 in humans) is an E3 ubiquitin ligase for p53, a tumor suppressor whose stabilization in response to various cell stresses results in cell-cycle arrest or apoptosis (Prives, 1998). This process is regulated by the nucleolar protein p19ARF (p14ARF in humans) whereby its association with Mdm2 prevents the ubiquitylation and degradation

of p53 (Wsierska-Gadek and Horky, 2003). It has been shown that Mdm2 not only relocalizes to nucleoli in these conditions, but is also detained there (Mekhail et al., 2005). Another example is the bHLH (basic helix-loop-helix) transcription factor Hand1, a protein involved in placentation and cardiac morphogenesis in the developing embryo. It has been shown that in proliferating trophoblast stem cells, Hand1 is sequestered in the nucleolus (Hamlin et al., 1994; Sahgal et al., 2006). During differentiation into trophoblast giant cells however, Hand1 is released from the nucleolus and activates downstream target genes (Martindill and Riley, 2008). Taken together, these examples demonstrate nucleolar sequestration as a means to regulate cellular processes within the cell.

1.7 Nucleolar Sequestration Maintains Energy Equilibrium

1.7.1 Importance of Energy Equilibrium

Within the cell, energy can simply be defined as the difference between supply and demand. It is believed that this basic equation must remain positive in order to maintain viability; the supply must be greater than the demand. If a cell's activities or environment results in energy disequilibrium, the cell could die. As a result, the cell tightly manages its metabolic pathways by controlling when and where various enzymes are active. It does this by switching on and off genes, for example HIF regulation of hypoxia-inducible genes, by regulating the activity of proteins directly through activation or inhibition, or by controlling protein localization, such as through nucleolar sequestration of VHL.

1.7.2 Sequestration of VHL Decreases Energy Consumption

When cells face a hypoxic environment, they undergo various modifications in order to maximize energy production. While studying the adaptive process to hypoxia/acidosis, our lab has demonstrated that an increase in the extracellular H^+ concentration causes the relocalization and static detainment of VHL within nucleoli (Mekhail et al., 2005). This has two major metabolic consequences. The first is an indirect increase in energy production through HIF stabilization. HIF is involved in various steps of the adaptation process to anaerobic metabolism such as through up-regulation of glycolytic enzymes and proteins involved in glucose uptake (Semenza, 1998). The second metabolic consequence of VHL detainment is a decrease in energy consumption through silencing of ribosomal biogenesis (Mekhail et al., 2006). Ribosomal biogenesis accounts for a majority of the total cellular energy consumption (Schmidt et al., 1999; Thomas, 2000), and therefore restriction of this process, when energy production is limited, would help maintain cell viability (Mekhail et al., 2006). Specifically, it was shown that nucleolar VHL interacts with the intergenic spacer (IGS), a region known to play key roles in the regulation of rDNA (Reeder, 1984). Failure of this interaction resulted in energy starvation and cell death (Mekhail et al., 2006).

1.8 Common Sub-Nuclear Localization Signal

1.8.1 Consensus Sequence Mediates Nucleolar Sequestration of VHL

Through mutagenesis studies, steady-state and protein dynamic analyses and computational investigation of VHL, our lab has discovered a position-independent sequence responsible for nucleolar targeting and detainment in acidosis $[RR(I/L)X_3r_{(n,n\geq 1)}$

+ $L(\phi/N)(V/L)_{(n,n>1)}$] (Mekhail et al., 2007). The 3 main criteria for this nucleolar detention signal regulated by H^+ ($NoDS^{H+}$) are:

1. At least one arginine domain known as the subnuclear targeting arginine domain (STAD, $RR(I/L)X_3r$)
2. More than one three-residue hydrophobic repeats called subnuclear targeting hydrophobic domain (STHD, $L(\phi/N)(V/L)$)
3. Low disorder character of STADs

1.8.2 The $NoDS^{H+}$ Sequence is Shared by Basal Metabolic Proteins

Knowledge of the consensus sequence responsible for the nucleolar detainment of VHL led to a variety of areas of investigation, one of which being whether other proteins contain this motif as well. Since shared localization signals are believed to allow for the coordinated response of different molecular networks, it is more than likely that nucleolar detention in response to acidosis is not an exclusive mechanism for VHL. Hence a bioinformatic search was performed using the $NoDS^{H+}$ consensus sequence to determine if proteins involved in energy consuming pathways are also detained in an effort to maintain energy equilibrium under non-permissive conditions. Our theory was suggested to be correct as the search resulted in 18 proteins involved in major metabolic pathways of the cell. Examples include POLD1, the major catalytic subunit of DNA polymerase delta involved in DNA replication, TAF1, the largest TATA box binding protein associated factor involved in transcription, eIF2B1, a GTP exchange factor essential for protein synthesis, and UAP56, a mRNA splicing and export protein. Some of the proteins that were found were tested to determine if they accumulated in the nucleolus in response to an extracellular accumulation in H^+. $NoDS^{H+}$-containing proteins including

apoptotic regulator cIAP2, heat shock protein HSC70 and transcription regulator RNF8 all demonstrated nucleolar detainment while other proteins which did not contain $NoDS^{H+}$ sequences did not (including lactate dehydrogenase, heat shock protein HSP110, and nucleolar phosphoprotein B23) (Mekhail et al., 2007).

1.9 Metabolic Rate Depression Maintains Energy Equilibrium

1.9.1 Insights from Animal Models

To maintain energy homeostasis, the cell's rate of ATP utilization must match the rate of production. When organisms face environmental circumstances which limit ATP production, mechanisms for reducing ATP-consuming processes are employed (Storey and Storey, 2004). These include decreasing the rate of protein synthesis, protein degradation and transcription. The exact mechanisms of the suppression of these processes are not fully understood.

Many studies have been performed using animal models in order to investigate how cells adapt to hypoxic conditions in order to maintain survival. Species living in water environments present an ideal model as water bodies consist of oxygen concentrations as much as 30 times lower than in air as well as a reduced rate of diffusion (Schmidt-Nielsen, 1997). This makes hypoxia a common occurrence in stagnant waters especially during the night when plants are not photosynthesizing. This problem is further compounded in the northern hemisphere when short day light is combined by thick ice cover causing anoxia in many small water bodies (Nilsson and Renshaw, 2004). One example is North American pond turtles, which are one of the most hypoxia tolerant vertebrates (Ultsch et al., 2004). One species, *Chrysemys picta*, has been shown to be

able to survive 160 days in anoxia at 3°C (Ultsch and Jackson, 1982). Another class of animal to face hypoxia is amphibians (Storey and Storey, 1986). Though aquatic and semi-aquatic species, such as frogs, do not have as great a tolerance to low oxygen conditions relative to fish and turtles, they have still been shown to survive as much as 4-7 days in extreme hypoxia (Knickerbocker and Lutz, 2001). Since these animals have all demonstrated increased survivability in response to low oxygen tension, study of the mechanisms which they employ to decrease energy demand would aid in the understanding of the maintenance of energy equilibrium in humans during anaerobic metabolism.

1.9.2 Suppression of Protein Synthesis

Protein synthesis is an energy dependent process consuming a large portion of the cells available ATP [ex 36% of ATP turnover in turtle hepatocytes; (Hochachka et al., 1996)]. This process is well known to be sensitive to energy availability and has been shown to be strongly reduced in response to severe hypoxia and anoxia (Casey et al., 2002; DeGracia et al., 2002). In a study on protein synthesis rates in hepatocytes of frogs, protein synthesis was observed to have decreased by 67% in hypoxia compared to normoxic condition (Fuery et al., 1998). In a study of anoxia tolerant turtles, researchers were able to show protein synthesis rates in various tissues to cease completely (Fraser et al., 2001). Further indication of protein synthesis depression as a mechanism to regulate energy utilization was shown in embryos of killifish where a decrease of over 93% was observed in response to a low oxygen environment (Podrabsky and Hand, 2000). In addition to a decreased rate of protein synthesis, a reduced rate of ATP turnover has also been demonstrated. In anoxic turtle hepatocytes, it has been shown that ATP use by

protein synthesis decreased from 24.4% to 1.6% compared to normoxic conditions (Land et al., 1993). This has been supported by studies on snails (*L. littorea*) where the ATP used by protein translation decreased by 50% in just 30 minutes upon exposure to anoxia and remained constant over 48 hours of exposure (Larade and Storey, 2002b). The speed at which this response occurred led the researchers to the hypothesis that a reduction in protein translation is not a response to a decreased energy supply but instead an active measure taken by cells to maintain viability. This idea was supported by further research on *L. littorea* showing that over 72 hours of anoxia exposure, ATP levels do not dramatically change (Churchill and Storey, 1996). There are two main possible causes for a decreased rate of protein synthesis observed in these circumstances. The first is a reduced amount of mRNA substrate available while the second is a specific inhibition of the translation machinery. In order to differentiate between the two, studies have been done looking at the global mRNA levels during hypoxia and anoxia. Studies of anoxic turtles found no change in RNA levels in all organs, and poly(A)$^+$ RNA levels were stable over 16h of submergence in hypoxia (Douglas et al., 1994). Similar results were observed in crucian carp and brine shrimp further suggesting that components of protein synthesis are directly inhibited (Hand, 1998; Smith et al., 1999).

1.9.3 Suppression of Protein Degradation

Net cellular protein level is determined by the rate of protein synthesis and degradation. Given that translation is suppressed in hypoxia/anoxia, and that upon reoxygenation organisms show no significant loss of cellular proteins, it is reasonable to suggest that protein degradation is also suppressed under these conditions. Not only would this be benficial through reduced ATP expenditure, but it would also prevent

accumulation of nitrogenous waste byproducts produced through degradation. This hypothesis has been supported by research with anoxic turtle hepatocytes showing urea synthesis to be reduced by 70% (Hochachka et al., 1996). Though there are fewer studies on protein degradation, the research seems to show a consensus of suppressed proteolysis in conditions of hypoxia and anoxia. One study involving brine shrimp gastrulae showed a 77-fold increase in the half-life of cytrochrome oxidase (Anchordoguy and Hand, 1995). A more detailed study was performed on anoxic turtle hepatocytes showing an increase in the half-lives of both the labile and stable protein pools by 40 and 50 percent respectively. The study also looked into the energy expenditure of protein degradation and showed it to decrease from 22% in normoxia to 0.7% in anoxia (Land and Hochachka, 1994). Taken together, these studies suggest that decreasing the rate of proteolysis is another mechanism employed to maintain energy equilibrium.

1.9.4 Suppression of Transcription

As previously discussed, suppression of protein synthesis is one method used to maintain survivability in conditions where oxygen is limited. It was also stated that under these circumstances, the global level of mRNA remained constant. This suggests that the rate of transcription is also decreased which would serve as another mechanism for conserving energy as this process has been estimated to account for 1-10% of energy expenditure in cells (Rolfe and Brown, 1997). The results from a nuclear runoff study using embryonic brine shrimp supported this theory by showing a reduction in transcription elongation by 79 and 88% after 4 and 24 hours in anoxia, respectively. This increase of approximately 8.5 times in mRNA half-life was returned to normal values after only 1 hour of reoxygenation. Additionally, it was further shown that artificial

acidification accounted for over half of the suppression observed through anoxia suggesting that the production of lactic acid may have played a key role (van Breukelen et al., 2000). Similar results were observed in a study on snail (*L. littorea*) hepatopancreatic cells showing a reduction in transcription by more than 67% (Larade and Storey, 2002a).

Taken together, the results from animal studies have demonstrated that in response to conditions of severe hypoxia or anoxia, cells remodel their metabolic pathways in order to decrease energy consumption, though the process by which this occurs is still being investigated.

1.10 Rationale

Energy within the cell can be simplified to the equation of supply minus demand, and it is believed that cell viability is dependent on the change in energy being positive. In conditions of hypoxia, energy production is dramatically reduced. Though the cell attempts to increase supply through the Pasteur Effect, this slight increase does not account for the energy consumption of our complex metabolic pathways. Therefore, it is reasonable to suggest that hypoxic cells must decrease energy demand in order to survive. Our lab has shown that acidosis confers a protective benefit to cells in hypoxia through nucleolar sequestration of VHL. First, it increases energy production through stabilization of the HIF transcription factor (Mekhail et al., 2004a). Secondly, nucleolar VHL is involved in silencing ribosomal biogenesis, a highly energy consuming process within the cell (Mekhail et al., 2006). Knowledge of the consensus sequence responsible

for the nucleolar sequestration of VHL led to production of a list of NoDS^{H+}-containing proteins involved in major metabolic pathways within the cell.

1.11 Statement of Hypothesis and Objectives

Since acidosis has been shown to play a protective role in hypoxia and detention of VHL has been shown to be involved in that role, we hypothesize that [H^+]-dependent nucleolar detention of NoDS^{H+}-containing proteins is involved in cellular adaptation to anaerobic metabolism. To test this hypothesis, the following objectives were formulated:

Objective 1. Identify and characterize NoDS^{H+}-containing proteins

In order to determine the predictive power of the NoDS^{H+} consensus sequence, the subcellular distribution of endogenous and exogenous NoDS^{H+}-containing proteins will be assessed through immunofluorescence and western blotting. In order to confirm the nucleolar localization observed is not simply a shift in steady-state distribution, photobleaching will be performed on GFP-tagged constructs to assess their nucleolar detainment potential.

Objective 2. Explore the functional changes of nucleolar detention on NoDS^{H+}-containing proteins

With respect to NoDS^{H+}-containing proteins involved in basal cellular functions, the effects of static detainment on their respective molecular networks will be studied.

Objective 3. Examine the effect of detention of NoDS^{H+}-containing proteins on cell survival and maintenance of energy equilibrium

The effects of nucleolar detainment on cell viability and maintenance of energy equilibrium will be studied. To study these effects the conditions of normoxia neutral,

hypoxia acidosis and hypoxia neutral will be studied. Additionally, dominant negative constructs will be used to discern between the effects of acidosis and nucleolar detainment. Energy equilibrium will be studied in three parts: cellular ATP levels, glucose uptake and lactate release.

METHODS

Chapter 2: Methods

2.1 Cells

MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). 786-0 cells were generated as previously described (Lee et al., 1999).

2.2 Cell Culture

Normoxic cells were incubated at 37°C in a 5% CO_2 environment. Hypoxia was achieved in a hypoxia chamber at 37°C under a 1%O_2, 5%CO_2, and N_2-balanced atmosphere. Acidosis experiments were conducted using standard (SD) or acidosis-permissive (AP) media. Buffer-free medium (DMEM; Invitrogen Carlsbad, CA) was freshly prepared, supplemented with 5% (v/v) fetal bovine serum (FBS), (v/v) penicillin-streptomycin and 44mM $NaHCO_3$, and adjusted to a specific pH value (SD pH 7.0; AP pH 6.0). Upon addition of media to the cells, the pH stabilizes at 7.0. Over time the media slowly acidifies to its set pH value (pH 6.0 for AP media whereas SD remains at pH 7.0). Coverslip hypoxia was performed using cells cultured in normoxic conditions with a glass coverslip on top of a portion of the cells. Transfected or infected cells were grown for 24 h under standard condition before any treatment.

2.3 Plasmid Construction

POLD1 and APC2 were cloned after a N-terminal GFP and FLAG-tag, into pcDNA3.1. Primers were designed with a 5' HindIII restriction site (GGATCC) and 3' Not1 restriction site (GCGGCCGC). UAP56 and $NoDS^{H+}$(POLD1) were cloned between a N-terminal FLAG-tag and C-terminal GFP, into pcDNA3.1. Primers were designed with a 5' Apa-1 restriction site (GGGCCC) and 3' Xho-1 restriction site (CTCGAG). Following reverse transcription and amplification, DNA templates were digested with

respective enzymes, purified in 1% agarose gels and inserted into vectors using T4 DNA ligase (Invitrogen). Cells were transiently transfected using Effectene Transfection Reagent (Qiagen, Mississauga, ON).

2.4 List of Primers

Gene	Primers 5' to 3'
wtPOLD1	**Fwd.**:GATAGGATCCGACTACAAAGACGATGACGATAAAAAGC TTGATGGCAAGCGGCGGCCA
	Rev.:GATAGCGGGCCGCTCACCAGGCCTCAGGTCCAGGGGG
wtAPC2	**Fwd.**:GATAAAGCTTGCGGCGGCAGTTGTGGTGGCGGAGGGGGA CAGCGACTCC
	Rev.:GATAGCGGCCGCTCAGCTGCAGTTCTTGGGCAGGCGGTAG ACCG
wtUAP56	**Fwd.**:GATAGGGCCCGCAGAGAACGATGTGGACAATG
	Rev.:GATACTCGAGCCGTGTCTGTTCAATGTAGG

2.5 RNA Isolation and Reverse Transcriptase Chain Reaction

RNA isolation was performed using the TRIPURE isolation reagent (Roche Diagnostics Co., Indianapolis IN) according to manufacturer's protocol. RT-PCR was performed using 1μg of RNA, 1.0μM of each primer, and the AccessQuick RT-PCR System kit (Promega, Madison, WI). Cycling conditions were as follows: A first strand synthesis of 45 min at 45°C and 2 min denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55-65°C for 30 sec and extension at 72°C for 1min/kb. A final elongation step was performed at 72°C for 5 min.

2.6 Triton Solubility Assay

Cells were harvested in 1X PBS (pH 7.4) composed of 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4 and 1.4mM KH_2PO_4. Cells were then incubated on ice for 10min

in a solution containing triton-X100 (1% v/v), leupeptin (2μg/ml), aprotinin (2μg/ml and pepstatin A (1μg/ml) in 1XPBS. This was followed by centrifugation to separate triton soluble and insoluble materials and lysed at a final concentration of 5% sodium dodecyl sulfate (SDS), maintaining equal volumes for both fractions. Samples were sheared with a 21-gauge needle.

2.7 Western Blot Analysis

Protein concentration of samples was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Equal amounts of soluble protein samples (with each respective insoluble sample loaded at equal volume) were resolved via SDS polyacrylamide gel electrophoresis. Proteins were transferred onto methanol-activated polyvinylidene fluoride membranes (PVDF; NEN, Boston, MA). Following blocking in a 5% (w/v) dried milk and 0.2% Tween 20-PBS solution for 1 hour, membranes were incubated in with their respective primary antibodies. Primary monoclonal antibodies recognizing HSC70 (1:1000), cIAP2 (1:1000), FLAG-M2 (1:500) and eEF1α (1:1000) (Abcam, Cambridge, MA) and primary polyclonal antibodies recognizing POLD1 (1:2000) and Fibrillarin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) were used. After washing with 0.2% Tween 20-PBS, membranes were incubated for 1h at room temperature with their respective horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and detected by Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer-Cetus, Boston, MA).

2.8 Immunofluorescence

Cells were seeded onto coverslips and fixed with pre-chilled methanol for 10 min followed by pre-chilled acetone at -20°C for 1 min. Polyclonal antibodies POLD1 (1:60)

and TAF1 (1:50) (Santa Cruz), as well as monoclonal antibody B23 (1:300) (Sigma, St. Louis, MO) were used. Cells were incubated at room temperature for 1h with a primary antibody solution containing 10% FBS and 0.1% Triton-X100 (v/v). Cells were then washed in PBS followed by incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch). Hoechst stain 33342 (Sigma) was added to visualize the nuclei and the coverslips were mounted onto slides using Fluoromount G (EMS, Hatfield, PA).

2.9 Photobleaching and Microscopy

Cells were cultured into 35mm dishes with coverslip bottoms and visualized with a confocal microscope (MRC 1024; Bio-Rad Laboratories, Richmond, CA) in an environmental chamber maintained at 37°C. A 60X plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to five rapid pulses of a 488nm argon laser at 100% power and image acquisition was conducted at 1% power. For FRAP, images were collected at 1 s or 5 s intervals. Recovery of fluorescence within a bleached region was calculated following $I_{rel}=(I_t/I_0)*(T_0/T_t)$ where T_t and T_0 are the total cellular intensities at time t or before bleach respectively and I_0 and I_t are the intensities of the bleached area at prebleach or time t respectively. For FLIP experiments, cells were repeatedly bleached at 20 s intervals and fluorescence loss in bleached areas was calculated according to $I_{rel}=(I_t/I_0)*(N_0/N_t)$ where I_t and I_0 are the average intensities of the unbleached nucleus or cell at time t or before bleach respectively and N_0 and N_t are the average total cellular fluorescence intensities of a neighboring cell at prebleach or time point t respectively. All results are based on the

analysis of 3 datasets. Average pixel intensities were quantified with ImageJ and normalized for background fluorescence.

Images from experiments that do not involve bleaching were captured with a microscope (Axiovert S100TV; Carl Zeiss MicroImaging, Inc.) equipped with a 40X C-Apochromat water immersion objective with a 1.2 NA using a digital charged-coupled device camera (Empix). Image acquisition was performed with the Pascal software and Adobe Photoshop CS was used for pseudocoloring.

2.10 BrdU Incorporation Experiments

Cells were seeded on coverslip-containing 35mm plates (350,000 cells/plate) and incubated overnight in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin (v/v). Following a day of growth, plates were replenished with either SD or AP media and incubated in hypoxia for 18 h. BrdU-labeling reagent (1μl/ml) was added for 2 h prior to fixation at -20°C with 70% ethanol in 50mM glycine (pH 2.0) for 20 min. Cells were then washed with PBS and incubated with anti-BrdU monoclonal antibody (1:30; Roche Diagnostic Co., Indianapolis, IN) for 30 min at 37°C. After a second wash, cells were incubated for 30min with a fluorescin-conjugated antibody at 37°C. Hoechst stain 33342 (Sigma) was added to visualize the nuclei and the coverslips were mounted onto slides using Fluoromount G (EMS). Results represent the average percentage of nuclei incorporating BrdU relative to the total number of nuclei.

2.11 Cell Death Assay

Cells were seeded on coverslip-containing 35mm plates (350,000 cells/plate) and incubated overnight in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin (v/v). The following day, cells were transfected or infected with their

appropriate constructs (where indicated) or left unaffected. Following 24 h incubation, plates were replenished with either SD or AP media and incubated in hypoxia for 18 h. Cells were then co-stained with propidium iodide (PI) and hoechst (Sigma) and incubated for 5 minutes. Cell death was determined by calculating the average number of cells incorporating PI relative to the total number of nuclei.

2.12 ATP, Glucose and Lactate Measurements

Cells were seeded on 60mm plates ($1x10^6$ cells/plate) and incubated overnight in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin (v/v). The following day, cells were infected with their appropriate constructs or left uninfected. Following 24 h incubation, plates were replenished with either SD or AP media and incubated in hypoxia for 18 h. For glucose and lactate quantification, substrate concentration was compared between start and final media, i.e. before and after hypoxic incubation. Measurements were performed using the Glucose Hexokinase assay (Sigma) and the Lactate Reagent and Lactate Standard Set (Trinity Biotech, Saint Louis, MO) respectively, according to manufacturers' protocol. For ATP measurement, cells were trypsinized and collected using DMEM media (final 3ml volume) and 50µl of the total was subsequently used. The CellTiter-Glo Luminescent Assay (Promega) was used according to manufacturer's protocol using a 96-well plate (Dynex Technologies, Chantilly, VA). All assays were done in triplicate and normalized to the number of cells.

RESULTS

Chapter 3: Results

3.1 Identification and Characterization of NoDS^{H+}-Containing Proteins

3.1.1 NoDS^{H+}-Containing Proteins are Involved in Basal Cellular Functions

A bioinformatics search was performed looking for human proteins conforming to the three rules of the NoDSH^{+} subnuclear targeting signal: at least one subnuclear targeting arginine domain (STAD; $[RR(L/I)X_3r]_{(n,n\geq 1)}$); more than one three-residue subnuclear targeting hydrophobic domain (STHD; $[L(\Phi/N)(L/V)]_{(n,n>1)}$); and low disorder character of the STAD domains. Through this search, 18 proteins were found to contain NoDS^{H+} signals (Table 2). These include POLD1, the major catalytic subunit of DNA polymerase delta involved in DNA replication, TAF1, the largest TATA box binding protein associated factor involved in transcription, eIF2B1, a GTP exchange factor essential for protein synthesis, APC2, promotes metaphase-anaphase transition through ubiquitination of mitotic cyclins and anaphase inhibitor, and UAP56, a pre-mRNA splicing and export protein. The discovery of NoDS^{H+}-containing proteins to be involved in major cellular pathways is suggestive of a coordinated response to reduce energy demand in a non-permissive environment. Because of their involvement in basal cellular functions, POLD1, TAF1, APC2 and UAP56 became the focus of our study.

3.1.2 Confirmation of Nucleolar Relocalization of NoDS^{H+}-Containing Proteins in Response to Acidosis

In order to confirm nucleolar accumulation of endogenous proteins in response to acidosis, Triton-X100(T-X)-based fractionation was employed. This detergent-based process produces two fractions, one containing soluble components including cytoplasmic and nucleoplasmic proteins, and an insoluble fraction which is enriched with

Table R1. Proteins containing NoDS^{H+} consensus sequence. Bioinformatics search for proteins conforming to NoDS^{H+} subnuclear targeting signal rules.

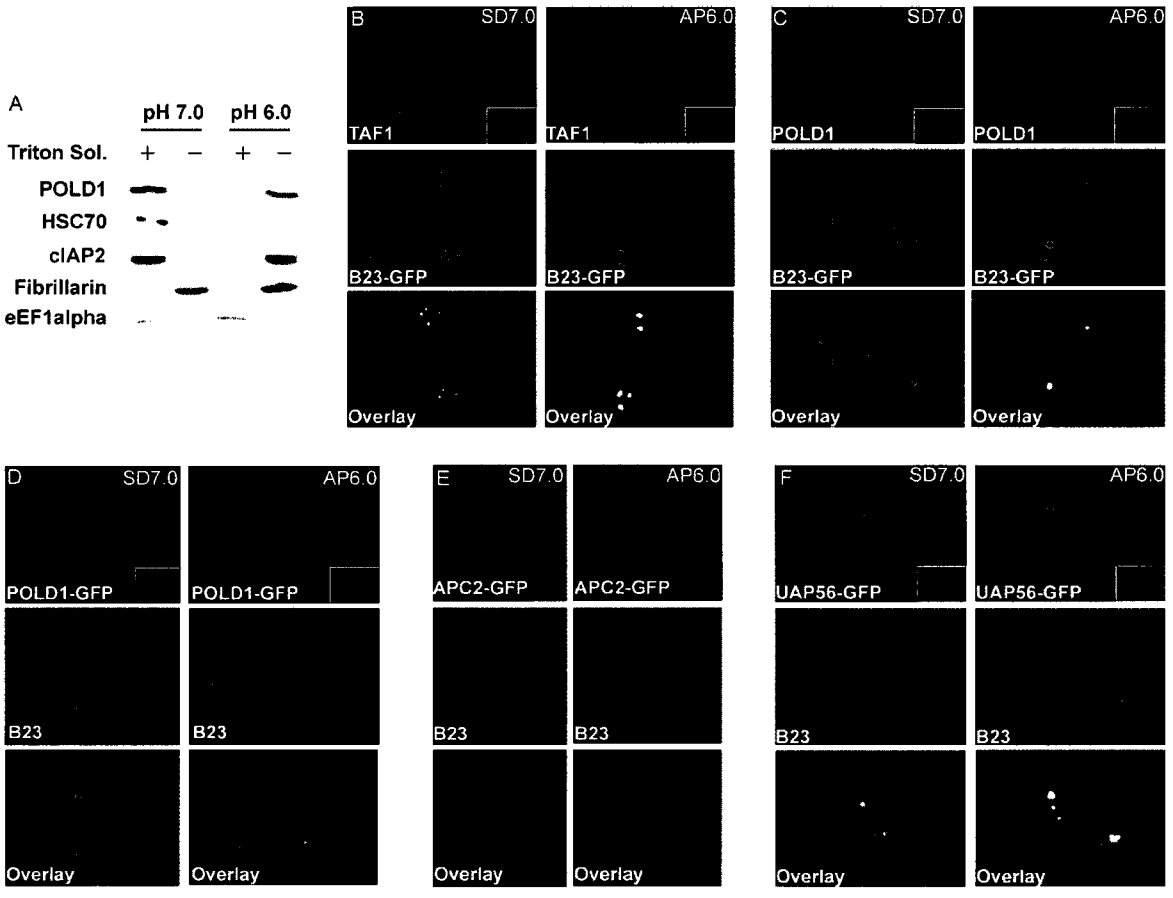
Accession Number	Protein Symbol	Full Name	STADs	STHDs
P28340	POLD1	DNA polymerase delta catalytic subunit	RRLLIDR	3LAL, 2LGL, 2LAV, LQV, LFV
P21675	TAF1	Transcription initiation factor TFIID subunit 1	RRIRMED, RRLSLKN, RRIQEQL, RRLKRNQ	LLL, LGL, LVL LGV, LQV
Q14232	eIF2B1	Translation initiation factor eIF2B alpha subunit	RRISLSR	LGV, LFL
Q9UJX6	APC2	Anaphase-promoting complex subunit 2	RRINANI	LLV, 2LLL, 2LGL, LLV
Q13838	UAP56	56 kDa U2AF65-associated protein	RRILVAT	LAL, LNL, LLV
P11142	HSC70	Heat shock 70kDa protein 8	RRLRTAC	LLL, 2LNV
Q13489	cIAP2	Inhibitor of apoptosis 1	RRLQEER	LVL, LLL, LFV, LVV, LPV
P40337	VHL	Von Hippel-Lindau tumor suppressor	RRIHSYR, RRLDIVR	LQV, LWL, LLV, LFV, LNV, 2LPV
O76065	RNF8	RING finger protein 8 ubiquitin ligase	RRIVLIR	LGV, LLL, LVL
P11142	HSC70	Heat shock cognate 71kDa	RRLRTAC	2LNV, LLL
Q16394	EXT1	Putative tumor suppressor protein EXT1	RRLGSFR	2LAL, LLV, LFV, LL
O14746	HEST2	Telomerase reverse transcriptase	RRLGCER	4LLL, 2LGL, 2FLF, LQL, LFV, LLV, LQV
Q09472	EP300	E1A binding protein p300	RRLSIQR	7LGL, LVL, LLL, LGV, LFV
P50749	RASSF2	Ras association domain-containing protein 2	RRIRRHR	2LLL, LQL
P19484	TFEB	Transcription factor EB	RRLEMTN	LNV, LWL
Q93074	MED12	Mediator of RNA POL-II transcription sub12	RRLALQL	2LQL, LNV, LVV, LQV, LVL
O76021	PBK1	Ribosomal L1 domain-containing protein 1	RRLLPSL	4LLL, 3LGL, 3LLV, LAV
Q86UFF	METTL3	Methyl transferase like 3	RRIINKH	LWL, LFL, LNL, LAL

nucleolar proteins such as fibrillarin (Mekhail et al., 2004a). Protein extracts were derived from cells incubated in hypoxia in either standard (SD) media, which mimics a natural cellular pH of 7.2, or acidification-permissive (AP) media, which permits a gradual acidification of the extracellular milieu to a set pH. Western blotting results showed that in neutral conditions tested proteins (POLD1, HSC70, cIAP2) were found in the T-X soluble fraction, as predicted due to their nuclear or nucleocytoplasmic distributions (Figure 6A). In response to acidosis (pH 6.0) however, all three proteins became relocalized to the insoluble fraction. Antibodies against control cytoplasmic (eEF1α) and nucleolar (fibrillarin) proteins, which do not contain $NoDS^{H+}$ sequences, showed the proteins to remain in their respective soluble and insoluble fractions regardless of pH.

In order to study the nucleolar accumulation of other proteins from Table 2, fluorescence microscopy and immunofluorescence co-localization was performed. In response to acidosis, endogenous proteins TAF1 and POLD1 shift from a nuclear to nucleolar distribution as determined by co-localization with B23 (Figure 6B and C). Exogenous GFP-tagged constructs of POLD1, APC2 and UAP56 also followed the predictive pattern of nucleolar accumulation under conditions of low extracellular pH (Figure 6D, E and F). These results emphasize the analytical power of the $NoDS^{H+}$ consensus sequence rules on subnuclear targeting in response to acidosis.

Coverslip hypoxia represents an alternative model to study nucleolar sequestration. The procedure involves placing a glass coverslip over a portion of the monolayer cells in a cell culture plate (Pitts et al., 2008; Pitts and Toombs, 2004). This restricts covered cells to a thin film of media while leaving uncovered cells access to the

Figure 6. NoDS^{H+}-containing proteins are relocalized to the nucleolus in response to extracellular accumulation of H^+. (A) Subcellular fractionation confirms nucleolar accumulation in response to acidosis. MCF7 cells were incubated in SD or AP conditions in hypoxia and Triton-X100 fractionation was performed to separate nuclear and cytoplasmic (soluble, +) from nucleolar (insoluble, –) proteins. Lysates were immunoblotted for POLD1, HSC70, cIAP2, Fibrillarin and eEF1α. (B-F) Immunofluorescence analysis shows nucleolar accumulation of NoDS^{H+} proteins in acidosis. MCF7 cells were cultured in hypoxia in either SD or AP conditions. (B,C) Endogenous protein TAF1 and POLD1 are relocalized to the nucleolus in response to acidosis. Cells transiently expressing nucleolar B23-GFP were fixed, hoechst stained and analyzed with TAF1 or POLD1 specific antibodies, respectively. (D-F) Exogenous GFP constructs of POLD1, APC2 and UAP56 show relocalization to the nucleolus in acidosis. Cells transiently expressing POLD1-GFP, APC2-GFP or UAP56-GFP were fixed, hoechst stained and analyzed using a B23 antibody.


A
pH 7.0
pH 6.0
Triton Sol.
+ − + −
POLD1
HSC70
clAP2
Fibrillarin
eEF1alpha
B
SD7.0
AP6.0
TAF1
TAF1
B23-GFP
B23-GFP
Overlay
Overlay
C
SD7.0
AP6.0
POLD1
POLD1
B23-GFP
B23-GFP
Overlay
Overlay
D
SD7.0
AP6.0
POLD1-GFP
POLD1-GFP
B23
B23
Overlay
Overlay
E
SD7.0
AP6.0
APC2-GFP
APC2-GFP
B23
B23
Overlay
Overlay
F
SD7.0
AP6.0
UAP56-GFP
UAP56-GFP
B23
B23
Overlay
Overlay

bulk of the media volume (Figure 7A). In addition to creating a hypoxic environment, this technique also reduces diffusion, exposing cells to high local concentrations of metabolites observed *in vivo*. It has also been shown that this causes the acidification of the extracellular environment for cells directly beneath the center of the coverslip. Because of this models ability to rapidly imitate ischemia, this method was subsequently used to study the subcellular distribution of both endogenous and exogenous POLD1. Through co-localization with VHL, known to be nucleolar in acidosis, POLD1 was confirmed to become relocalized to the nucleolus in response to extracellular H^+ accumulation (Figure 7B).

3.1.3 NoDS^{H+}-Containing Proteins Show Static Detention in the Nucleolus

A change of localization of a protein doesn't necessarily translate into a loss of function. Therefore, even though a protein may shift its localization to the nucleolus, it could still remain highly mobile and shuttle between cellular compartments in a rapid, energy-independent fashion. Therefore, photobleaching was used in order to assess the mobility of NoDS^{H+}-containing proteins in the nucleolus (see Section 1.5.1). FRAP analysis of POLD1-GFP (Figure 8A and C) and of APC2-GFP (Figure 8B and D) indicated that not only did acidosis cause relocalization to the nucleolus, but also loss of the dynamic mobility observed in neutral conditions. FRAP of UAP56-GFP (Figure 9A and C) showed recovery of fluorescence in acidosis, though it was greatly reduced compared to neutral conditions. This suggests that acidosis has a prominent effect on decreasing the kinetics of UAP56, but does not cause it to be detained. FRAP analysis of B23-GFP (Figure 9B and D) was performed as a control and showed that it maintains its dynamic mobility regardless of extracellular pH.

Figure 7. POLD1 accumulates in the nucleolus in the reverse coverslip model of ischemia. (A) Graphical representation of coverslip hypoxia in a cell culture plate. The coverslip creates three distinct zones: ischemic zone (cells under the center of the coverslip), border zone (cells under the periphery of the coverslip) and non-ischemic zone (cells not under the coverslip). (B) Immunofluorescence analysis shows accumulation of POLD1 in nucleoli in the reverse coverslip model. MCF7 cells were cultured in normoxic conditions, and a coverslip was placed over cells in a region of the plate. Cells were either co-transfected with VHL-BFP and POLD1-GFP followed by fluorescence analysis, or singly transfected with VHL-GFP followed by fixing, hoechst staining and analysis with a POLD1 specific antibody. All cells observed were those directly under the center of the coverslip.

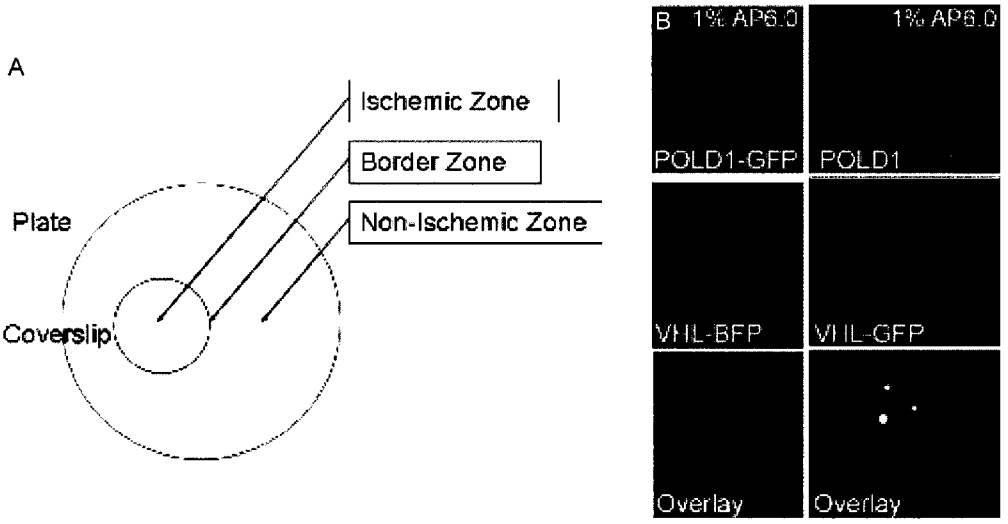

A
Ischemic Zone
Border Zone
Non-Ischemic Zone
Plate
Coverslip
B
1% AP6.0
1% AP6.0
POLD1-GFP
POLD1
VHL-BFP
VHL-GFP
Overlay
Overlay

Figure 8. FRAP: Acidosis shifts POLD1 and APC2 from a dynamic to static state. (A-D) MCF7 cells were transiently transfected to express POLD1-GFP or APC2-GFP and incubated in pH7.0 SD or pH6.0 AP media in hypoxia. FRAP was subsequently performed on the cells. (A,B) Cells were imaged before and after bleaching of indicated nucleoplasmic or cytoplasmic regions (square/arrows). Dashed circles represent nuclei. Post-bleach time is indicated in seconds. Pseudocolored panels better illustrate changes in fluorescence. (C,D) Corresponding quantification of recovery kinetics. Fluorescence intensity in the bleached region was measured and expressed as a relative intensity. Results are based on the analysis of 3 datasets.

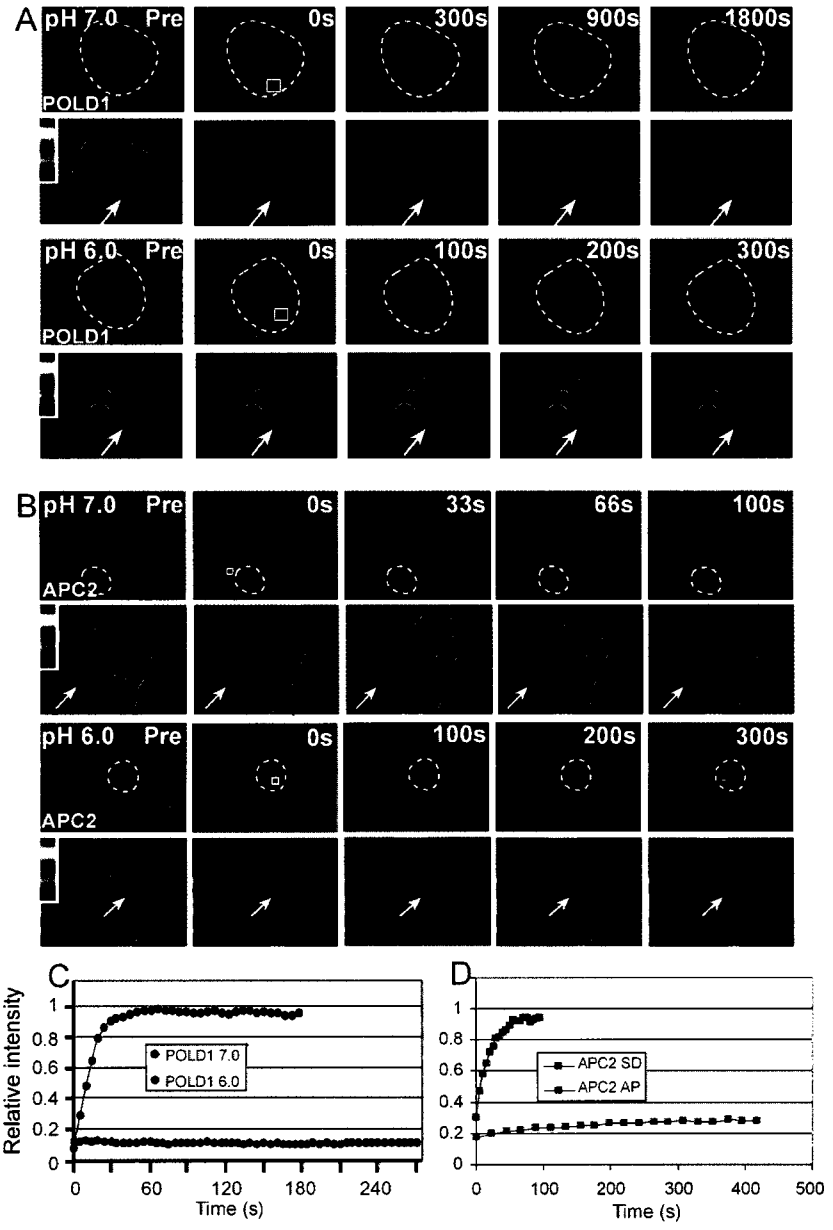
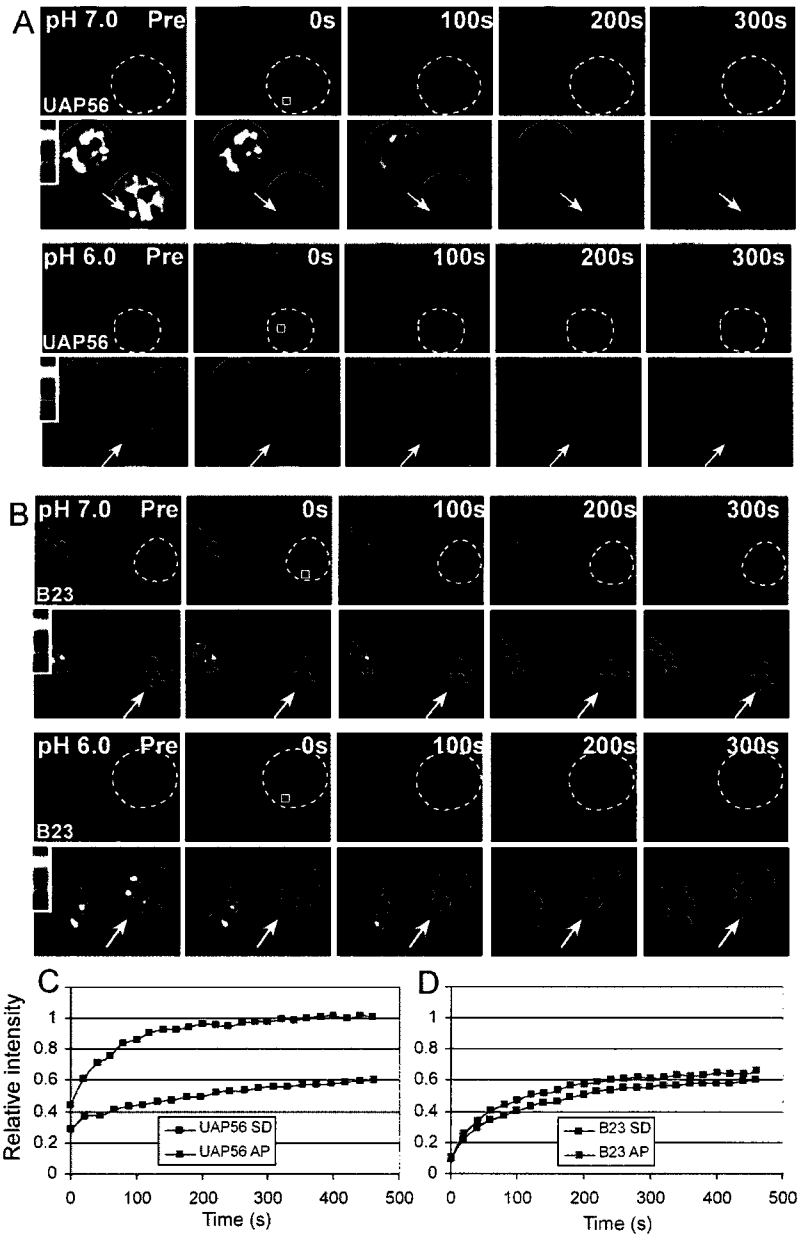

A
pH 7.0 Pre
0s
300s
900s
1800s
POLD1
pH 6.0 Pre
0s
100s
200s
300s
POLD1
B
pH 7.0 Pre
0s
33s
66s
100s
APC2
pH 6.0 Pre
0s
100s
200s
300s
APC2
C
Relative intensity
POLD1 7.0
POLD1 6.0
Time (s)
D
APC2 SD
APC2 AP
Time (s)

Figure 9. FRAP: Acidosis reduces the dynamic mobility of UAP56. (A-D) MCF7 cells were transiently transfected to express UAP56-GFP or B23-GFP and incubated in pH7.0 SD or pH6.0 AP media in hypoxia. FRAP was subsequently performed on the cells. (A,B) Cells were imaged before and after bleaching of indicated nucleoplasmic regions (square/arrows). Dashed circles represent nuclei. Post-bleach time is indicated in seconds. Pseudocolored panels better illustrate changes in fluorescence. (C,D) Corresponding quantification of recovery kinetics. Fluorescence intensity in the bleached region was measured and expressed as a relative intensity. Results are based on the analysis of 3 datasets.


A
pH 7.0 Pre
0s
100s
200s
300s
UAP56
pH 6.0 Pre
0s
100s
200s
300s
UAP56
B
pH 7.0 Pre
0s
100s
200s
300s
B23
pH 6.0 Pre
0s
100s
200s
300s
B23
C
Relative intensity
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
Time (s)
UAP56 SD
UAP56 AP
D
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
Time (s)
B23 SD
B23 AP

To complement the FRAP results, FLIP (fluorescence loss in photobleaching) was also performed. FLIP involves the repeated bleaching of a small area within the cell, resulting in a decrease in total fluorescence for dynamic proteins through eventual passing of the bleaching area. FLIP of POLD1 and APC2 resulted in loss of nuclear and cytoplasmic fluorescence respectively in pH 7 but no loss of nucleolar fluorescence in pH 6 (Figure 10) confirming a loss of dynamic mobility in acidosis. Results obtained in the case of UAP56-GFP however demonstrated only a slight loss in mobility between pH 7 and pH 6, but still indicated that acidosis decreases the mobility of UAP56 (Figure 11A and C). FLIP analysis of B23-GFP (Figure 11B and D) was performed as a control and showed that it maintains its dynamic mobility regardless of extracellular pH.

Taken together, these results for the most part support previous findings that $NoDS^{H+}$-containing proteins not only cause relocalization, but also detention within the nucleolus (Mekhail et al., 2007). One exception was the protein UAP56-GFP which, although having reduced mobility, was still dynamic in acidosis.

3.2 Nucleolar Sequestration of POLD1 Prevents DNA Synthesis

We next examined the effect of nucleolar sequestration on the native function of $NoDS^{H+}$-containing proteins, specifically POLD1. Since we have demonstrated that POLD1 is statically detained within the nucleolus, it is reasonable to suggest that its function is inhibited in acidosis in an effort to maintain energy equilibrium. To test the function of POLD1, DNA synthesis, BrdU incorporation was measured and compared between cells in AP media of various pH values. Results showed a near arrest in BrdU incorporation at approximately pH 6.3 (Figure 12A and B). In order to correlate this

Figure 10. FLIP: Acidosis shifts POLD1 and APC2 from a dynamic to static state. (A-D) MCF7 cells were transiently transfected to express POLD1-GFP or APC2-GFP and incubated in pH7.0 SD or pH6.0 AP media in hypoxia. FLIP was subsequently performed on the cells. (A,B) Specific nucleoplasmic or cytoplasmic regions (square) were repeatedly bleached and cells were imaged between pulses. Pseudocolored panels better illustrate changes in fluorescence. (C,D) Corresponding kinetics of loss of fluorescence. Fluorescence intensity of the nucleus (POLD1) or whole cell (APC2) was measured and expressed as a relative intensity. Results are based on the analysis of 3 datasets.

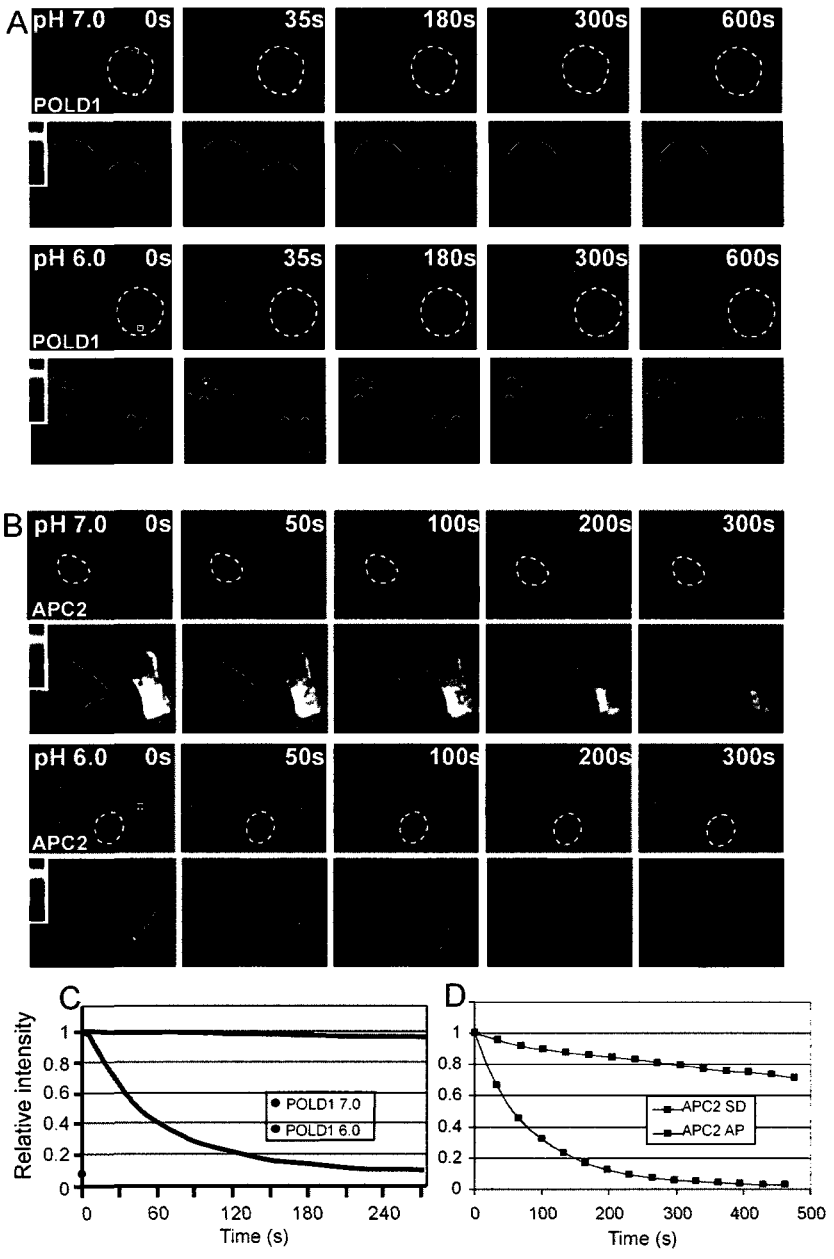

A
pH 7.0 0s
35s
180s
300s
600s
POLD1
pH 6.0 0s
35s
180s
300s
600s
POLD1
B
pH 7.0 0s
50s
100s
200s
300s
APC2
pH 6.0 0s
50s
100s
200s
300s
APC2
C
Relative intensity
1
0.8
0.6
0.4
0.2
0
0
60
120
180
240
Time (s)
POLD1 7.0
POLD1 6.0
D
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
Time (s)
APC2 SD
APC2 AP

Figure 11. FLIP: Acidosis reduces the dynamic mobility of UAP56. (A-D) MCF7 cells were transiently transfected to express UAP56-GFP or B23-GFP and incubated in pH7.0 SD or pH6.0 AP media in hypoxia. FLIP was subsequently performed on the cells. (A,B) Specific nucleoplasmic (square) were repeatedly bleached and cells were imaged between pulses. Pseudocolored panels better illustrate changes in fluorescence. (C,D) Corresponding kinetics of loss of fluorescence. Fluorescence intensity of the nucleus was measured and expressed as a relative intensity. Results are based on the analysis of 3 datasets.

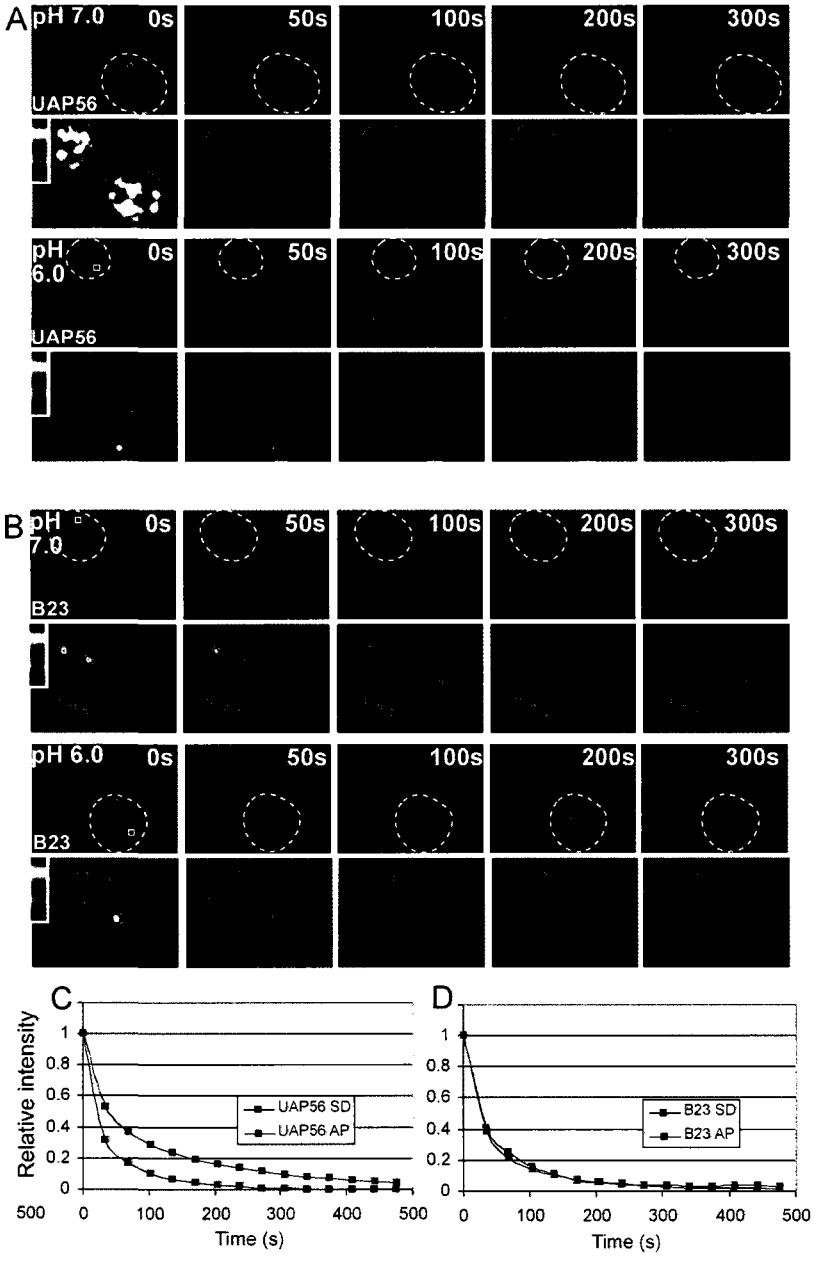
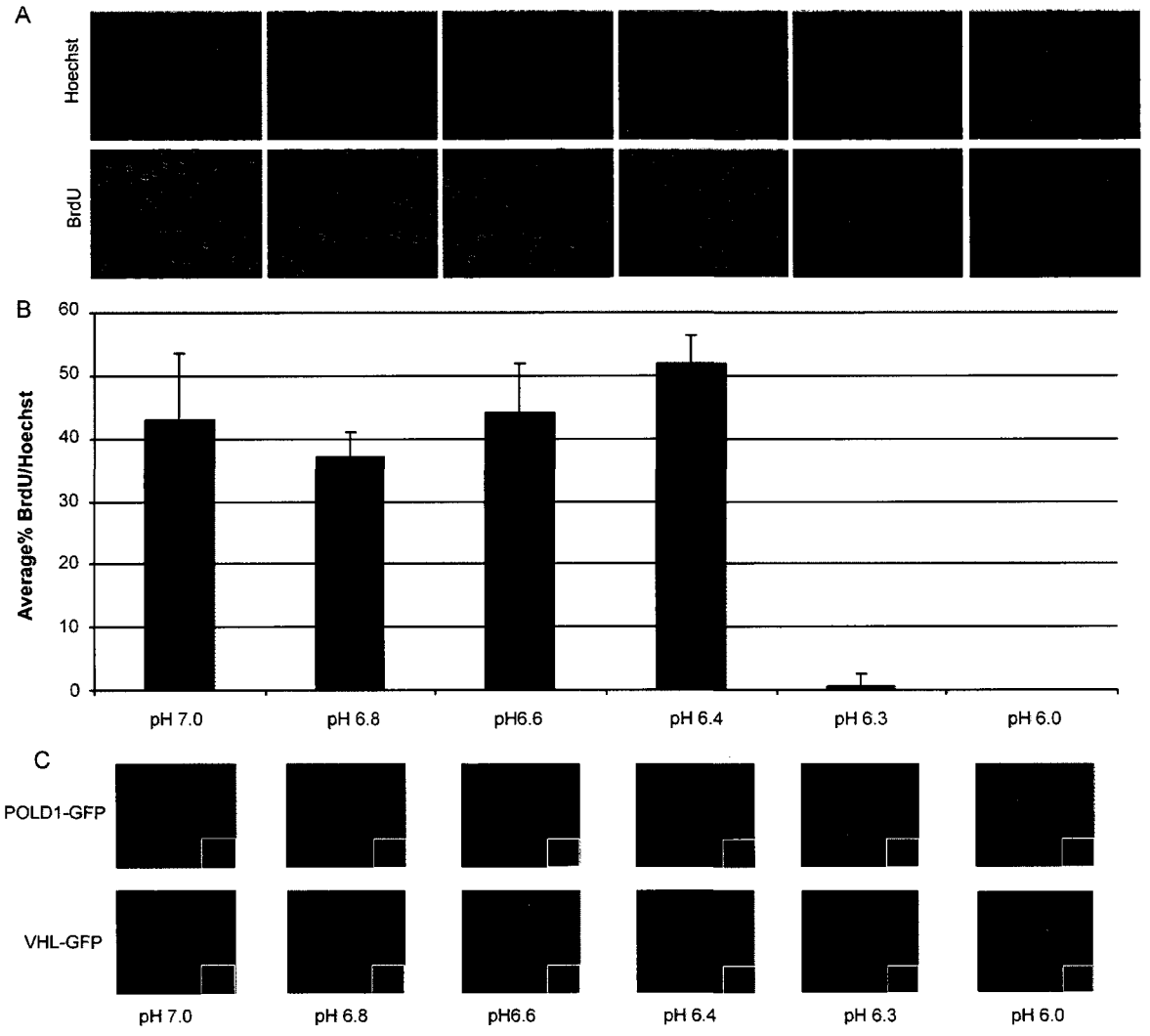

A
pH 7.0
0s
50s
100s
200s
300s
UAP56
pH 6.0
0s
50s
100s
200s
300s
UAP56
B
pH 7.0
0s
50s
100s
200s
300s
B23
pH 6.0
0s
50s
100s
200s
300s
B23
C
Relative intensity
1
0.8
0.6
0.4
0.2
0
UAP56 SD
UAP56 AP
500
0
100
200
300
400
500
Time (s)
D
1
0.8
0.6
0.4
0.2
0
B23 SD
B23 AP
0
100
200
300
400
500
Time (s)

Figure 12. Acidosis decreases BrdU incorporation correlative with a pH at which nucleolar detention of POLD1 occurs. (A,B) Acidosis decreases levels of DNA synthesis. MCF7 cells were cultured in AP media (pH6.0-7.0) in hypoxia for 18 hours, after which BrdU labeling reagent (1ul/ml) was added. Cells were incubated for 2 hours then immunofluorescence was performed using a BrdU-specific antibody along with Hoechst staining. (A) Representative immunofluorescence images. (B) The average percentage of BrdU incorporating cells relative to Hoechst-stained cells was calculated. (C) POLD1 accumulates at the same pH which decreases DNA synthesis. Corresponding POLD1 and VHL localization was monitored using Hoechst-stained MCF7 cells transiently transfected with POLD1-GFP or infected with adenovirus-introduced VHL-GFP and cultured in AP media (pH6.0-7.0) in hypoxia for 18 hours.


A
Hoechst
BrdU
B
Average% BrdU/Hoechst
60
50
40
30
20
10
0
pH 7.0
pH 6.8
pH6.6
pH 6.4
pH 6.3
pH 6.0
C
POLD1-GFP
VHL-GFP
pH 7.0
pH 6.8
pH6.6
pH 6.4
pH 6.3
pH 6.0

decrease in DNA synthesis with POLD1, cells were transiently transfected with POLD1-GFP or infected with VHL-GFP and placed in the same AP pH range as those in the BrdU experiment. Fluorescence microscopy demonstrated that POLD1-GFP accumulates in the nucleolus at the same pH that BrdU synthesis decreases (Figure 12C). Additionally, VHL-GFP is not detained at this pH suggesting that the diminished level of DNA synthesis is caused by the nucleolar detention of specific protein(s), likely POLD1.

3.3 Effect of Nucleolar Detention on Cell Survival and Maintenance of Energy Equilibrium

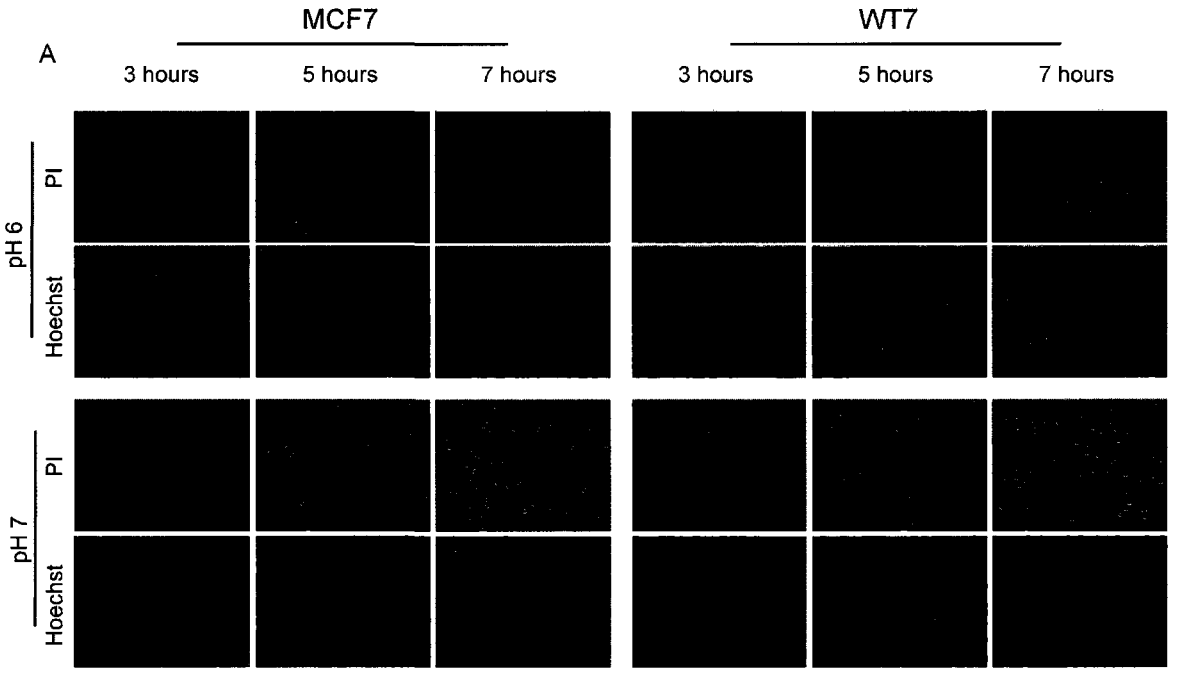
3.3.1 Acidosis Maintains Cell Viability in Hypoxia

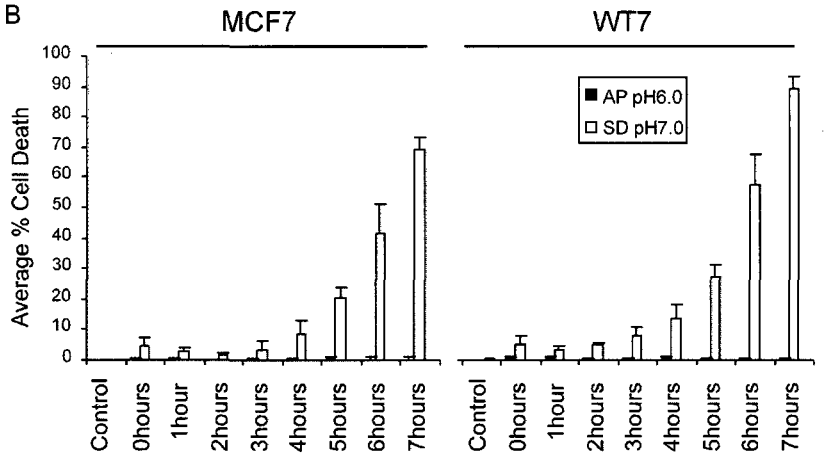
We next looked at cell viability in hypoxia through the use of the intercalating fluorescent stain propidium iodide (PI), since it is only able to penetrate dying cells lacking an intact plasma membrane. Cells were cultured in hypoxia in either SD or AP media, followed by hoechst and PI staining. Results showed a dramatic increase in cell death in hypoxia if the cells are prevented from acidifying their extracellular environment (Figure 13). This confirmed the protective effects of extracellular acidosis in hypoxic conditions.

3.3.2 VHL Mutant Prevents Nucleolar Accumulation of NoDS^{H+}-Containing Proteins

Since we have demonstrated that acidosis maintains cell viability in hypoxic conditions, we next wanted to determine what was responsible for this effect. According to our hypothesis, nucleolar detention is required to maintain energy equilibrium and hence cell viability in hypoxia. Therefore, in order to determine if the protective effects

Figure 13. Acidosis plays a protective role in hypoxia. (A, B) MCF7 or WT7 cells were put in either SD or AP media in hypoxia. Control consists of cells in normoxia SD media. After 18 hours, cells were stained using PI and hoechst every hour for 7 hours. (A) Representative photos taken at 3, 5 and 7 hours. (B) The average percentage of PI incorporating cells to hoechst stained cells was determined.


A
MCF7
WT7
3 hours
5 hours
7 hours
3 hours
5 hours
7 hours
pH 6
PI
Hoechst
pH 7
PI
Hoechst

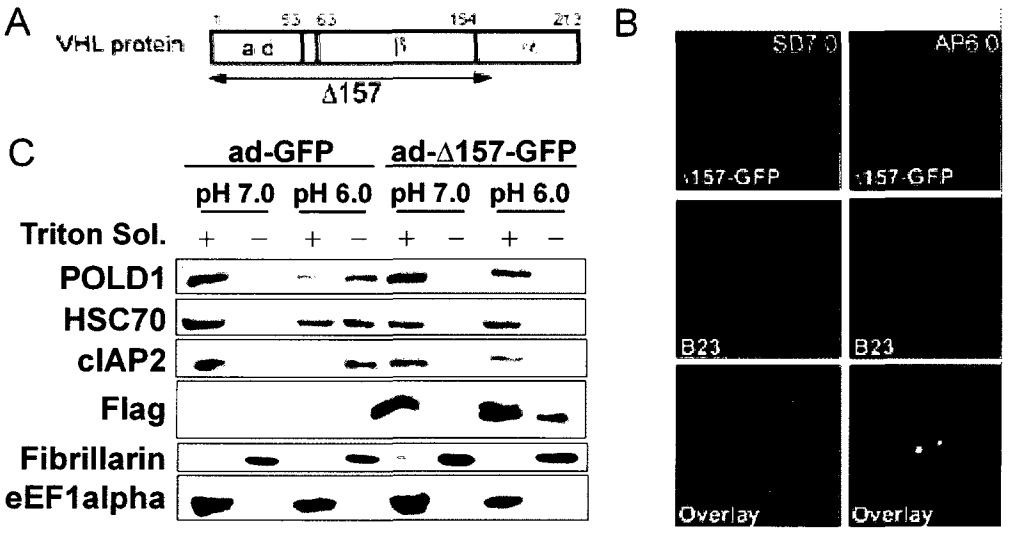

B
MCF7
WT7
Average % Cell Death
100
90
80
70
60
50
40
30
20
10
0
AP pH6.0
SD pH7.0
Control
0hours
1hour
2hours
3hours
4hours
5hours
6hours
7hours
Control
0hours
1hour
2hours
3hours
4hours
5hours
6hours
7hours

observed in acidosis were due to nucleolar sequestration of $NoDS^{H+}$-containing proteins, we sought to prevent acidosis-induced nucleolar sequestration in acidosis, and observe the resulting effects. Previous research has shown that the C-terminal mutant of VHL, Δ157 (Figure 14A), is able to be targeted to the nucleolus in response to extracellular H^+ accumulation and act as a dominant negative for VHL (Figure 14B). In acidosis, exogenous expression of Δ157 competes with nucleolar VHL, but is unable to perform its function of inhibiting ribosomal biogenesis (Mekhail et al., 2004a). Because of these effects, it was hypothesized that Δ157 might not only be able to compete with VHL, but also other nucleolar proteins. To test this, immunoblotting for POLD1, HSC70 and cIAP2 was performed on T-X fractionated FLAG-Δ157-GFP infected cells cultured in hypoxia neutral and hypoxia acidosis conditions. GFP infected cells served as a control. Results from this experiment indicated that FLAG-Δ157-GFP was able to efficiently prevent $NoDS^{H+}$-containing proteins POLD1, HSC70, and cIAP2 from becoming nucleolar in acidosis (Figure 14C). Antibodies against FLAG served to confirm nucleolar localization of FLAG-Δ157-GFP in acidosis. Antibodies against control cytoplasmic (eEF1α) and nucleolar (fibrillarin) proteins confirmed proper fractionation. These results indicate that because Δ157 is able to prevent some $NoDS^{H+}$-containing proteins from accumulating in the nucleolus, and that it might be a good tool to differentiate between the effects of acidosis and nucleolar sequestration.

3.3.3 $NoDS^{H+}$ (POLD1) Prevents Nucleolar Accumulation of POLD1

Because a truncated protein containing the $NoDS^{H+}$ of VHL was able to prevent the sequestration of other nucleolar proteins, it was reasoned that the $NoDS^{H+}$ of other proteins would act similarly. Therefore, the $NoDS^{H+}$ of POLD1 was cloned with FLAG

Figure 14. The Δ157 mutant of VHL is able to prevent the relocalization of some NoDS^{H+}-containing proteins to the nucleolus. (A) Schematic diagram of Δ157 VHL mutant which lacks one of the two STADs of VHL and one of the seven STHDs. It also lacks the alpha domain which is responsible for elongin C binding and subsequent HIFα degradation. (B) The Δ157 mutant accumulates in the nucleolus in response to acidosis, like VHL. Immunofluorescence was performed on Δ157-GFP infected cells incubated in either SD or AP media, in hypoxia. Cells were fixed, hoechst stained and analyzed through immunofluorescence using a B23 antibody. (C) Δ157 prevents nucleolar accumulation of some NoDS^{H+}-containing proteins. Cells were infected with adenovirus GFP or adenovirus FLAG-Δ157-GFP and placed in either SD or AP conditions in hypoxia. Triton-X100 fractionation was performed which separates nuclear and cytoplasmic fractions (soluble, +) from the nucleolar fraction (insoluble, –). Lysates were immunoblotted for NoDS^{H+}-containing proteins POLD1, HSC70, and cIAP2. Immunoblotting of FLAG was performed to ensure the nucleolar localization of Δ157 in acidosis. Fibrillarin and eEF1α were used as nucleolar and cytoplasmic markers.


A
VHL protein
1
65
154
213
α
β
Δ157
B
SD7 0
AP6 0
Δ157-GFP
Δ157-GFP
B23
B23
Overlay
Overlay
C
ad-GFP
ad-Δ157-GFP
pH 7.0
pH 6.0
pH 7.0
pH 6.0
Triton Sol.
+ − + − + − + −
POLD1
HSC70
cIAP2
Flag
Fibrillarin
eEF1alpha

and GFP tags and stably expressed within cells. Immunofluorescence results demonstrated that like POLD1, it was able to become relocalized to the nucleolus in response to acidosis (Figure 15A). Next, immunoblotting was performed using T-X fractionated cells stably expressing either FLAG-NoDS^{H+}(POLD1)-GFP or FLAG-GFP and cultured in either hypoxia neutral and hypoxia acidosis conditions. Though the NoDS^{H+} of POLD1 wasn't able to compete with a variety of NoDS^{H+}-containing proteins like Δ157, it was able to efficiently prevent endogenous POLD1 from becoming nucleolar (Figure 15B).

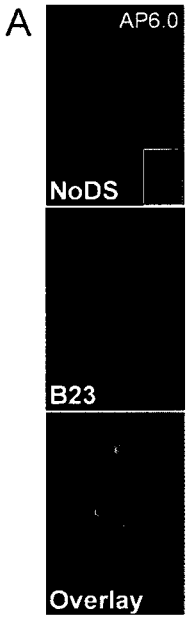
3.3.4 Acidosis-Induced Nucleolar Sequestration Maintains Cell Viability in Hypoxia

Next we wanted to determine if nucleolar sequestration is specifically responsible for maintaining cell viability in hypoxia. To address this, cells were infected with either adenovirus GFP or adenovirus FLAG-Δ157-GFP and incubated in hypoxia in either SD or AP media. We observed an increase in cell death in Δ157-infected cells in hypoxia acidosis (Figure 16A). This suggests that detention of NoDS^{H+}-containing proteins, and not acidosis, is responsible for maintaining cell survival in hypoxia. This experiment was repeated with the use of the NoDS^{H+} of POLD1. In this case, cells were stably expressing FLAG-NoDS^{H+}(POLD1)-GFP or FLAG-GFP, or left untransfected. Results suggest that preventing sequestration of POLD1 is enough to inhibit the protective effects of acidosis in hypoxia (Figure 16B). This supports the Δ157 results, further indicating that nucleolar sequestration is involved in maintaining cell viability in hypoxia.

3.3.5 Maintenance of Energy Equilibrium through Nucleolar Sequestration

According to our model, nucleolar sequestration is involved in decreasing the energy demand during hypoxia, enabling cell survival in an environment where energy

Figure 15. The NoDS^{H+} of POLD1 is able to prevent nucleolar relocalization of POLD1. (A) NoDS^{H+} (POLD1) accumulates in the nucleolus in response to acidosis. Immunofluorescence was performed on FLAG-NoDS^{H+}(POLD1)-GFP stably expressing cells incubated in AP media in hypoxia. Cells were fixed, hoechst stained and analyzed through immunofluorescence using a B23 antibody. (B) NoDS^{H+} (POLD1) is able to prevent endogenous POLD1, but not other tested NoDS^{H+}-containing proteins, from accumulating in the nucleolus. MCF7 cells were stably expressing either FLAG-GFP or FLAG-NoDS^{H+}(POLD1)-GFP and incubated in either SD or AP conditions in hypoxia. Triton-X100 fractionation was performed which separates nuclear and cytoplasmic fractions (soluble, +) from the nucleolar fraction (insoluble, –). Lysates were immunoblotted POLD1, HSC70, cIAP2, FLAG, Fibrillarin, and eEF1α. Immunoblotting of FLAG was performed to ensure the nucleolar localization of NoDS^{H+} (POLD1) in acidosis. Fibrillarin and eEF1α were used as nucleolar and cytoplasmic markers respectively.


A
AP6.0
NoDS
B23
Overlay

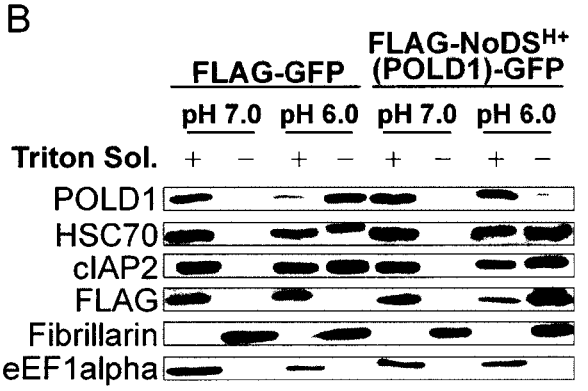
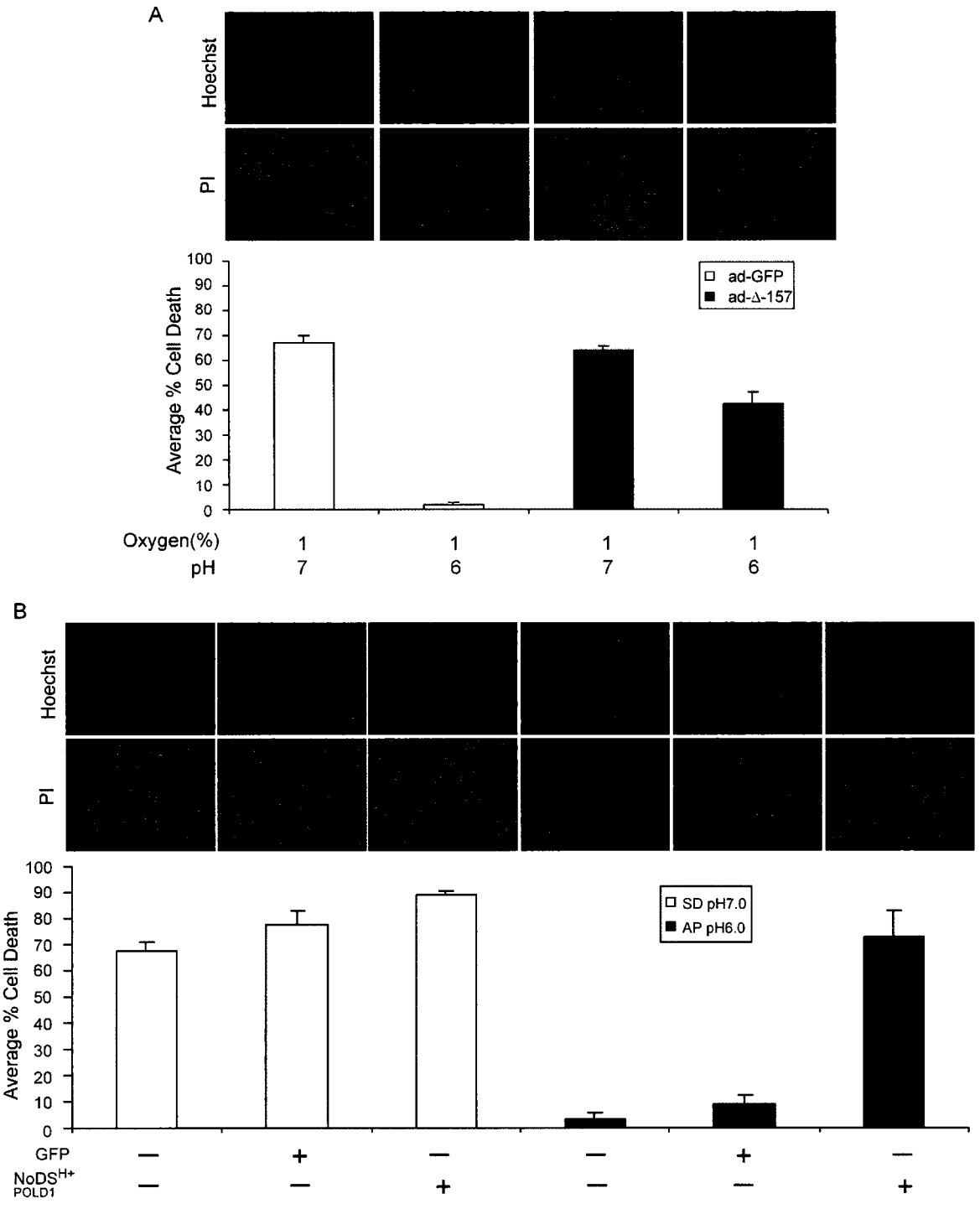

B
FLAG-GFP
FLAG-NoDS^{H+} (POLD1)-GFP
pH 7.0
pH 6.0
pH 7.0
pH 6.0
Triton Sol. + − + − + − + −
POLD1
HSC70
cIAP2
FLAG
Fibrillarin
eEF1alpha

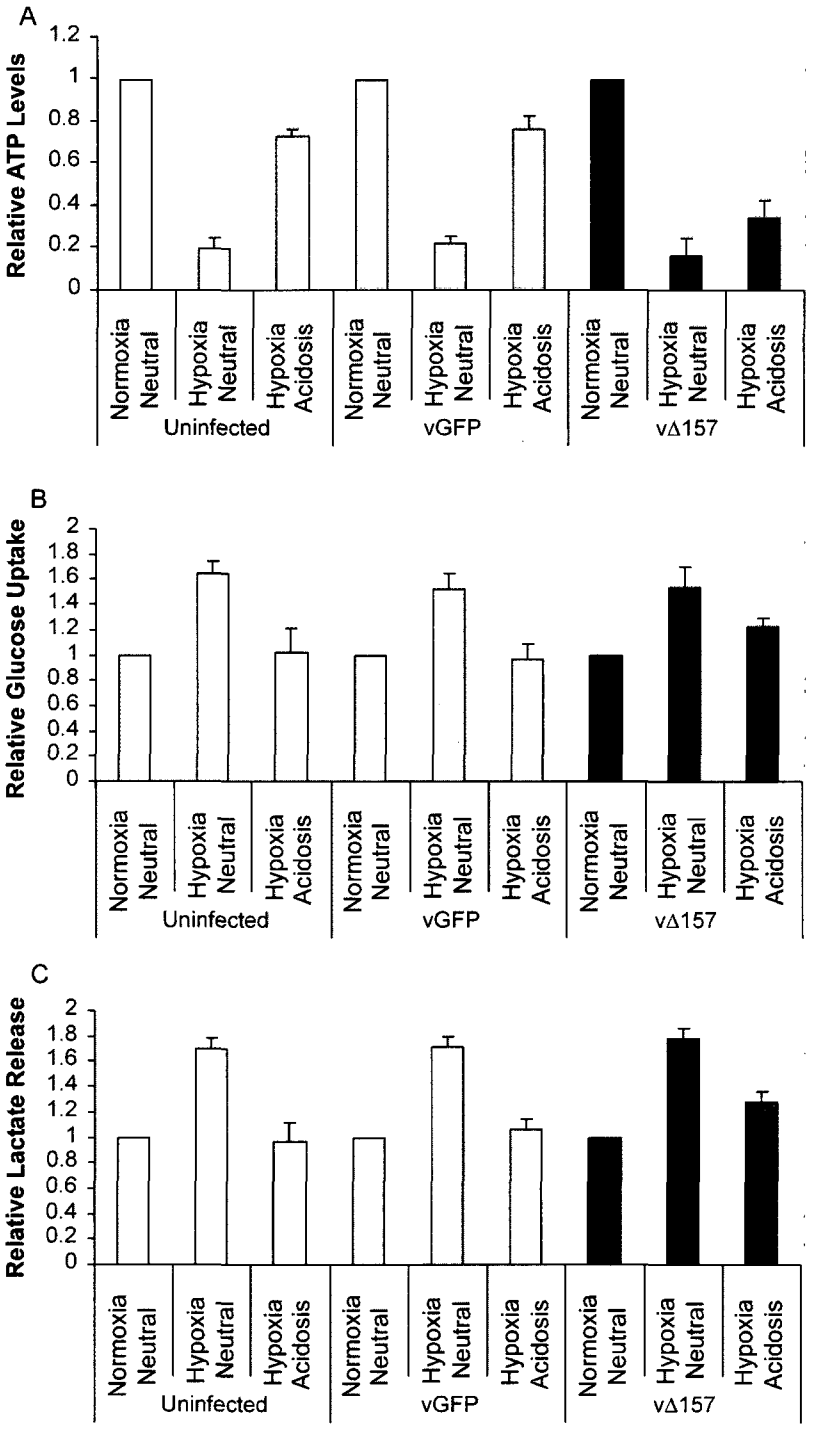
Figure 16. Acidosis-induced nucleolar sequestration maintains cell viability in hypoxia. (A, B) MCF7 cells were cultured in hypoxia in either SD or AP media. After 18 hours, cells were stained using PI and hoechst. The average percentage of PI incorporating cells to hoechst stained cells was calculated. Representative fluorescence pictures are shown. (A) Interfering with nucleolar sequestration reduces cell viability in acidosis. MCF7 cells were infected with either GFP or FLAG-Δ157-GFP. (B) Interfering with nucleolar sequestration of POLD1 reduces cell viability in acidosis. MCF7 cells were stably expressing either FLAG-GFP or FLAG-NoDS^{H+}(POLD1)-GFP, or left untransfected.


A
Hoechst
PI
Average % Cell Death
100
90
80
70
60
50
40
30
20
10
0
ad-GFP
ad-Δ-157
Oxygen(%)
pH
1
7
1
6
1
7
1
6
B
Hoechst
PI
Average % Cell Death
SD pH7.0
AP pH6.0
GFP
NoDS^H+
POLD1

supply is reduced. To study the effects of hypoxia, acidosis and nucleolar sequestration on cellular energy equilibrium, we measured total levels of ATP within the cell. Cells were left uninfected, infected with GFP, or infected with FLAG-Δ157-GFP. They were then put in normoxia in SD media, or in hypoxia in SD or AP media. Similar to previous findings, exposure to hypoxia resulted in a sharp decrease in total ATP levels (Figure 17A). If the extracellular environment was permitted to acidify, total ATP within the cell returned to near-normoxia levels. However, introduction of Δ157 reduced total ATP to near normoxia levels, inhibiting any protective effects conferred by acidosis.

In order to determine if high levels of ATP in acidosis are due to increased ATP production, glucose uptake and lactate release were measured to determine rates of glycolysis and lactic acid fermentation respectively. In hypoxia neutral conditions, there was a marked increase in glucose uptake, which is expected due to the Pasteur effect; a shift from slow aerobic to rapid anaerobic consumption of glucose (Figure 17B). Supporting this shift was an increase in lactate release, indicating a switch from oxidative metabolism to lactic acid fermentation (Figure 17C). Taken together with the ATP data, this suggests that under normoxia neutral conditions, the increased production of ATP from the Pasteur effect is not enough to satisfy the high demand that remains in hypoxia. In hypoxia acidosis, cells had normoxia-like levels of glucose uptake and lactate release (Figure 17B and C). Compared to hypoxia neutral cells, this indicates that even with a lower level of energy production, hypoxia acidotic cells are able to maintain much higher total ATP levels. This suggests that energy consumption under these conditions is significantly reduced. Introduction of Δ157 appeared to have no effect on glucose uptake and lactate release (Figure 17B and C) suggesting that prevention of nucleolar

Figure 17. Nucleolar sequestration maintains energy equilibrium. (A-C) MCF7 cells were cultured under normoxia neutral, hypoxia neutral or hypoxia acidosis conditions. Cells were either left uninfected, infected with GFP or infected with FLAG-Δ157-GFP. Measurements were taken after 20 hours in each condition and endpoint pH was measured. (A) While acidosis preserves ATP levels in hypoxia, the presence of the Δ157 mutant prevents this protective effect. ATP levels were measured and normalized for cell number. Results represent the average of 3 datasets. (B and C) Hypoxia increases glucose uptake and lactate release while acidosis returns these to near normoxia levels. Glucose uptake and lactate release were measured and normalized to cell number. Results represent the average of 3 datasets.


A
Relative ATP Levels
1.2
1
0.8
0.6
0.4
0.2
0
Normoxia Neutral
Hypoxia Neutral
Hypoxia Acidosis
Uninfected
vGFP
vΔ157
B
Relative Glucose Uptake
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
Relative Lactate Release

sequestration reduces cellular ATP levels through resumption of energy expending pathways in the cell.

DISCUSSION

Chapter 4: Discussion

4.1 Summary of Major Findings

Through immunofluorescence and western blotting, we have been able to confirm the H^+-dependent nucleolar localization of $NoDS^{H+}$-containing proteins involved in basal cellular pathways including DNA replication (POLD1), transcription (TAF1), the cell cycle (APC2) and mRNA export (UAP56). Photobleaching analysis was utilized to confirm their static detention within nucleoli. This data has demonstrated the predictive ability of the $NoDS^{H+}$ consensus sequence in indentifying proteins sequestered within the nucleolus in response to extracellular acidosis. Investigation of the functional consequences of nucleolar detention was also examined and demonstrated a substantial decrease in DNA synthesis at a pH correlative to accumulation of POLD1 in the nucleolus. This result suggests that sequestration of POLD1 is involved in the reduced DNA replication observed in acidosis. Study of cell death showed increased cell viability in hypoxia if cells are allowed to acidify their extracellular milieu and suggested acidosis-induced nucleolar sequestration to be responsible for increased cell survival in hypoxia. Examination of the consequences of hypoxia and hypoxia-induced acidosis on energy metabolism associated nucleolar sequestration with the reduction in energy demand observed in hypoxia. Taken together, these results suggest that nucleolar detention of $NoDS^{H+}$-containing proteins is involved in maintaining energy equilibrium through inhibiting major energy consuming pathways within the cell.

4.2 The Nucleolus Regulates Protein Activity through Detention of $NoDS^{H+}$-Containing Proteins

The nucleolus is the largest sub-nuclear domain and has been shown to be involved in a variety of processes including ribosomal biogenesis, cell cycle regulation, modification of small RNAs, control of aging and signal recognition particle assembly (Lyon and Lamond, 2000; Olson, 2004; Olson et al., 2002). Proteins within the nucleolus are highly dynamic and involved in rapid associaton/dissocation reactions with nucleolar components and the nucleoplasm (Phair and Misteli, 2000). The nucleolus has also been implicated in regulating protein function through protein detention. Illustrating this role is the example of the protein Cdc14, whose detention in the nucleolus prevents it from performing its role in the cell cycle (D'Amours and Amon, 2004). Though the nucleolus has a distinct set of resident proteins, in response to different metabolic cues, the nucleolar proteome has been shown to undergo significant changes including loss and gain of various proteins (Andersen et al., 2005).

Through studying the effects of hypoxia/acidosis, our lab has demonstrated that nucleolar sequestration of the protein VHL is an adaptive response of the cell to decreased energy supply (Mekhail et al., 2004b). Bioinformatic analysis of the consensus sequence responsible for its detention, $NoDS^{H+}$ (Mekhail et al., 2007), revealed that 18 proteins, each involved in major cellular pathways, contained this sequence. Through biochemical analysis, we were able to show the nucleolar accumulation of four of those proteins in response to acidosis; POLD1, TAF1, APC2 and UAP56.

DNA replication is a process cells use to transmit genetic information to daughter cells and offspring. To date, 14 different eukaryotic DNA polymerases have been discovered (Burgers et al., 2001; Francesconi et al., 1993; Garg and Burgers, 2005; Pavlov et al., 2006). Three in particular, DNA polymerase α (Polα), DNA polymerase δ

(Polδ), and DNA polymerase ε (Polε), are believed to be directly involved in nuclear DNA replication (Garg and Burgers, 2005; Hubscher et al., 2002). Polα plays a priming role in the process, forming a Polα/RNA primase complex which synthesizes RNA-DNA primers. Polδ and Polε are thought to be involved in elongation of these short primers, where Polδ is associated with lagging strand synthesis and Polε synthesizing the leading strand (Garg and Burgers, 2005; Pursell et al., 2007). The Polδ holoenzyme is believed to consist of various subunits including POLD1 and two to four smaller subunits (Li and Lee, 2001). The importance of the POLD1 subunit is illustrated by the demonstration that it is essential for proper replication and is highly conserved in eukaryotes (Plaster et al., 2006). Through our studies, we have shown POLD1 to be detained within the nucleolus in response to extracellular acidosis. DNA synthesis induces a heavy demand on metabolism and bioenergetics, and therefore it is logical for this process to be sensitive to various environmental factors, including oxygen availability (Zhang et al., 2006). Arrest in DNA synthesis in response to anaerobic metabolism has been well studied over the past 20 years with consistent data observed in the vast majority of cell lines (Hammond et al., 2002; Hammond et al., 2003; Martin, 2007; Probst et al., 1999). This effect has also been shown to be reversible following reoxygenation (Probst and Gekeler, 1980). Our data presents itself as a mechanism for the cessation of DNA synthesis observed in hypoxia. By sequestering POLD1 away from its site of action, it is more than likely that this causes a disruption in Polδ function, resulting in an arrest in DNA replication.

The production of RNA from DNA, termed transcription, is a highly coordinated process regulated by RNA polymerase. Three main eukaryotic RNA polymerases have

been identified: RNA polymerase I (pol I) is involved in transcribing 18S and 28S ribosomal RNA, RNA polymerase II (pol II) mainly transcribes mRNAs, and RNA polymerase III (pol III) is involved in transcription of cellular 5S rRNA and tRNAs (Roeder and Rutter, 1970). Through biochemical study of pol II, it has been shown that this enzyme requires accessory factors, termed general transcription factors (GTFs) for site-specific initiation of transcription (Weil et al., 1979). The GTFs along with pol II form the preinitiation complex (PIC) which aids in positioning pol II over transcription start sites, denaturing the DNA, and positioning DNA in the active site of pol II for transcription. Six GTFs have been identified, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH which have been shown to play different roles in PIC formation and activity (Gerard et al., 1991). TFIID in particular has been shown to be involved in three main activities: core promoter-binding in TATA-containing and TATA-less promoters, mediating interactions between activators and the GTFs to enhance PIC assembly, and post-translationally modifying chromatin in transcriptional control (Thomas and Chiang, 2006). TFIID is itself a multiprotein complex composed of the TATA-box binding protein (TBP) and at least 14 TBP-associated factors (TAFs) (Green, 2000; Tora, 2002). TAFs are not obligatory coactivators and not required for transcription activation, but instead are involved in gene specificity through their promoter selective functions (Thomas and Chiang, 2006). Thereby it has been suggested that TAFs are required for activated transcription while TBP alone mediates basal transcription (Mizzen et al., 1996). TAF1 is the largest subunit of TFIID and has been shown to be responsible for transcription of at least 18% of genes in hamster cells (O'Brien and Tjian, 2000). Our results have demonstrated that in response to extracellular accumulation of H^+, TAF1

accumulates within nucleoli suggesting an inhibition of its function. Transcription is a very intensive process and in hypoxic conditions where energy production is reduced, it is logical for the cell to inhibit unnecessary mRNA production in order to maintain cell viability. This hypothesis is consistent with previous studies of transcription in hypoxia. As mentioned before, a major mediator of transcription in low oxygen tension is the HIF transcription factor, whose activity is associated with the hypoxic response. Therefore in these conditions, it is important for the cell to maintain transcription of specific genes, such as those mediated by HIF, while reducing or eliminating transcription of others which are not immediately essential. Preventing the function of transcription factors involved in activated transcription, such as through nucleolar sequestration of TAF1, would aid in reducing energy demand while also allowing transcription of a subset of genes necessary for adapting to environmental stresses.

The cell cycle has been traditionally divided into the following stages: 1) gap phase 1 (G1), where genes important in DNA replication are activated and necessary proteins are accumulated; 2) S phase, where DNA replication occurs; 3) gap phase 2 (G2) where proteins required for mitosis are accumulated; 4) mitosis (M phase), where chromatin condensation, chromatid separation and cytokinesis occur; and 5) G0, during which cells can exit the cell cycle for differentiation or quiescence. Cell cycle activity is controlled at various stages and transition points (Douglas and Haddad, 2003). Mitosis is divided into four stages: prophase, metaphase, anaphase and telophase. The metaphase-anaphase transition is particularly dependent on regulation by the APC/C complex, the largest multi-subunit E3 ubiquitin ligase (Lindon, 2008). During metaphase, sister chromatids are held together at the equatorial plate through several protein complexes.

The APC/C is responsible for degradation of these cohesion factors in order for proper chromatid separation (De Antoni et al., 2005). The complex also participates in mitotic exit through degradation of specific cyclins and cyclin-dependent kinases (CDKs), which together compose the principle regulatory proteins responsible for passage through the cell cycle (Saha et al., 1998). The APC/C is composed of at least 13 different subunits, one of which is APC2. This protein acts as a cullin-like subunit (Yu et al., 1998b) and together with an additional subunit, APC11, is sufficient to perform ubiquitin ligase activity in vitro (Leverson et al., 2000; Tang et al., 2001). We have shown APC2 to accumulate and become statically detained within the nucleolus in response to hypoxia-induced acidosis. Besides saving on the energy requirements of cell division, inhibition of APC/C, through APC2 sequestration, would prevent separation of sister chromatids in mitosis. This would protect cells from errors associated with abnormal spindle migration, improper chromatin alignment and irregular separation of chromatids in hypoxia (Steen et al., 2008; Uhlmann, 2004). In has been well demonstrated that during anaerobic metabolism, cells undergo arrest in G1 phase (Graeber et al., 1994; Schmaltz et al., 1998) and that this is dependent on HIF expression (Hackenbeck et al., 2009). It has been suggested that HIF increases transcriptional expression of two cyclin-dependent kinase inhibitors as well as regulation of cyclin protein levels (Gardner et al., 2001). In addition to G1 arrest, studies have also shown that hypoxia can arrest cells in metaphase. Through study of fruit flies, it has been demonstrated that in response to hypoxic or anoxic exposure, embryos arrests at two positions in the cell cycle: in metaphase where cells showed aligned, non-segregated chromatids; and just prior to S phase entry. The type of arrest observed was dependent on the cell cycle stage of the embryo exposed to low

oxygen tension. Metaphase arrest was observed if hypoxia exposure occurred slightly before metaphase, while G1 arrest occurred if hypoxia occurred after metaphase. It has been suggested that the prevention of APC/C activity could be involved in this hypoxia-induced metaphase arrest (Douglas and Haddad, 2003; Holloway et al., 1993; Kotani et al., 1999). These studies support a role for APC2 sequestration in hypoxia acidosis, providing a mechanism for inhibiting APC/C activity and subsequently reducing unnecessary energy expenditure as well as errors associated with abnormal chromatid separation.

UAP56 is an ATP-dependent RNA helicase essential for pre-mRNA splicing through facilitating small nuclear ribonucleoprotein (snRNP) attachment to the splicing branchpoint (Fleckner et al., 1997; Peelman et al., 1995). It has also been shown to be critical for export of messenger RNA from the nucleus. mRNA export is more complex than other nuclear trafficking pathways as it is important to ensure transcription, splicing and processing steps have completed before exit from the nucleus (Moore, 2005; Rodriguez et al., 2004). As a result, RNA splicing in the cell is coupled with nuclear export (Luo and Reed, 1999). The essential role of UAP56 in cells is exemplified by the fact that a reduction in its expression results in increased retention of poly(A)$^+$ RNA and partial inhibition of gene expression (Kapadia et al., 2006). Through studying hypoxia, we have been able to show the nucleolar accumulation of UAP56 in response to an extracellular accumulation of H^+. Nucleolar sequestration of UAP56 would aid in conditions of low energy supply through reducing unnecessary mRNA splicing and export, both of which are ATP dependent processes. But since UAP56 has been shown to be crucial for mRNA export (Kapadia et al., 2006), it would be important for an

alternate, and perhaps more selective and energy conservative splicing/export process to be active for ongoing HIF-dependent transcription. Studying the dynamics of UAP56 has demonstrated significantly reduced mobility within acidosis. While this suggests a negative impact on its function, it is surprising that it is not completely sequestered within nucleoli like other $NoDS^{H+}$-containing proteins. There are a few possibilities for this anomaly. The first is that the exogenous UAP56-GFP protein used in these studies is non-functional, even though it demonstrates correct localization to speckled domains within nuclei. Fusion of GFP disrupting functionality is not uncommon and has been shown to interfere with protein folding and interaction (Krautwald et al., 2008; Meyer and Fromherz, 1999). Another possibility is that the decreased mobility observed is enough to reduce its role in splicing and export to cause a significant decrease in energy demand while still maintaining enough activity for hypoxia-induced genes. As previously mentioned, acidosis-induced sequestration of VHL serves the dual functions of inhibiting HIF-α degradation as well as interfering with ribosomal biogenesis within the nucleolus. Along the same logic, it is possible that UAP56 maintains its mRNA splicing and export functions in acidosis, but additionally gains a new function within the nucleolus. While all these are possibilities, further investigation into the role of nucleolar sequestration of UAP56 in acidosis needs to be performed.

Taken together, the nucleolar accumulation of POLD1, TAF1, APC2 and UAP56, clearly demonstrate the important role of the nucleolus in regulating protein activity in response to environmental conditions. In addition to sequestering VHL, we have shown these proteins to accumulate within the nucleolus and that in most cases this results in

static detainment. By preventing proteins from reaching their sites of action, their respective functions are more than likely inhibited.

4.3 Nucleolar Detention Functions to Control DNA synthesis

Arrest in DNA synthesis has been widely reported in anaerobic metabolism as well as cells located within the core of tumors (Martin, 2007; Probst et al., 1999; Probst and Gekeler, 1980; Probst et al., 1988). The mechanism underlying this effect, however, remains unclear. As previously mentioned, POLD1 plays a major role in DNA synthesis, acting as the major catalytic subunit for Polδ, a polymerase involved in primer elongation as well as DNA repair (Garg and Burgers, 2005; Pursell et al., 2007). In order to investigate the implications of sequestration of POLD1 on its function associated with DNA replication, BrdU incorporation experiments were performed. The results from this experiment demonstrated an arrest in DNA synthesis which occurred at a pH value correlative with sequestration of POLD1. This suggests that pH-responsive nucleolar sequestration of POLD1 prevents a key protein in DNA synthesis from locating to its site of action, resulting in inhibition of DNA replication. We have demonstrated that expression of a $NoDS^{H+}$(POLD1) construct is able to prevent nucleolar accumulation of POLD1. Therefore we attempted to rescue BrdU synthesis through expression of this construct at low pH values. Through this method, we were not able to measure DNA synthesis in acidosis due to high levels of cell death. One possibility for this is that $NoDS^{H+}$(POLD1) expression allows an un-sequestered pool of POLD1 to perform its role in DNA replication resulting in increased energy expenditure under conditions of low energy supply causing high levels of cell death.

4.4 Nucleolar Sequestration is Prevented through the use of Dominant Negative Constructs

In order to investigate the mechanism of nucleolar sequestration of $NoDS^{H+}$-containing proteins on regulating energy demand, dominant negative constructs were used to prevent nucleolar accumulation and study the resulting effects. Previous research from our lab has shown that exogenous expression of a C-terminal-mutant of VHL, Δ157, is capable of preventing nucleolar accumulation of endogenous VHL. This truncation mutant contains one of the two STAD domains and six of the seven STHD domains of VHL and is unable to inhibit ribosomal biogenesis upon nucleolar accumulation (Mekhail et al., 2004a). Through western blotting experiments, we were able to demonstrate that Δ157 is able to prevent nucleolar accumulation of additional $NoDS^{H+}$-containing proteins. Since a truncated protein containing the $NoDS^{H+}$ of VHL was able to prevent sequestration, a $NoDS^{H+}$ construct containing the STAD and two of the seven STHD domains of POLD1 was tested to determine if it could act similarly. Surprisingly, it was found that it was only able to prevent nucleolar accumulation of POLD1. One possible reason for this is the expression levels of the two proteins. A virus was used to express Δ157 compared to cells stably expressing $NoDS^{H+}$(POLD1) leading to the possibility that the lower expression level of $NoDS^{H+}$(POLD1) reduced its efficacy as a dominant negative construct. While this remains a possibility, the fact that $NoDS^{H+}$(POLD1) was able to efficiently prevent nucleolar accumulation of at least one protein, POLD1, suggests that the composition of Δ157 led to a more efficient competitor of $NoDS^{H+}$-containing proteins. Besides containing different STAD and STHD domains, compared

to the NoDS^{H+}(POLD1), the Δ157 also contains both the acidic and beta domains of VHL. Further mutational analysis is required in order to determine whether the difference in STAD/STHD domains or the presence of the VHL domains was responsible for the increased efficacy of Δ157 as a dominant negative.

4.5 Acidosis Maintains Cell Viability through Reducing Energy Consumption

Hypoxia occurs in a variety of physiological and pathological events including strenuous exercise, embryonic development, ischemia and the well studied tumor microenvironment (Chen et al., 1999; Donnan et al., 2008; Fang et al., 2008; Li and Foote, 1993; Loria et al., 2008). Decreased oxygen tension causes cells to modify energy metabolism shifting towards anaerobic metabolism. One consequence of this is increased production of lactic acid resulting in accumulation of H^+ in the extracellular milieu. Though in the past, the effect of acidosis has been contested, currently there is a general consensus towards its beneficial effects. A second consequence of hypoxia is an increased glycolytic rate, the Pasteur Effect, to compensate for decreased energy production in anaerobic metabolism. The increase in energy production, though, is almost negligible compared to energy produced through aerobic metabolism. Ultimately, hypoxia reduces energy supply and is thought to be deleterious to cells. Through studying cell viability in anaerobic metabolism, we were able to demonstrate that establishment of acidosis maintains cell viability in hypoxia. This result supports previous studies which have observed similar effects (Bonventre and Cheung, 1985; Gores et al., 1988). In addition, through the use of the dominant negative constructs Δ157 and NoDS^{H+}(POLD1), we were able to clearly show that nucleolar sequestration is

responsible for the increase in cell survival observed during acidic conditions. This suggests that nucleolar sequestration is an essential adaptation and responsible for the protective effect of acidosis in hypoxia.

We next wanted to confirm that the increased cell survival observed in acidosis and through nucleolar detention was due to decreased energy consumption. Similar to previous reports, hypoxia resulted in significant reduction in ATP levels (Liu et al., 2006), however when cells were permitted to acidify, ATP concentrations were preserved. We were additionally able to show that disrupting nucleolar sequestration caused a significant decrease in cellular energy levels suggesting that the maintained energy equilibrium observed in acidosis is due to sequestration of $NoDS^{H+}$-containing proteins.

To verify that the increase in ATP levels observed in acidosis was due to increased energy production (Mekhail et al., 2004a), glucose uptake and lactate release, processes which are increased in hypoxia, were studied. Through these results we were surprised to show a decrease in these processes to near normoxia levels in acidosis. This suggests that compared to hypoxia, energy production in hypoxia acidosis is actually decreased indicating that energy consumption must be greatly reduced in order to maintain ATP concentrations similar to those observed in normoxia. One possibility is that the increased glycolytic rate observed in hypoxia, the Pasteur Effect, is a mechanism used by the cell in order to acidify the extracellular milieu, rather than increase energy production. Once cell type specific thresholds are achieved and $NoDS^{H+}$-containing proteins have accumulated within the nucleolus, there is no longer a need to continue producing lactic acid and consequently the glycolytic rate is reduced. In addition,

decreased lactic acid production would prevent the pH of the extracellular milieu from decreasing too low to become detrimental to the cell.

Taken together, these results suggest cells rely on lactic acid fermentation and subsequent acidosis to preserve cellular energy levels and viability. We have also shown that this protective effect is directly linked to nucleolar sequestration and abrogation of this process leads to increased cell death. This is likely due to a resumption of energy consuming pathways in an environment of low supply, leading to imbalance in energy equilibrium and loss of viability.

4.6 Potential Clinical Applications of Preventing Nucleolar Sequestration

Hypoxia and acidosis play a major role in cancer progression. Early tumor cell proliferation on epithelial surfaces is separated from the underlying blood supply. This results in diffusion-reaction kinetics of substrate and metabolite flow as cells proliferate further from the basement membrane, leading to regional hypoxia and acidosis (Fang et al., 2008). While these environmental conditions are detrimental for most cells, it has been proposed that cancer cells utilize this environment for selection, resulting in the development of a more invasive phenotype of malignant cancers (Trosko et al., 2004). Hypoxic cells can acquire a mutator phenotype consisting of decreased DNA repair, increased mutation rate and increased chromosomal instability (Bristow and Hill, 2008). Acidosis, as we have shown, increases cell survival through maintenance of energy equilibrium by decreasing consumption.

Our research has demonstrated that H^+-dependent nucleolar sequestration involves proteins in various major metabolic pathways of the cell and that regulation of

sequestration is controlled by a specific consensus sequence ($NoDS^{H+}$). In addition, we have also shown that interfering with the nucleolar sequestration process, through the use of a dominant negative construct such as Δ157, increases cellular energy demand and results in high levels of cell death. Therefore, use of this construct would prove a valuable method of inhibiting cellular adaptations occurring in tumors. This is especially so since cells in neutral conditions would not be affected by such a construct. This area of research presents itself as an interesting subject of investigation in the future.

4.7 Future Directions

1-Further Study of the Nucleolar Accumulation and Detention of UAP56

We were able to demonstrate that UAP56 accumulates in the nucleolus in response to an extracellular accumulation of H^+. Upon study of its dynamic character however, we were surprised to discover that it was able to maintain mobility, though reduced, in acidosis. This was unlike other $NoDS^{H+}$-containing proteins which have demonstrated static detainment within the nucleolus in response to acidosis. As mentioned, one possibility for this is that the UAP56-GFP construct used is non-functional. One way to test its activity would be to determine if it is capable of splicing and exporting a reporter plasmid. For this, we will first silence endogenous UAP56, then transiently co-transfect UAP56-GFP with an intron-containing β-globin minigene in 293T cells. After 24 hrs, cytoplasmic and nuclear RNA fractions will be separated and extracted. Quantitative reverse transcriptase PCR will be performed giving the relative concentrations of spliced and unspliced β-globin RNA in each fraction. If it is functional, the β-globin RNA would

be expected to accumulate in the cytoplasmic fraction. Additionally, the nuclear PCR product will be separated on a 1% agarose gel; a smaller product would be expected if the RNA was spliced. In addition to exogenous UAP56, it would also be important to investigate whether endogenous UAP56 is capable of accumulating in the nucleolus through immunofluorescence and western blot analysis. Furthermore, it would be interesting to examine the effects of hypoxia-induced acidosis on mRNA export. To do this, FISH (fluorescent in situ hybridization) analysis will be performed in order to observe the nuclear-cytoplasmic distribution of poly(A)$^+$ RNA. Localization will be determined through the use of a fluorescent oligo(T) tag.

In humans, two proteins correspond to UAP56, the other being URH49 which is 90% identical. Both proteins have similar functions but exhibit relatively different expression profiles in different tissues as well as in response to different growth conditions (Pryor et al., 2004). Each contains the same STAD, but URH49 lacks one of the STHDs found within UAP56. It would be of interest to pursue investigation into URH49; determining whether it too can demonstrate nucleolar accumulation in response to acidosis and whether it displays a similar mobility profile to that of UAP56.

2-Functional Repercussions of Nucleolar Sequestration

While in our study we assessed the effects of acidosis on DNA replication, it would be interesting to investigate the consequences on other cellular pathways. TAF1 is the largest subunit of TFIID and has been shown to be responsible for transcription of at least 18% of genes in hamster cells (O'Brien and Tjian, 2000). Additionally we have shown it to accumulate in the nucleolus in response to an extracellular accumulation of H^+. Therefore, we will investigate the consequences on transcription through performing

microarray analysis, comparing gene expression between hypoxia neutral and hypoxia acidotic conditions. The results from this experiment would help gain insight into how transcription is regulated under acidosis. APC2 is one of at least 13 subunits of the APC/C complex, which has been shown to be involved in metaphase-anaphase transition (Yu et al., 1998b). In order to investigate the effects of nucleolar detention of APC2, the effect of acidosis on the cell cycle will be studied. This will be accomplished through flow cytometry of propidium iodide stained cells in hypoxia neutral and hypoxia acidosis. Another protein which was identified through bioinformatic analysis to contain an $NoDS^{H+}$ sequence was eIF2B1, a subunit of the protein eIF2B. eIF2B is a GTP exchange factor essential for protein synthesis and acts to convert eIF2 from the inactive -GDP to active -GTP state. eIF2 in turn is responsible for the binding of methionyl-tRNA to the ribosome. Therefore it would be interesting to investigate if eIF2B1 accumulates and becomes detained in the nucleolus in response to acidosis, especially since inhibition of eIF2B has been shown to prevent translation initiation altogether (Mohammad-Qureshi et al., 2008). If we can demonstrate nucleolar detention, we will then investigate the effects of acidosis on translation initiation. This will be done through performing a polysome assay. In this method, the rate of overall translation is measured through quantifying the level of polysomes–mRNA molecules attached to several ribosomes. Polysome profiles will be produced through centrifugation of cell lysates on sucrose gradients. Since polysome size is proportional to the rate of initiation and inversely proportional to the rate of elongation, this method would allow us to thoroughly examine the effects of acidosis-induced nucleolar sequestration on translation.

3-Investigation of the Pasteur Effect

As stated, we have demonstrated that glucose uptake and lactate release increases in hypoxia neutral conditions but appears to decrease back to normoxia-like levels in hypoxia acidosis. One possibility for this effect is that once the cells have acidified, the increased glycolytic rate observed in hypoxia (Pasteur Effect) is decreased. In order to test this idea we will incubate cells in normoxia SD, hypoxia SD or hypoxia AP media. Once the AP plate has acidified, the media in all plates will be replaced; normoxia SD and hypoxia SD plates with fresh SD media and the AP media plate with pH6 crash media, which is made to maintain its pH value at 6. We will then perform a time course experiment removing plates every hour and examining their glucose uptake and lactate release. If our theory that acidosis inhibits glycolysis is correct, we should see some glucose uptake/lactate release in normoxia neutral, much more in hypoxia neutral and very little in hypoxia acidosis over the course of the experiment. This experiment will help determine the reason for the differences previously observed between these conditions.

4-Therapeutic Potential of Δ157 in Preventing Tumor Formation

We have shown that Δ157 is capable of preventing accumulation of $NoDS^{H+}$-containing proteins to the nucleolus in response to acidosis. Furthermore, we have demonstrated that this results in increased energy production in conditions when supply is greatly reduced, which results in increased cell death. Therefore it would be interesting to determine if this construct could be used to disrupt nucleolar sequestration to cause tumor cell death. To investigate this possibility, we will carry out nude mouse tumor assays. In this method, one flank of the animal is subcutaneously injected with a regular cancer cell line, while the opposite is injected with the same cells transfected to express Δ157. The

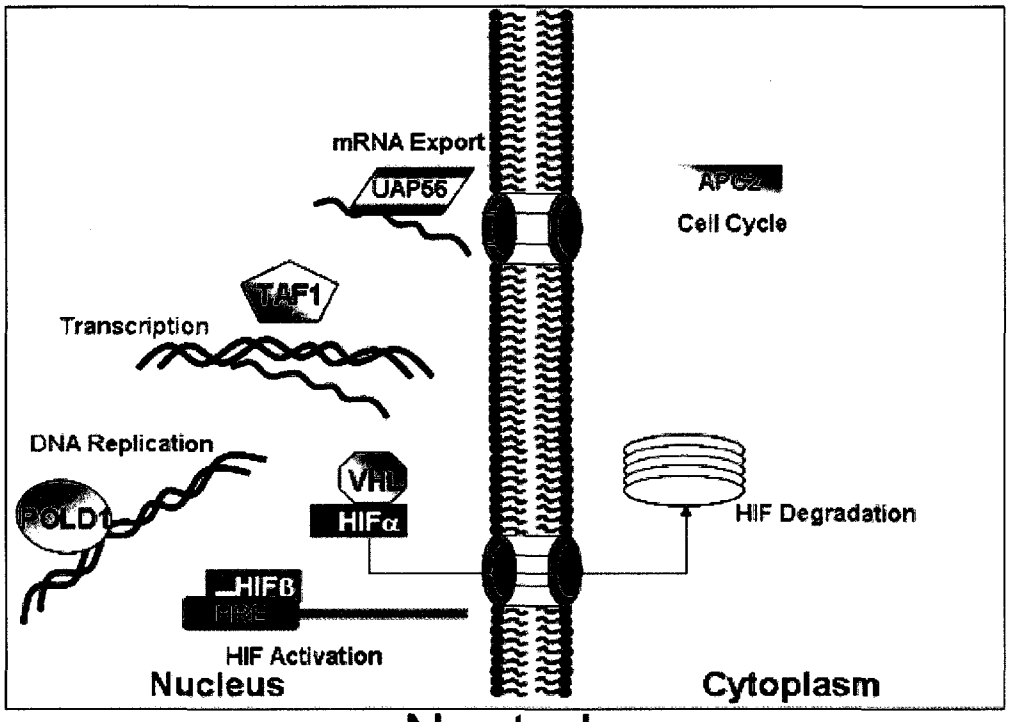
absence of tumor formation would indicate that such a peptide could be a useful therapeutic tool to cause cancer cell death and inhibit tumor formation.

4.8 Conclusions

Anaerobic metabolism occurs under a wide range of physiological and pathological settings. We have shown that regulation of energy demand within the cell is linked to acidosis and specifically nucleolar sequestration. In order to maintain viability in hypoxia, cells maintain energy levels through acidosis dependent nucleolar sequestration of proteins. We propose a model whereby lactic acid fermentation, in addition to regenerating the co-factor NAD^+, is involved in generating extracellular acidosis. This accumulation of H^+ in the cellular milieu causes the nucleolar accumulation of proteins involved in major metabolic pathways and it is the detention of these molecules that separates them from their sites of action. This inhibits their respective pathways resulting in a major reduction in cellular energy consumption. This alteration in energy demand allows the cell to maintain energy equilibrium and subsequently viability under restrictive conditions when energy supply is reduced (Figure 18).

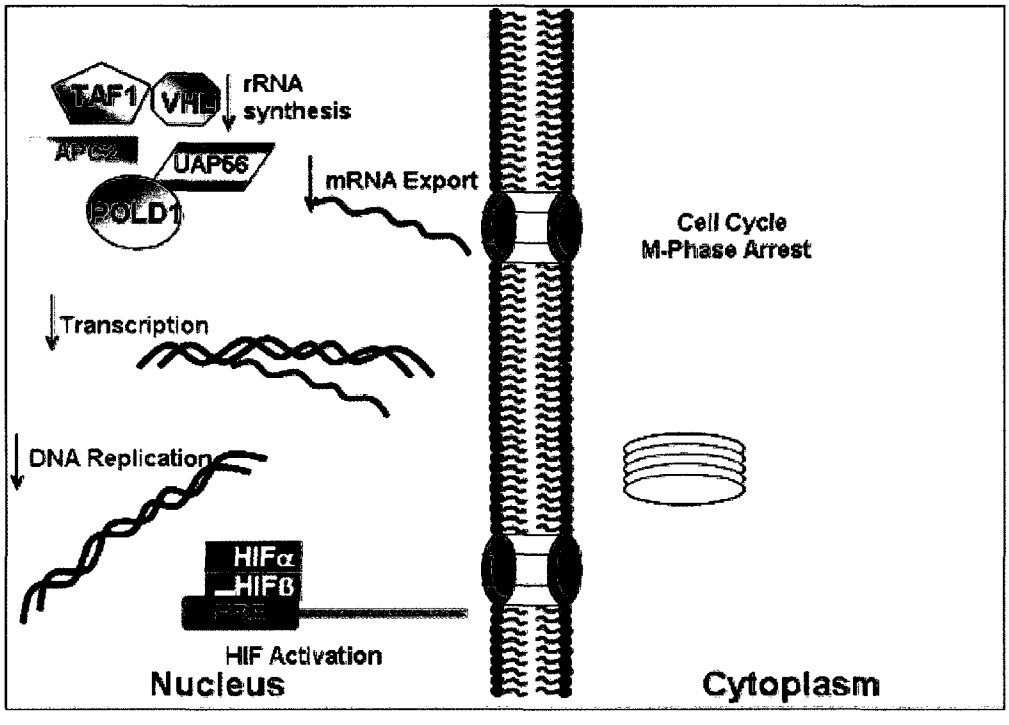
Figure 18. Nucleolar sequestration of NoDS^{H+}-containing proteins is involved in cellular adaptation to anaerobic metabolism. (A) In normal (neutral) conditions, proteins within the cell are highly dynamic enabling them to associate with their various partners to perform their biological functions. (B) In acidosis however, the cell undergoes a complex reorganization of its molecular networks in an attempt to reduce energy demand under conditions when energy supply is drastically reduced. This is accomplished by nucleolar sequestration of proteins involved in major metabolic pathways within the cell through the NoDS^{H+} consensus sequence. This process decreases energy demand and helps maintain the change in energy within the cell (ΔE) at a positive value, ensuring cell viability. ΔE can be simplified to basic equation: energy supply minus energy demand.

A



Neutral

B



Acidosis

$$\Delta E = E_{supply} - E_{demand}$$

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