A novel role for the pro-apoptotic protein Noxa in survival and glucose metabolism

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Dedication

I dedicate this thesis to my parents—Paul Lowman Jr. and Lori (Yellowhorse) Lowman—and my grandparents—Paul Lowman Sr. and Helen Lee Lowman and Woody Yellowhorse and Gloria Semalie Yellowhorse. My grandparents were always my first cheerleaders and they taught my parents as well as they could. Through my parents example of hardwork and great faith, I was able to keep moving forward at a steady pace.

Abstract

The studies described in this dissertation focus on a novel mechanism of post-translational control as well as a novel pro-survival role for a canonical tumor-suppressor BH3-only protein, Noxa, in human hematopoietic cells and cancers. An investigation into regulatory mechanisms underlying the constitutive expression of this pro-apoptotic Bcl-2 family member in leukemias, led to the identification of a phosphorylated serine on the protein. The phospho-modification suppressed its pro-apoptotic function and was regulated by glucose levels. Glucose deprivation promoted Noxa dephosphorylation and activated its apoptotic function. The atypical cyclin dependent kinase, Cdk5, was identified as the glucose-sensitive Noxa kinase. Further investigation pointed to a role for phospho-modified Noxa in glucose metabolism; cells over-expressing Noxa increased their uptake of glucose and promoted its utilization in anabolic metabolic pathways that are favored in proliferating cells. Finally, studies showed Noxa and its anti-apoptotic binding partner Mcl-1 associated within cytosolic multi-protein complexes in proliferating T-leukemia and activated primary T-cells. Early characterization and identification of the components point to a novel glucose-responsive signaling role for these complexes. Taken together, these studies offer fresh insights into cancer cell metabolism and the regulation of Bcl-2 proteins and are expected to contribute to novel therapeutic strategies aimed at activating Noxa's pro-apoptotic function in leukemias.

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Chapter 1
Introduction

Programmed cell death (PCD) is a fundamental cellular process required for tissue homeostasis and development. Aberrant regulation of PCD can accelerate or impede cell death and is at the basis of many pathologies, including neurodegenerative diseases and cancer. Apoptotic PCD is an orderly form of cellular suicide that is dependent upon the activation of proteases known as caspases. Apoptosis can follow 'extrinsic' or 'intrinsic' routes depending on the trigger (Debatin, 2004). The extrinsic pathway is initiated through the stimulation of transmembrane death receptors, such as the Fas receptors, on the cell membrane. In contrast, the intrinsic pathway is initiated through the release of signaling factors by mitochondria within the cell. Stress stimuli, such as starvation, hypoxia, UV or gamma radiation, and bacterial infection, initiate the signaling cascade that disrupts the mitochondrial membrane potential, causing mitochondrial outer membrane permeability (MOMP) and leading to the release of toxic molecules from the inter membrane space into the cytoplasm. This release triggers a cascade of events leading to caspase activation that culminate in the internal deconstruction of the cell (Favaloro et al., 2012). Here we focus on the intrinsic pathway and the proteins that regulate it.

Bcl-2 family of apoptosis regulator proteins

The 'mitochondrial pathway of apoptosis' is overseen by a family of B-cell lymphoma 2 (Bcl-2) proteins that function as sentinels to either prevent (anti-apoptotic proteins) or to promote (pro-apoptotic proteins) the release of cytotoxic contents from the mitochondria (Adams and Cory, 2001). In keeping with their role as cell death regulators, the proper expression and control of Bcl-2 proteins is important during embryonic development and for tissue homeostasis in adults. The deregulation of these proteins can promote tumor formation, resistance to anti-cancer therapy, and autoimmune, infectious or neurodegenerative disorders (Garcia-Saez, 2012). Bcl-2 proteins harbor one or more Bcl-2 homology (BH) domains. Anti-apoptotic multi-domain proteins, such as Bcl-2, Bcl-x_L and Mcl-1_L, protect mitochondrial integrity and promote cell survival (Chipuk et al., 2010). They often function by interacting with pro-apoptotic multi-domain proteins Bax and Bak, keeping them from promoting MOMP (Chipuk et al., 2010). In addition to their roles as gatekeepers of MOMP, novel functions are being attributed to Bcl-2 proteins in regulation of mitochondrial morphology and in metabolism (Martinou and Youle, 2011).

BH3-only proteins - their regulation and function

BH3-only proteins represent a major subset of the Bcl-2 family (Huang and Strasser, 2000; Kelekar and Thompson, 1998). These proteins promote cell death by interacting with multi-domain Bcl-2 proteins via their BH3-domain. Many of the BH3-only proteins are expressed only in response to a stress stimulus. Those proteins that are constitutively expressed are either present at low levels or kept inactive by transcriptional and translational regulatory mechanisms in a tissue and stimuli-specific manner (Puthalakath and Strasser, 2002; Shamas-Din et al., 2011). BH3-only proteins bind with varying affinity to multi-domain partners (Elkholi et al., 2011) and are unstructured unless bound to another member of the Bcl-2 family (Hinds et al., 2007). The proteins have been further classified based on how they promote cell death (Elkholi et al., 2011; Letai et al., 2002). *Direct activators* bind to anti-apoptotic Bcl-2 proteins to inhibit their function and to multi-domain pro-apoptotic proteins to activate MOMP. *Sensitizers/de-repressors* inhibit anti-apoptotic Bcl-2 proteins, thereby sequestering them away from the pore-forming Bcl-2 proteins, Bax and Bak (Elkholi et al., 2011). BH3-only proteins bear little resemblance to each other outside the BH3 domain, and phylogenetic analyses suggest that many of these proteins may have biological activities beyond regulation of cell death. Indeed, research on these proteins in the past decade has revealed roles for some of these proteins in cellular pathways related to metabolism, inflammation, and autophagy (Chipuk et al., 2012; Happo et al., 2012).

The BH3-only protein: Noxa

Human Noxa (hNoxa) was originally identified in an adult T-cell leukemia library as a PMA-responsive protein (Hijikata et al., 1990). It was only a decade later that it was recognized to have a role in DNA damage and hypoxia (Kim et al., 2004; Oda et al., 2000). It promotes apoptosis by selectively binding to the anti-apoptotic protein, Mcl-1, and facilitating its degradation (Czabotar et al., 2007; Ploner et al., 2008). Significant structural and regulatory differences exist between the murine and human proteins. Murine Noxa is a 103 aa protein, harbors two BH3 domains and appears to be a product of gene duplication, whereas hNoxa, the smallest known BH3-only protein, comprises 54 residues and a single BH3 domain (see Figure 1-1) (Ploner et al., 2008). *NOXA* knock out mice are viable and do not exhibit a distinctive phenotype (Shibue et al., 2003; Villunger et al., 2003). Murine Noxa is not constitutively expressed but is activated by

transcriptional induction, and the human protein is also transcriptionally silent in most epithelial cells in the absence of a stress stimulus. However, hNoxa was observed to be stably expressed alongside Mcl-1 in proliferating lymphoid and myeloid leukemia cells (Alves et al., 2006). These observations, in light of the differences between the murine and human protein structures, made it difficult to discern Noxa's specific function and relevance in human biology (Ploner et al., 2009), and underscored the need to study the regulation of the human protein in hematological malignancies.

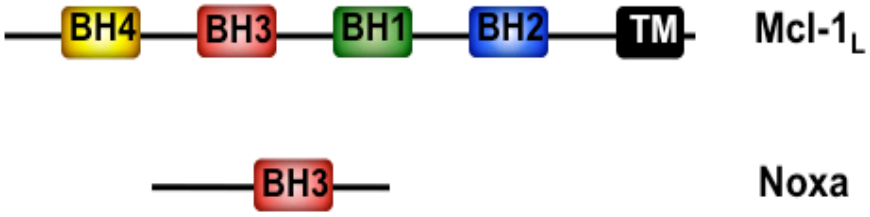


Figure 1-1. BH domains of Mcl-1L and Noxa. Mcl-1 is a multi-domain Bcl-2 protein with many isoforms. BH domains 1, 2, and 4 create an amphipathic pocket by which the BH3-only protein Noxa can interact with Mcl-1_L, the ~37 kDa long isoform via its BH3-domain.

Mcl-1 is an essential and unique Bcl-2 protein

Mcl-1 (myeloid cell leukemia-1) is a unique anti-apoptotic Bcl-2 protein. It was identified in differentiating myeloid cells, ML-1 (Kozopas et al., 1993) and has since been shown to play an essential role in the survival of many hematopoietic lineages (Opferman et al., 2005; Opferman et al., 2003; Perciavalle et al., 2012). Structurally, Mcl-1 is different from other anti-apoptotic Bcl-2 proteins due to its length and carboxy-terminal core that confers selective affinity for promiscuous binding to multiple BH3-only proteins (Day et al., 2005; Day et al., 2008). Mcl-1 is regulated by a myriad of mechanisms that are both transcriptional and translational; its expression can be stimulated by growth factors cytokines and glucose (Maurer et al., 2006; Zhao et al., 2007), and regulated by microRNAs (Mott et al., 2007; Steele et al., 2010; Xiong et al., 2010) and post-translation modifications (Quinn et al., 2011). Mcl-1 is distinct from other Bcl-2 proteins in having a short half-life (Nijhawan et al., 2003) that is regulated by a variety of mechanisms. Its degradation is found to be both ubiquitin dependent and independent (Stewart et al., 2010; Zhong et al., 2005). Furthermore, Mcl-1 may be modified by the addition of a negative charge (phosphorylation), which influences its stability in both negative and positive ways (Quinn et al., 2011).

Mcl-1[1] is required for the maintenance of immature B and T lymphocytes and for the survival of hematopoietic stem cells and myeloid cells. BH3-only proteins Bim, Puma and Noxa interact with Mcl-1_L but it is the Noxa/Mcl-1_L interaction, in particular, that is believed to play a prominent role in the survival and death of lymphoid and other cells of hematopoietic lineage. A variety of human cancer cell lines express elevated levels of Mcl-1 (Placzek et al., 2010), including the majority of leukemias and lymphomas (Beroukhim et al., 2010; Warr and Shore, 2008). Increased expression of Mcl-1_L has been observed in human B-CLL cells and in bone marrow cells from patients with AML and ALL. Mcl-1_L is also an important survival factor for multiple myeloma. Surprisingly, many of these hematopoietic cancers also persist with high, constitutive levels of Noxa (Alves et al., 2006; Hallaert et al., 2007). Thus, post-translational modifications could play important roles in regulating the pro- and anti-apoptotic functions of Noxa and Mcl-1 as well as their stability in response to various signaling cues and are areas currently under investigation.

Cdk5 - Post-translational regulation by an atypical cyclin dependent kinase

Kinases represent a large family of proteins that significantly change the behavior of a protein by phosphorylation (Knight et al., 2012) at serine, threonine, and tyrosine residues. More than 500 kinases are reported in the human kinome and approximately 40% are associated with disease (Knight et al., 2012; Manning et al., 2002). Cyclin dependent kinases (Cdk) when coupled with a cyclin drive a cell through the cycles of growth and division. Cdk5 a kinase of interest for the research presented here is a proline-directed serine/threonine kinase highly homologous to members of the Cdk family. However, it is referred to as an atypical cyclin dependent kinase being neither activated by cyclins nor playing a cell cycle role (Sandal et al., 2002). It has a diverse array of substrates, including pRb, ErbB, src, β-catenin, STAT3, and its own binding partners and activators, p35 and p39 (Dhavan and Tsai, 2001). Until recently, Cdk5 activity was primarily associated with neuronal development and survival (Dhavan and Tsai, 2001; Liebl et al., 2011) and has been studied extensively in murine models of

[1] Mcl-1 undergoes alternative splicing to yield different isoforms that often vary in their subcellular localization, function, and levels of expression. Mcl-1 has three alternative splicing variants of the human MCL-1 gene denoted as, MCL-1_L and MCL-1_S (Craig, 2002). Mcl-1_{ES} has also been identified recently (Kim et al., 2009). Mcl-1_L is the binding partner to Noxa. We observe two Mcl-1_L bands, which we label as Mcl-1La and Mcl-1Lb.

Alzheimer's and Parkinson's disease. In recent years, however, numerous Cdk5 targets of non-neuronal origin have been identified, and mounting evidence is attributing regulatory functions in differentiation, senescence, and apoptosis to this kinase. Furthermore, Cdk5 has been detected in "non-neuronal" cells of hematopoietic origin, adipocytes, and pancreatic β-cells (Rosales and Lee, 2006). Of particular relevance for the current research is the contribution of active Cdk5/p35 complexes to glucose-dependent regulation in hematopoietic cells.

Glucose cell metabolism in cancers and proliferating normal cells

Fundamental to all cells is an energetic requirement to grow, to generate biomass and to maintain homeostasis. Energy is acquired through the metabolism of sugars and proteins, the six-carbon sugar glucose being the main metabolic source. Under aerobic conditions, normal differentiated cells will take up glucose and metabolize it through the glycolytic pathway to produce pyruvate, which can be further used in oxidative phosphorylation to generate high amounts of energy in the form of ATP (Lunt and Vander Heiden, 2011; Vander Heiden et al., 2009). In oxygen-limiting conditions, glucose is broken down to pyruvate and then fermented to lactate in an energetically inefficient manner known as 'anaerobic glycolysis'. Otto Warburg was the first to note that cancer cells rapidly consume high amounts of glucose and secrete high levels of lactate (Warburg, 1956), and he postulated that it was due to a defect in mitochondrial respiration. This was an erroneous assumption and many groups have since shown that mitochondrial respiration is not compromised in cancer cells (Fantin et al., 2006; Zu and Guppy, 2004). Interestingly, normal actively proliferating cells, like lymphocytes and certain microbes, use 'aerobic glycolysis' to meet the biosynthetic demands of cell growth (Lunt and Vander Heiden, 2011). Renewed interest in metabolism, especially as it pertains to cancer cells, seeks to address how proliferating cells benefit from 'reprogrammed' metabolism.

Warburg revisited

Proliferating cells (much like cancer cells) differ from resting cells in three major areas- they must generate adequate energy to support cell division, satisfy the demands of biosynthesis for macromolecules, and maintain the cellular redox balance (Cantor and Sabatini, 2012). Glycolysis is not a one-dimensional pathway; rather the conversion of

glucose to pyruvate is composed of carbon intermediates that fuel protein, lipid, and nucleotide synthesis. Figure 1-2 shows the significant biosynthetic pathways that are maintained by the metabolism of glucose via glycolysis. Early in this process, glucose-6-phosphate can enter the pentose phosphate pathway (PPP) to generate NADPH and ribose-5-phosphate (R5P). NADPH defends the cells against oxidative stress, which is increased in cancer cells (Jeon et al., 2012) and R5P is key to nucleotide biosynthesis. Fructose-6-phosphate and glyceraldehyde-3-phosphate may also generate R5P by entering the PPP downstream. Glycerol-3-phosphate provides substrates for lipid synthesis. 3-phosphoglycerate may be diverted to serine biosynthesis pathways for the generation of nonessential amino acids and lipid synthesis (Lunt and Vander Heiden, 2011).

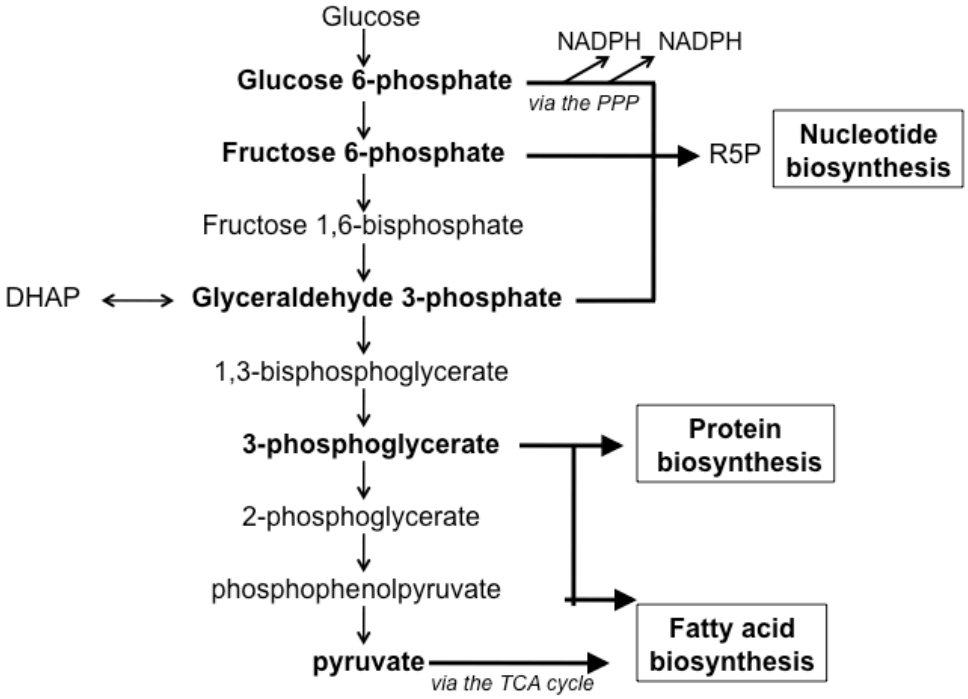


Figure 1-2. Glycolytic intermediates sustain macromolecular biosynthesis. Glycolysis is a series of reactions that converts the six-carbon sugar, glucose, into two molecules of the three-carbon metabolite, pyruvate. Intermediates within the pathway may contribute to nucleotide synthesis, such as glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde 3-phosphate. 3-phosphoglycerate has been shown to be important for serine synthesis (protein), which serves as a backbone for other proteins. Pyruvate may enter the tricarboxylic acid (TCA) cycle to generate cytosolic citrate that will be used for fatty acid synthesis. *DHAP, dihydroxyacetone phosphate; PPP, pentose phosphate pathway; R5P, ribose-5-phosphate.*

Although, cancer cells secrete excessive amounts of lactate the upstream intermediate, pyruvate, may still enter the TCA cycle and contribute to fatty acid synthesis (Jones and Thompson, 2009).

Indeed, as already mentioned mitochondrial respiration proceeds as usual in cancer and proliferating cells and retains its capacity to generate ATP (DeBerardinis and Cheng, 2010; Ward and Thompson, 2012). A major reason for this may be the utilization by cancer cells of the nonessential amino acid, glutamine. Cancer cells are addicted not only to glucose, but also to glutamine (Medina, 2001).

Proliferating cells depend upon glutamine as a nitrogen source for the synthesis of nucleotides and proteins (DeBerardinis and Cheng, 2010). Glutamine may also replenish the carbon intermediates of the TCA cycle, thereby contributing to fatty acid synthesis. Moreover, production of reductive NADPH molecules increases with glutamine metabolism. The altered metabolism that cancer cells adopt in order to survive and continue proliferating indefinitely is currently the subject of intense exploration. Questions remain regarding the significance of elevated lactate levels, the diversion of metabolic flux towards NADPH generation and oxidative stress control, and, most importantly, the triggers that cause the cells to switch metabolic pathways (Vander Heiden et al., 2009).

An investigation of regulatory mechanisms underlying the expression of the Bcl-2 protein, Noxa, in human leukemias and proliferating lymphocytes has led to an intriguing role for this protein in metabolic reprogramming that is dependent upon its post-translational modification in a glucose-dependent manner by the kinase, Cdk5. The investigation is described in this dissertation.

Chapter 2

Noxa is post-translationally regulated by the kinase Cdk5

Introduction

Noxa, a BH3-only member of the Bcl-2 family of apoptosis regulator proteins, is a canonical tumor suppressor that promotes cell death in response to stimuli, such as DNA damage and hypoxia. Noxa functions as a death promoter in part by interacting with its pro-survival binding partner, Mcl-1 and facilitating its degradation (Czabotar et al., 2007). Both human and mouse Noxa proteins are typically activated by transcriptional mechanisms in a cell type-and stimulus-dependent manner. However, as described in Chapter 1, there are significant structural differences between the murine and human proteins. Human Noxa has 54 residues and one BH3 domain; mouse Noxa comprises 103 residues and has two BH3 domains. In most human epithelial cells Noxa is transcriptionally induced by *p53, E2F, (HIF)-1*α, *c-myc*, and *ATF3* in response to apoptotic stimuli (Ploner et al., 2008). Two studies had suggested that Noxa was required for glucose deprivation-induced apoptosis of proliferating immune cells and observed that steady-state levels of the protein were detected in proliferating human leukemia cells (Alves et al., 2006; Hallaert et al., 2007).

The expression of Mcl-1, Noxa's canonical binding partner, is elevated in a majority of human leukemias and lymphomas (Warr and Shore, 2008) and many of these cancers appeared to express Noxa at high levels. Given these paradoxical observations, we hypothesized that Noxa protein expression in proliferating primary and malignant human hematopoietic cells was likely post-translationally regulated.

Results

Human Noxa is constitutively expressed in human leukemia cells

First, we determined the steady-state expression levels of the Noxa protein in human cancer cell lines of epithelial and hematopoietic origin. A western blot of a selection of established human tumor cell lines is shown in Figure 2-1A. The majority of tumors that express high levels of Noxa are derived from hematopoietic lineages. We then obtained fresh lymphoid and myeloid cancer samples from T-ALL and AML patients. Western blots showed detectable steady levels of Noxa in the patient samples (Figures 2-1B and 2-1C).

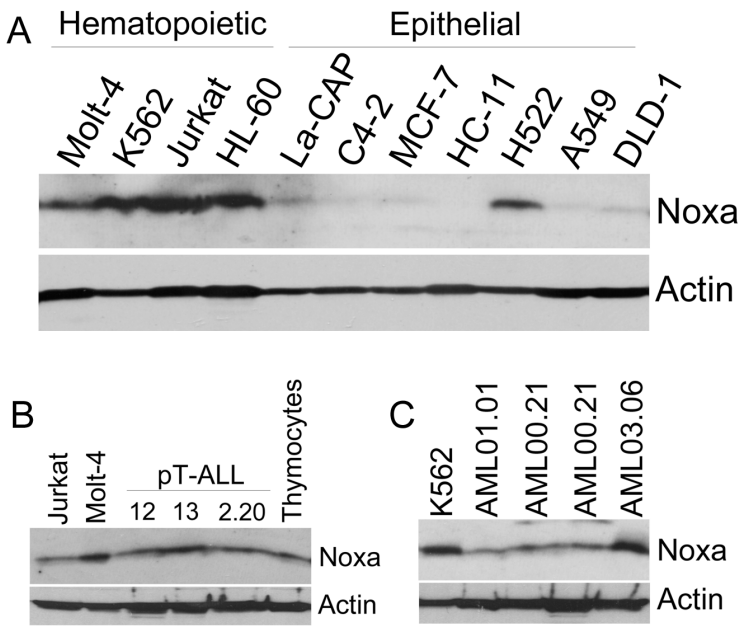


Figure 2-1. Noxa is constitutively expressed in hematopoietic cancers. Western blots of Noxa and Actin expression from the indicated cell lines. (A) Noxa is constitutively expressed in selected human leukemia cell lines. From left to right established lines derived from human tumors: Molt-4 (T-ALL), K562 (CML), Jurkat (T-ALL), HL-60 (AML), LN-CAP, C4-2 (prostate cancer), MCF-7 (breast cancer), H522 (small cell lung cancer), A549 (alveolar basal epithelial carcinoma), and DLD1 (colon cancer). (B) Noxa is constitutively expressed in patient-derived lymphoid cells and proliferating cells (thymocytes). From left to right, T-ALL patient derived samples: two established lymphoid cell lines (Jurkat and Molt-4), three patient (pT-ALL) cell lines and fresh thymocytes from isolated normal human thymus. (C) Noxa is constitutively expressed in patient-derived myeloid cells. From left to right, AML patient derived samples: an established CML line (K562) and four patient-derived AML cell lines.

Human Noxa is phosphorylated on a serine residue

BH3-only proteins are death promoters and, as a rule, members of this group that are constitutively expressed in cells are often post-translationally modified to prevent them from exerting their pro-apoptotic function (Danial et al., 2008; Datta et al., 2000). We hypothesized that Noxa was likely post-translationally modified in leukemia cells to prevent it from functioning as a cell death promoter. We scanned the 54 amino acid sequence of Noxa for potential regulatory sites (Figure 2-2). The CBS Prediction Server NetPhos as well as Predikin (http://www.cbs.dtu.dk/services/) identified serine 13 (S^{13}) embedded within the QPSPARA sequence as a highly probable target for at least three kinases—Cdk5, GSK-3α/β, and p38MAPK—with Cdk5 identified as the kinase most likely to phosphorylate this residue. The remaining two serines and single threonine appeared to be poor kinase targets. Noxa does not contain a tyrosine.

BH3

MPGKKARKNA**QPSPARA**PAELEVECATQ**LRRFGDKL**NFRQKLLNLISKLFCSGT

Figure 2-2. The 54 amino acid sequence of human Noxa. Noxa protein sequence with the BH3 domain and putative kinase substrate motif (QPSPARA) underlined. Human Noxa retains a putative phosphorylation site at S13. We reasoned that Noxa could be post-translationally regulated by phosphorylation. We scanned the Noxa sequence with the NetPhos and Predikin software. These programs identified the QPSPARA consensus sequence as a probable phosphorylation site. QPSPARA is a strong consensus motif for the cyclin dependent kinase Cdk5 and a weaker target for GSK-3α/β and p38MAP kinases.

In order to investigate the putative post-translational regulation of Noxa, we first determined whether Noxa could be phosphorylated. To do this we carried out *in vitro* kinase assays using recombinant (r) Noxa tagged with a FLAG epitope as substrate and extracts from a variety of leukemic cell lines as kinase sources. All cellular extracts tested labeled rNoxa-FLAG in the assay (Figure 2-3A). Having demonstrated that Noxa was indeed a kinase target, we then wanted to identify the phosphorylated residue. We mutated S13 on Noxa-FLAG to either Alanine (A) or Glutamic acid (E), to generate Noxa SA- and SE-FLAG. The SA mutant would serve as a nonphosphorylatable protein and SE would serve to mimic a constitutively phosphorylated serine. We used the cellular extract from the promyelocytic leukemia HL-60 cell line in an *in vitro* kinase assay with the wild-type (WT) Noxa-FLAG and Noxa SA-FLAG mutant. Noxa SA-FLAG remained unlabeled (Figure 2-3B), suggesting S13 was the likely phosphorylated residue. We extended the scope of our experiments by generating an additional point mutation distant from the S13 site to one within the BH3-domain (D34). In addition, we switched to experiments in cellular systems. For this purpose, we transiently transfected human embryonic kidney cells, HEK293, with FLAG-tagged WT Noxa and DA (D34), SA, and SE (S13) constructs. The cells were incubated with [^{32}P]-orthophosphate. The transfected proteins were expressed at comparable levels, but only WT Noxa-FLAG and the Noxa DA mutant were labeled (Figure 2-3C). These data strongly suggested the S13 site was the phosphorylating target. To continue with these studies and to ascertain the physiological relevance of the phosphorylation we proceeded to make a phosphospecific antibody against this motif in Noxa.

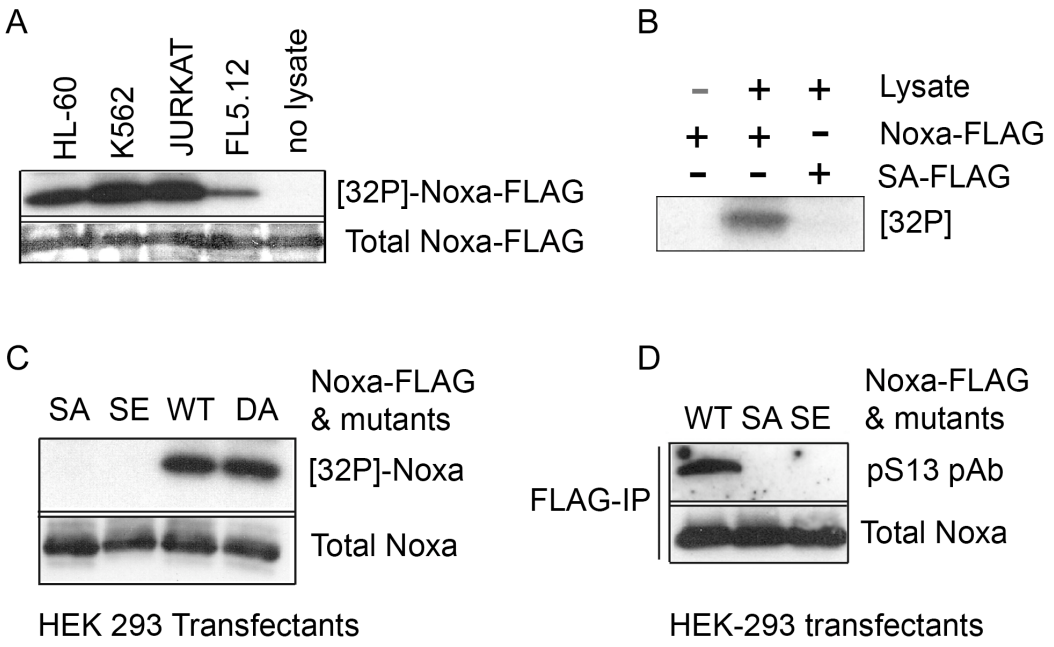


Figure 2-3. Noxa is phosphorylated at Serine 13. (A) Recombinant (r) FLAG-tagged Noxa is phosphorylated by endogenous kinases. Cell lysates from HL-60, K562, Jurkat, and murine FL5.12 cells were incubated with Noxa-FLAG, kinase buffer, and [γ-^{32}P]-ATP per reaction. Top panel is an autoradiograph of labeled Noxa-FLAG immunoprecipitated with anti-FLAG mAb. Lower panel is a Western blot showing total Noxa levels. (B) HL-60 cell lysates were incubated with rNoxa-FLAG or SA-FLAG in kinase buffer and [γ-^{32}P]-ATP. The reactions were processed and resolved by SDS-PAGE; the gel was dried and autoradiographed. (C) Noxa is phosphorylated on S13 in transiently transfected HEK293 cells. Cells transfected with Noxa-FLAG plasmids and point mutants were labeled with [γ^{32}-P]-orthophosphate for 36 h post-transfection. FLAG-tagged proteins were immunoprecipitated from cell lysates; autoradiograph of labeled [γ^{32}-P]-Noxa is shown in the top panel, Western blot of total immunoprecipitated Noxa in the lower panel. (D) Phospho-S13-specific Noxa rabbit pAbs (rAK2) detect pS13 Noxa expression in transiently transfected 293 cells. Data show an immunoprecipitation/Western of lysates from 293 cells transfected with WT Noxa-FLAG, or SA/SE mutants immunoblotted with the phospho-specific rAK2 pAb (top), then stripped and reblotted for total Noxa detection.

In order to do this we collaborated with Dr. Ron Jemmerson, an expert in monoclonal and polyclonal antibody production at the University of Minnesota. With his help we were able to generate phosphospecific monoclonal and polyclonal antibodies (mAbs and pAbs) against a synthetic peptide encompassing the S13 motif. The rabbit pAbs (rAK2) were affinity purified against the nonphosphopeptide to eliminate any cross-reactivity. Our tests showed that it was useful for Western blot applications. In Figure 2-3D, we were able to specifically detect exogenously introduced WT Noxa, but not the SA or SE Noxa, in Western blots of extracts from transiently transfected HEK293 cells.

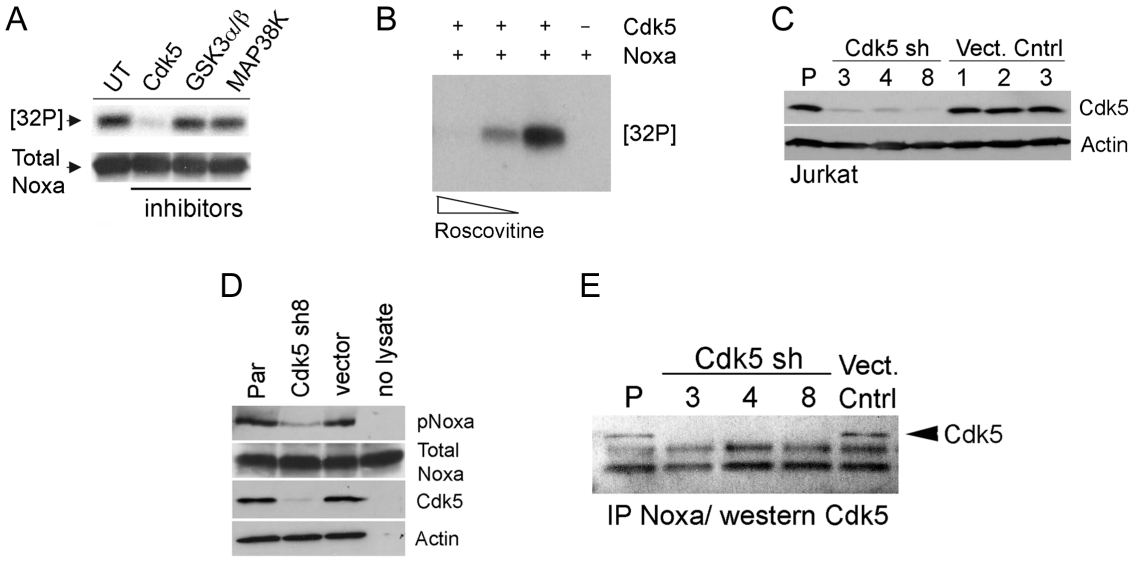


Figure 2-4. Cdk5 is the kinase that phosphorylates Noxa at S13. (A) Cdk5 inhibition decreases *in vitro* phosphorylation of the Noxa peptide by Jurkat cell lysates. Jurkat cells were pretreated with various kinase inhibitors prior to harvesting. Lysates were incubated with the Noxa peptide in kinase buffer and [γ^{32}P]-ATP. Noxa was immunoprecipitated with Noxa pAbs, resolved by SDS-PAGE, transferred to membrane, and autoradiographed (top panel). The blot was then processed for total Noxa detection (lower panel). *From left to right*: Roscovitine (Cdk5 inhibitor), SB216763 (GSK3α/β inhibitor), SB203580 (MAP38K inhibitor). (B) Recombinant Cdk5 phosphorylates synthetic untagged full-length Noxa peptide. Kinase reactions included incubation with roscovitine in the two left lanes at the respective concentrations, 25 and 10 μM. (C) Western blot showing Cdk5 levels in clonal lines of Jurkat cells knocked down for Cdk5 expression. From left to right: Jurkat parental, three Cdk5 shRNA knocked down clones, three vector control clones (D) Cdk5 knockdowns show a reduced ability to phosphorylate Noxa. Jurkat parental, Cdk5 sh8, and vector control lysates were incubated with kinase buffer, synthetic Noxa, and cold ATP. Phosphorylation was detected using the phospho-S13 specific Noxa pAb, rAK2 (top panel). The membrane was later immunoblotted for total Noxa. The lower half panels show Cdk5 and Actin levels in the lysates. (E) Noxa and Cdk5 interact endogenously. Immunoprecipitation/Western showing that endogenous Cdk5 is immunoprecipitated with anti-Noxa IP antibody from lysates of untreated Jurkat parental and vector controls.

Noxa is phosphorylated by the atypical cyclin-dependent kinase, Cdk5

We had conclusively identified serine 13 as the phosphorylated residue in Noxa. Our next goal was to identify the kinase that phosphorylated Noxa at S13. The QPSPARA motif is a strong consensus for Cdk5 target sites, and a weaker target for the serine/threonine kinases GSK-3α/β and MAP38K. We decided to first look at the effect of pharmacologically inhibiting Cdk5 and the other target kinases. To remove any potential interference of the FLAG-tag on *in vitro* assays we used a *de novo* synthesized full length Noxa peptide for these studies. We had concerns that the small size of Noxa could increase the likelihood for the FLAG tag to perturb the protein's secondary

structure and compromise its ability to serve as a kinase substrate. Peptide synthesis, purification and characterization were carried out by Dr. Christine Karim in the BMBB Department at the University of Minnesota. Next we determined whether the lysates from Jurkat cells, pretreated with specific inhibitors to Cdk5, GSK-3 α/β, and MAP38K, could phosphorylate the synthetic Noxa peptide in a kinase assay (Figure 2-4A). Radioactive [^{32}P] labeling of Noxa was markedly reduced in extracts from cells exposed to the Cdk5 inhibitor. We then confirmed that phosphorylation of the full-length peptide was sensitive to concentrations of roscovitine inhibitory to Cdk5 (Figure 2-4B). These results strongly suggested that Cdk5 was the Noxa-modifying kinase. We continued to validate the *in vitro* results by generating stable clonal populations of Jurkat cells knocked down for Cdk5 (Figure 2-4C). Extracts from knock-downs demonstrated a reduced ability to phosphorylate Noxa compared to parental and vector control extracts (Figure 2-4D), further establishing Cdk5 as the Noxa kinase. Notably, in this experiment, pS13 Noxa was detected using the phosphospecific pAb, rAK2 in a cold *in vitro* kinase assay. It has been shown that active Cdk5 stably associates with its target (Luo et al., 2005). Using a commercially available Noxa antibody we were able to pull-down endogenous Cdk5 from Jurkat cells (Figure 2-4E) and to verify that Cdk5 did indeed interact with Noxa. Thus, using a variety of approaches we were able to identify Cdk5, a proline-directed serine/threonine kinase, as the enzyme that phosphorylated human Noxa. A previous study had shown that r-Roscovitine promoted apoptosis of human lymphoid cancer cells and that cell death was preceded by Mcl-1_L degradation, in a Noxa-dependent manner (Hallaert et al., 2007). Since roscovitine is an effective inhibitor of Cdk5, the dependence on Noxa for its cell death effect had indirectly suggested a role for Cdk5 in regulating Noxa. Our studies had now identified a direct role for this atypical kinase in phosphorylating Noxa.

Noxa accelerates glucose deprivation-Induced death in leukemia cell lines

Noxa had been shown to interact with the prosurvival protein Mcl-1_L in response to stimuli such as DNA damage and hypoxia and to promote cell death by facilitating the degradation of Mcl-1 (Czabotar et al., 2007). We were interested in determining how phospho-modification influenced Noxa's cell death function. Prior to this, we wanted to identify the cell death stimuli that were dependent on Noxa to promote apoptosis in leukemia cell lines. In order to identify relevant death pathways that might be regulated

by Noxa, we generated stable Jurkat clones (Figure 2-5A) that either overexpressed Noxa or were knocked down for expression, and determined their viability in response to various death stimuli. Camptothecin (CPT) and etoposide (Etop), two DNA damage agents known to induce Noxa's death function, were selected to treat the cells. In epithelial cells, Noxa is induced in a p53-dependent manner following exposure to CPT and is required for the DNA damage response. In the p53-null Jurkat cells, Noxa levels did not significantly influence the rate of death in response to CPT or Etop (Figures 2-5B and 5C). However, expression of this Bcl-2 protein affected the death response to glucose-deprivation (Figure 2-5D); Noxa-overexpressing Jurkat clones accelerated the death response to 36 h of glucose deprivation, while loss of Noxa imparted partial protection. The withdrawal of glucose also promoted cell death in the chronic myelogenous leukemia (CML) cell line, K562, albeit at a slower rate. Similarly death was delayed in Noxa shRNA knockdowns of K562 cells (data not shown, supplemental data in (Lowman et al., 2010)).

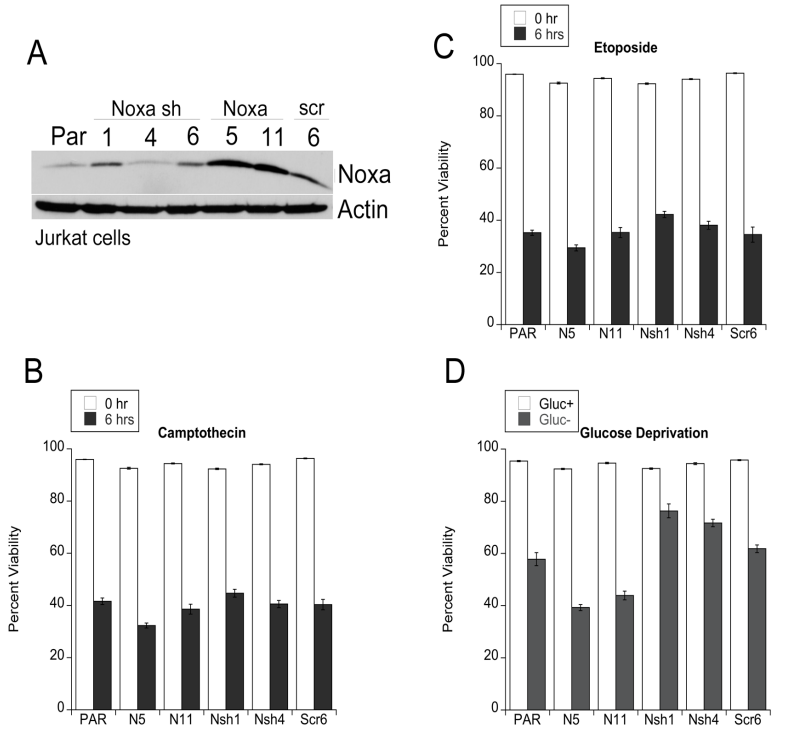


Figure 2-5. Noxa is required for the glucose-deprived induced death response in hematopoietic cells. (A) Western blot of cell lysates from transfected, cloned Jurkat cell lines overexpressing Noxa (Noxa 5 and 11) or knocked down for Noxa with shRNA (Noxa sh 1, 4, 6) and scrambled control (Scr6). Selected cell lines, including parentals (PAR), were treated with camptothecin (B), etoposide (C), or grown in RPMI with 25 mM glucose or no glucose for 36 h (D).

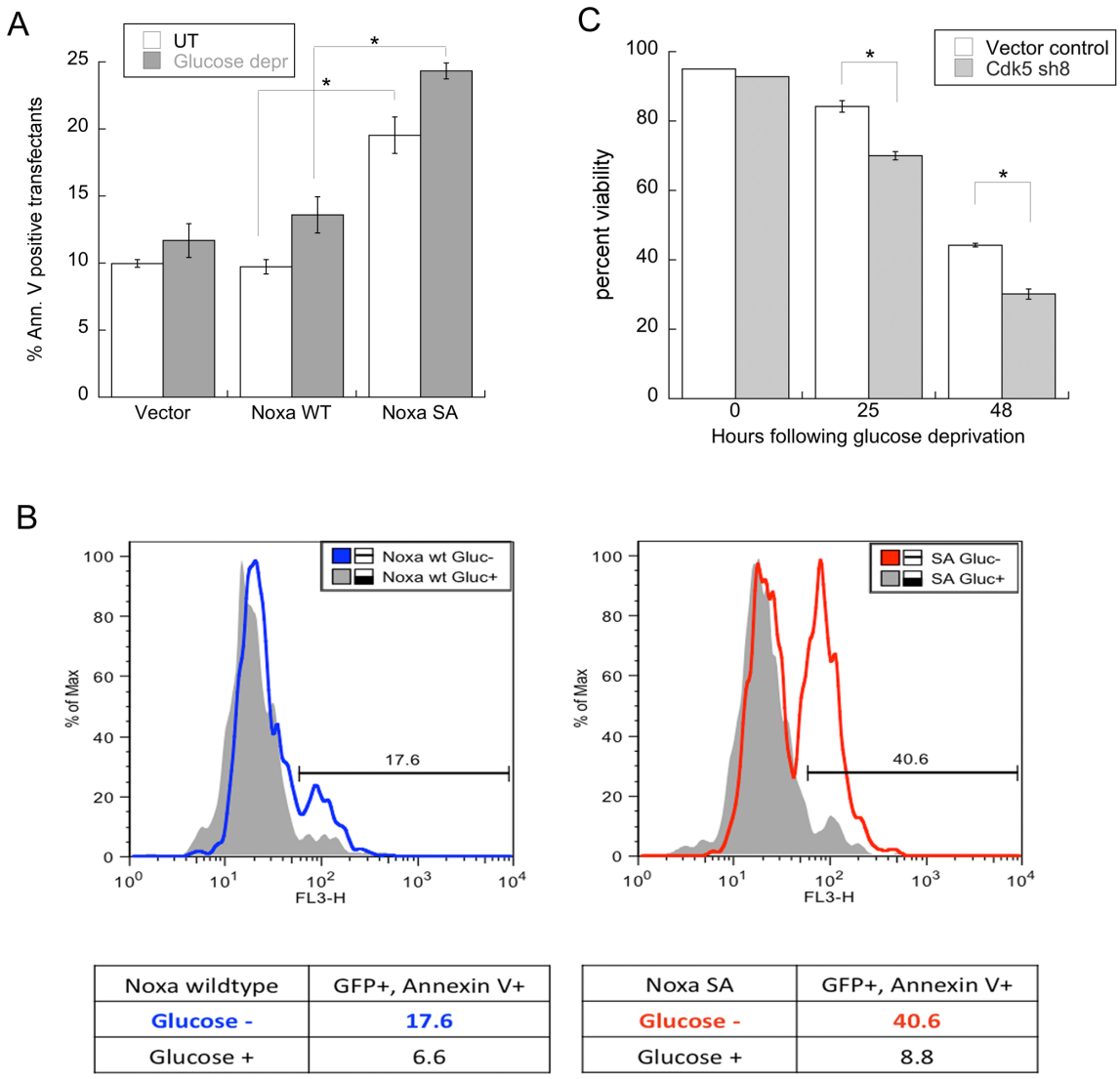
Phosphorylation of Noxa by Cdk5 controls its apoptotic function

Noxa was required for the apoptotic response to glucose loss. Did the phosphorylation of Noxa influence its role in the glucose-stress response? We reasoned that if this were the case, a non-phosphorylatable mutant (e.g. S13A) should promote apoptosis more potently than the WT Noxa that retained its serine. Indeed, Jurkat cells transiently transfected with IRES-EGFP vectors expressing SA-Noxa showed increased cell death compared to WT-Noxa expressers even in the absence of a death stimulus (Figure 2-6A, compare UT bars). Moreover, Jurkat and Molt-4 cells subjected to transient transfection with the SA-Noxa mutant showed elevated cell death rates following glucose deprivation compared to cells expressing WT Noxa or the empty vector (Figure 2-6A, 2-6B). These data along with our failure, despite many attempts, to generate stable Jurkat clones expressing SA-Noxa suggested that the phosphorylated S13 was protective. The lethality of the transiently transfected SA mutant along with its ability to accelerate apoptosis following glucose stress underscores the protective role of the S13 modification. Finally, Jurkat cells knocked down for Cdk5 also demonstrated increased sensitivity to glucose deprivation compared to controls (Figure 2-6C). Altogether, these results indicated that the phosphorylation of Noxa by Cdk5 effectively rendered the BH3-only protein inactive as a promoter of apoptosis. However, the link between Cdk5 activity and the accelerated response of Noxa expressing cells to glucose-induced cell death remained to be investigated.

Conclusions

Our interest in the death-promoting, tumor-suppressor protein Noxa originated from its stable expression in human leukemias. We hypothesized that Noxa's death function was likely to be suppressed by post-translational regulation. Indeed, our studies demonstrated conclusively that Noxa was phosphorylated on a serine residue (S13) by Cdk5, an atypical cyclin dependent kinase. We also showed that Noxa was required for glucose deprivation-induced, rather than DNA damage-induced, death in leukemia cells. Phosphorylation of Serine 13 on Noxa suppressed its apoptotic function. Knockdown of Cdk5, or expression of a Noxa SA mutant increased sensitivity to glucose deprivation, confirming that the phosphorylation was protective. Both glucose deprivation and Cdk5 inhibition promote apoptosis by dephosphorylating Noxa. We propose that Noxa plays a

dual role, promoting either survival or cell death in hematopoietic cancers, with phospho-S13 as an important switch controlling these opposing functions.



Noxa wildtype	GFP+, Annexin V+
Glucose -	17.6
Glucose +	6.6

Noxa SA	GFP+, Annexin V+
Glucose -	40.6
Glucose +	8.8

Figure 2-6. Absence of Noxa phosphorylation results in accelerated cell death. (A) Jurkat and (B) Molt-4 cells electroporated with pIRES2-EGFP vector alone, or pIRES2-EGFP plasmids expressing Noxa WT or Noxa SA, were Ficoll purified to eliminate dead cells and cultured in glucose-rich or glucose-free medium. Viability of the GFP positive population (mean and SE, n=3) was measured after 16 h by flow cytometric analysis of Ann V-PE-Cy5 uptake (*ρ<0.03). (C) Loss of Cdk5 increases sensitivity of Jurkat cells to glucose deprivation. Cells were cultured in glucose-free medium and viability was assessed by flow cytometric analysis of Ann V-PE-Cy5 uptake (*ρ = 0.02, ** ρ=0.01).

Chapter 3

Post-translational regulation of Noxa facilitates glucose uptake and metabolism

Introduction

In the previous chapter, we showed that constitutively expressed human Noxa was phosphorylated in leukemia cell lines and identified Cdk5 as the Noxa kinase. We also showed that the glucose-deprivation induced death response of the leukemia cells was dependent on Noxa. A role for Mcl-1_L and Noxa in glucose sensing had previously been suggested (Alves et al., 2006). The two proteins are distinctly upregulated in activated human primary T cell populations in the presence of adequate nutrients and glucose. This raised an important question of whether Noxa/Mcl-1 interactions could have either canonical 'apoptotic' or novel 'pro-growth' consequences depending on the availability of glucose. The requirement for Noxa and Mcl-1_L during this period of expansion and high-energy utilization also suggested a role for the Noxa/Mcl-1_L axis in glucose metabolism. One other BH3-only protein, Bad, has been shown to regulate glucose metabolism. Phosphorylation of S155 in its BH3 domain controls this function by directly regulating BAD's binding properties. The metabolic function of BAD involves mitochondria and glycolysis in insulin-secreting pancreatic and liver cells (Danial, 2008). Serine 13 on Noxa, however, is not located within the BH3 domain, raising the question as to whether phosphorylated S13 on Noxa regulates Noxa/Mcl-1 interactions in addition to the canonical BH3 domain. In the studies described below we investigate the regulation of Noxa function by Cdk5 and by glucose.

Results

Glucose regulates the accessibility of Noxa

To further understand the mechanism underlying the accelerated cell death observed in Noxa overexpressing Jurkat cells (N5 and N11) cultured in glucose-free medium (see Figure 2-5D), we compared protein levels of cells grown in glucose-free or high-glucose medium. We observed no significant induction in protein levels (Figure 3-1A, left). However, the Western blot of Noxa immunoprecipitated with anti-Noxa antibody (Figure 3-1A, right) showed that Noxa pull-down was impeded in cells grown in high-glucose medium, but not in cells grown in glucose-free medium. This suggested that Noxa was either in an altered conformation or sequestered in a complex with other proteins in a manner that rendered the epitopes allowing for immunoprecipitation (IP) inaccessible to

the antibody when glucose was present. The data further implied that these epitopes became exposed following the withdrawal of glucose. We tested whether this 'release' of Noxa could be reversed by the addition of glucose from cells previously treated in glucose-free media. To do this we deprived Jurkat and N11 cells of glucose for 12 h and then transferred the cells to glucose-rich medium (Figure 3-1B). A Western blot shows comparable Noxa expression levels under all conditions. However, the IP/Western of Noxa indicated that immunoprecipitation of the protein was greatly reduced in the presence of glucose (lane 1= 12h, lane 3= 12h glucose-free/ 12h glucose-rich) in comparison with glucose starved cells (lane 2= 12h, lane 4= 24h).

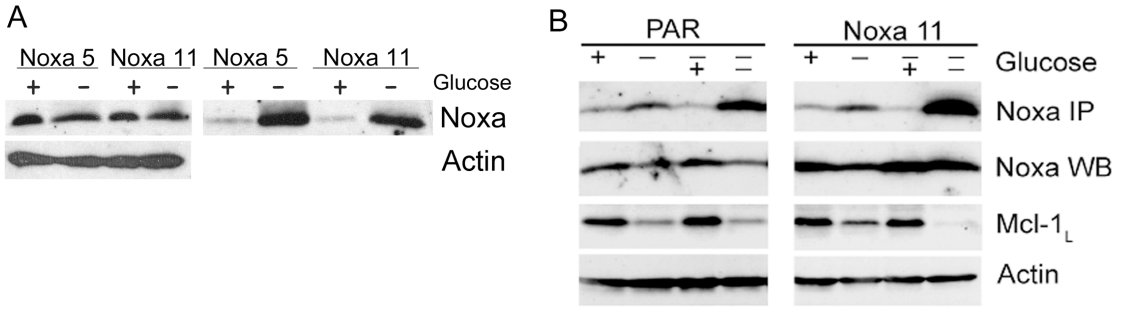


Figure 3-1. Noxa precipitation is dependent upon the withdrawal of glucose. A. Noxa over-expressing cells, Noxa 5 and Noxa 11, were cultured in high glucose (+, 10 mM) or glucose-free (-) RPMI media for 24 h. The left panel shows a Western blot of 50 μg of cellular extract immunoblotted for Noxa expression using anti-Noxa monoclonal antibody. The right panel shows an immunoprecipitation/Western of Noxa immunoprecipitated from 200 μg of the same lysate shown on the right. B. Noxa is post-translationally regulated following glucose starvation and glucose re-addition. Jurkat parental and N11 cells were cultured in glucose-free medium for 12 h (-) or 24 h (=), or moved back to high glucose media for 12 h after 12 h of glucose deprivation (-/+). Shown are Noxa immunoprecipitation/Western (top panel) and Western blots of Noxa (second from top), Mcl-1_L (second from bottom), and Actin (bottom panel).

These results supported the data in Fig 3-1A, which had suggested that Noxa was somehow inaccessible in the presence of glucose. Was Noxa sequestered in a complex in a glucose dependent manner? Or did the presence of glucose cause Noxa to have an altered conformation? It is relevant to mention that these questions were partly addressed in two experiments not shown here, but published alongside the described studies (Lowman et al., 2010). First, *in vitro* immunoprecipitations of full length phosphorylated and unphosphorylated Noxa revealed no differences in the ability of the IP antibody to pull down the peptides (Figure 5-2, will be elaborated on later). This

suggested it was unlikely that phospho-Noxa was adopting a conformation that shut out the relevant epitopes. Confocal microscopy and imaging flow cytometry using monoclonal phosphospecific antibodies against Noxa (also generated in collaboration with R. Jemmerson), showed that pS13 Noxa was detected in the cytosol of dividing HL-60 (a promyelocytic leukemia) and Jurkat cells, but did not co-localize with mitochondria. The punctate nature of the fluorescence suggested that Noxa could be a component of large multi-protein particles. Studies to characterize these complexes and identify their components will be further discussed in a later chapter.

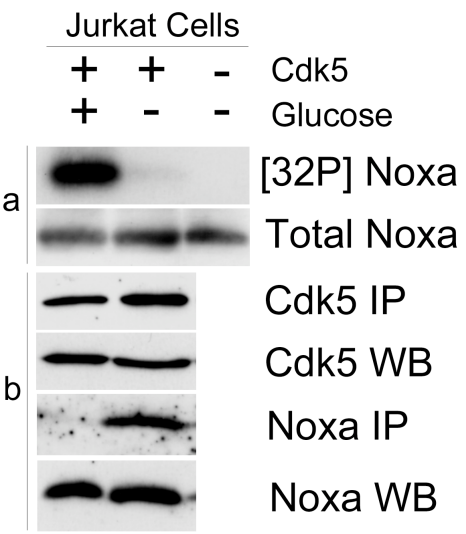


Figure 3-2. Phosphorylation of Noxa by Cdk5 regulates its accessibility in a glucose-dependent manner. Cdk5 was immunoprecipitated from lysates of Jurkat cells grown in high- glucose or glucose-free medium for 36 h and used in an *in vitro* kinase assay with synthetic Noxa peptide as substrate and a Western blot of total Noxa (a) Lower panel set (b) An IP/Western (top) and Western blot of Cdk5 (second from top) from control and glucose-deprived Jurkat lysates (50 μg). The lower two panels are IP/Westerns and Western blots of endogenous Noxa with 200 μg and 50 μg of lysate, respectively, from the same experiment.

Cdk5 controls Noxa's apoptotic function and is regulated by glucose

We had yet to establish a connection between Cdk5 phosphorylation of Noxa, and the glucose-sensitivity of Noxa's pro-apoptotic function. To investigate this we subjected cellular extracts from Jurkat cells, cultured in the presence or absence of glucose, to *in vitro* kinase assays using endogenous immunoprecipitated Cdk5 as the kinase. Figure 3-2a shows that Cdk5 immunoprecipitated from Jurkat lysate phosphorylated the full length Noxa peptide *in vitro* only if it was derived from cells grown in glucose-rich medium, a clear indication that its activity was glucose-dependent. The data further suggested that the Cdk5 under glucose-starved conditions was inactive. Cdk5 IP/Westerns from the same experiment (Figure 3-2b) confirmed that similar levels of Cdk5 were immunoprecipitated from cells under either condition (+/- glucose) and that there were no differences in total Cdk5 levels following glucose deprivation. Similar

levels of endogenous Noxa were also observed and, again, it was only in glucose-deprived cells that Noxa was accessible to immunoprecipitating antibody, supporting our previous observations using Noxa overexpressing cells (Figure 3-1). Taken together, these studies allowed us to conclude that modification of Noxa by Cdk5 in the presence of glucose was promoting its sequestration and masking its apoptotic function.

Noxa is post-translationally regulated by glucose in primary human T cells

Previous studies (Alves et al., 2006) had shown that Noxa transcripts were induced in activated primary human T cells grown in glucose-rich medium. We were interested in whether the post-translational regulation of Noxa we had observed in Jurkat cells also occurred in human primary T cells under these conditions.

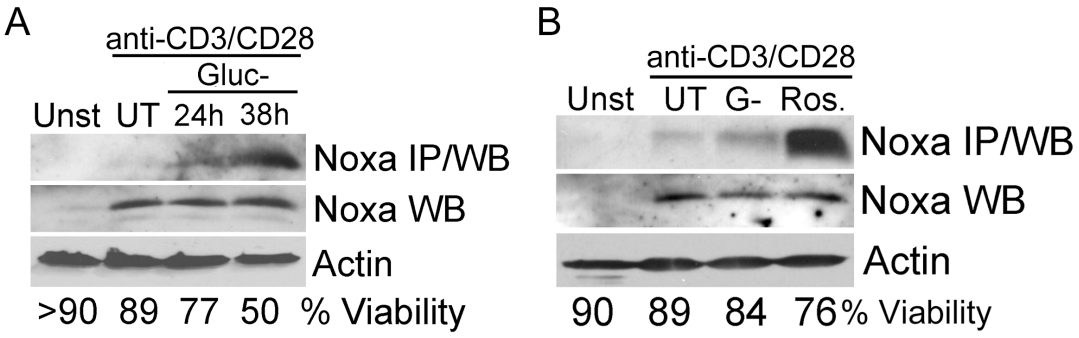


Figure 3-3. Noxa accessibility is also observed in proliferating activated T cells. A. A mixture of $CD4^+$ and $CD8^+$ human T cells were left unstimulated or were stimulated with anti-CD3 and anti-CD28 Abs for 24 h and then cultured in glucose-free medium for the indicated times. An IP/Western of Noxa from 200 μg cell lysate protein and Western blots of total Noxa and Actin levels are shown. Cell viability at the time of harvesting is indicated under each lane. B**.** Cdk5 inhibitor, roscovitine (Ros.), increases accessibility of Noxa to IP in primary T cells. Stimulated $CD4^+/CD8^+$ human T cells were cultured either in glucose-free medium or high-glucose medium in the presence of 25 μM Ros. for 15 h. Viability was assessed prior to harvesting; 200 μg of cell protein were used for the Noxa IP/Western and 40 μg for the Western blot.

For these experiments we purified T cells from peripheral normal human blood and activated them *in vitro* using anti-CD3 and anti-CD28 antibodies (described under Materials and Methods). First and foremost, western blots detected high levels of Noxa in the activated cells compared to controls, confirming the Alves et al (2006) observation that Noxa expression was highly induced in activated T cells (Figure 3-3A). Consistent with our data from Jurkat cell studies we observed that, following 24-38 h of glucose

deprivation, Noxa was not further induced in these cells but was more accessible to immunoprecipitating antibodies compared to Noxa from activated cells cultured in the presence of glucose. The Cdk5-specific inhibitor, roscovitine, promoted apoptosis and increased levels of immunoprecipitable Noxa in Jurkat and activated primary T cells (Figure 3-2, 3-3B). In Figure 3-3B, 15 h of roscovitine exposure, but not 15 h of glucose deprivation, was sufficient to promote both Noxa release and cell death in primary T cells. These results suggested that the glucose-dependent Cdk5 regulation of Noxa function that we had observed in Jurkat T-ALL cells was a physiologically significant regulatory mechanism. The results further suggested that this was a tissue specific mechanism, since elevated Noxa levels were associated primarily with proliferating cells of hematopoietic lineage. Therefore, Cdk5 phosphorylation and sequestration of Noxa in proliferating T cells were also regulating its function by effectively rendering it inactive as a proapoptotic protein.

Glucose dependence, uptake and flux in Noxa-overexpressing Jurkat Cells

Thus far, our data pointed to (a) a novel pro-growth or survival role for human Noxa, provided it was phosphorylated and (b) a dependence on glucose for maintaining the phosphorylated state of Noxa in T-ALL cells. We sought to investigate the dependence on glucose for Noxa's novel pro-growth function by simply comparing the growth properties of Noxa overexpressing Jurkat cells to control cells in culture. Otto Warburg (Warburg, 1956) had proposed that cancer cells preferentially metabolize glucose through the glycolytic pathway despite sufficient oxygen levels in a process now termed the 'Warburg effect'. This metabolic diversion of glucose to 'aerobic' glycolysis, rather than to the mitochondria for oxidative phosphorylation, causes cancer cells to consume more glucose and produce more lactate than normal cells to ensure the production of adequate ATP for cellular processes (Vander Heiden et al., 2009). Jurkat parental and Noxa overexpressing cells were compared for their cell accumulation, survival, glucose consumption, and lactate production properties. Although all three lines were >95% viable at initial seeding, cell viability in culture without passage became compromised after 4 days in Noxa overexpressing cell lines (Figure 3-4A). Both N5 and N11 cells exhausted their media of glucose more rapidly than the controls, which may account for the lower viability at later times (Figure 3-4C). The levels of secreted lactate were also high in N5 and N11 culture media (Figure 3-4B). Finally, glucose re-addition almost

completely abrogated the decline in viability of N5 cells (Figure 3-4D). This suggested that Noxa was being metabolized at a greater rate in Noxa overexpressing cells than in controls. Indeed, these results supported the 'Warburg Effect' allowing us to predict that the glycolytic rate of Noxa overexpressing cells would be higher than that of control cells. We measured the glycolytic rate by the conversion of [3H] glucose to [3H] water per hour and found that it was significantly lower in the N5, N11 (ρ<0.0003), and the phospho-mimic SE-expressing lines, compared to controls (Figure 3-5A). Thus, although overexpression of Noxa was increasing the dependence on glucose in T-ALL cells and suggesting a stimulatory role for Noxa in glucose metabolism, not all the glucose was being converted to H_2O via aerobic glycolysis.

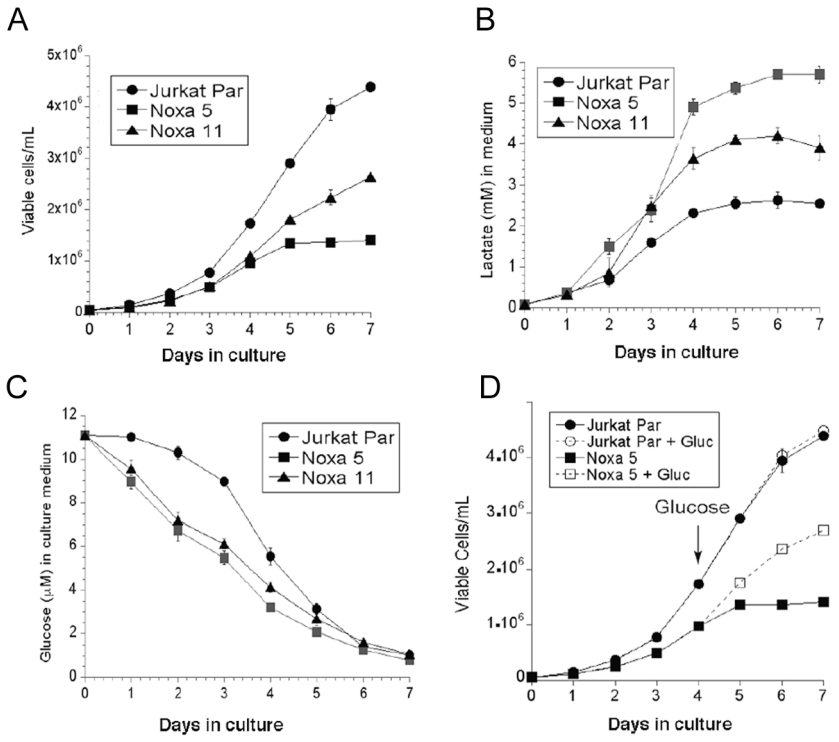


Figure 3-4. Noxa overexpressing cells possess distinctive growth properties. Cells were seeded in triplicate at 5 x 10^4/mL and > 95% viability (values shown are the mean with SE, n=3). Cell counts and automated trypan blue viability measurements were carried out using a Vi-CELL Series Analyzer. A. Viable cell counts were carried out over 7 days in culture without passage. B and C. Lactate and glucose in the culture media (normalized to cell numbers.) were quantified using colorimetric reaction kits (BioAssay Systems). D. Cultures of Par and N5 cells were either supplemented, or not, with 5 mM glucose after 4 days.

Noxa overexpression imparts resistance to 2-deoxyglucose

Concurrent with the growth assays described above, we measured the response of the cell lines to non-hydrolyzable glucose analog, 2-deoxyglucose (2-DG). Although 2-DG can be taken up by the cells and phosphorylated by the enzyme hexokinase (HK), it was reported to be halted from further metabolic breakdown as 2-DG-6-phosphate and therefore exert a condition similar to glucose-withdrawal.

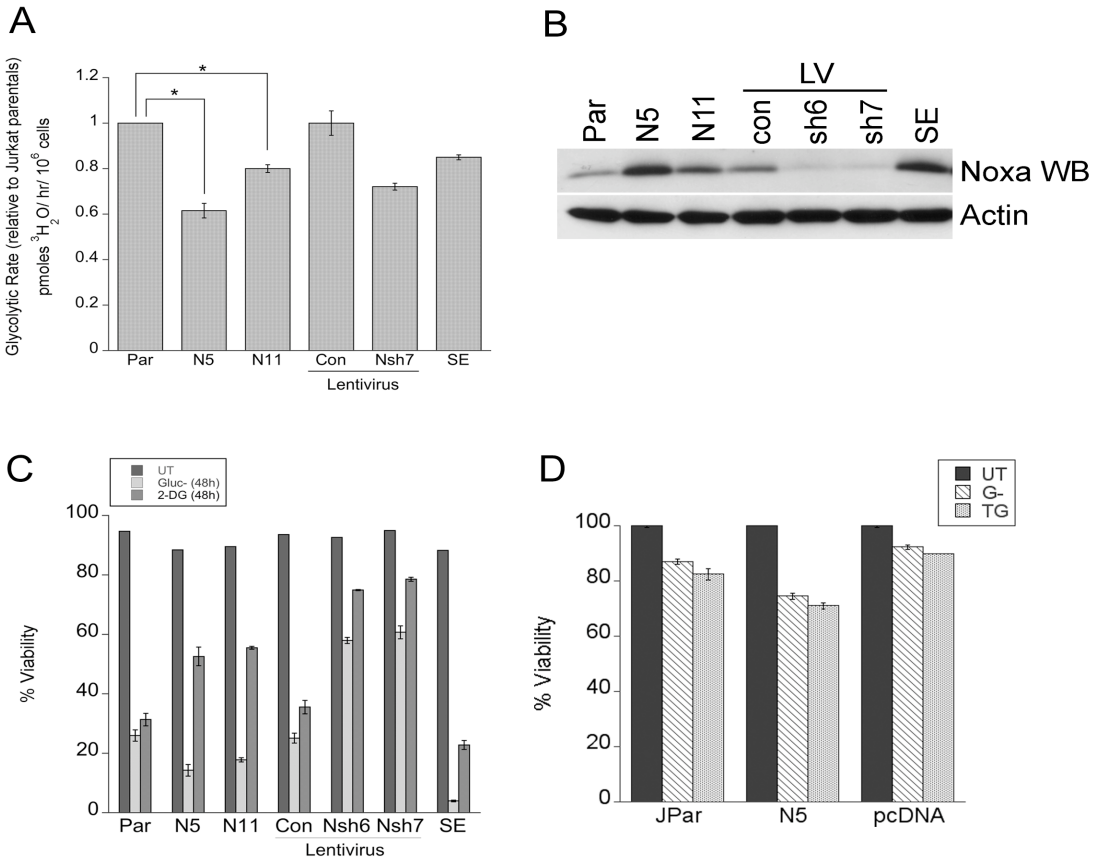


Figure 3-5. Noxa overexpressing cells are resistant to 2DG but not TG. A. Noxa overexpressers exhibit lower glycolytic rates than control cells. Glycolysis was measured by monitoring the conversion of 5-[^{3}H]-glucose to [^{3}H]$_2$O, as described under Materials and Methods. The rates were determined in pmoles $^3H_2O/h/10^6$ cells and values were plotted as a fraction of the rate determined by Jurkat Parental (Par) cells (*ρ <0.0003). B. Western blots of lysates from stable lines used in experiments shown in 5A and 5C. Con - lentiviral control; sh6 and sh7 - clonal lines infected with lentiviral constructs of Noxa shRNA; SE and DA – clones expressing S[13] and BH3 domain point mutants, respectively. C. Jurkat controls and selected clonal lines were cultured in glucose-free medium with or without 2-DG (50 μM) for 48 h. Viability was determined by Ann V/ PI uptake (mean and SE, n=3). *ρ < 0.005, **ρ < 0.0005. D. Jurkat parental cells, the Noxa overexpressing cell line, N5, and the Noxa knockdown transfected with an empty vector (pcDNA) were cultured in glucose-free medium with or without thioglucose (5 mM) for 21 h. Viability was determined by Ann V/ PI uptake (mean and SE, n=3).

Jurkat cells and indicated clonal lines were cultured in glucose-free medium with or without 2-DG for 48 h (Figure 3-5C). While controls and Noxa knockdowns exhibited 5-10% higher viability in 2-DG than in glucose-free medium, Noxa overexpressers, N5 and N11, which respond poorly to the absence of glucose (Figures 2-4D and 3-5C) to our surprise survived significantly better in the presence of 2-DG than did control cells. The SE-expressing line also demonstrated higher tolerance to medium with 2-DG than without; however, the generally poor response of SE cells to glucose deprivation suggested that regulatory phosphorylation/dephosphorylation at S13 may contribute to Noxa's proapoptotic function.

We were initially puzzled by the ability of 2-DG to impart resistance to cell death in the Noxa overexpressers, since this compound is often used as a mimic for the glucose-deprived state. To further investigate this we treated Jurkat control cells, N11 cells and Noxa knockdown cells with a different non-hydrolyzable glucose analog, thioglucose. In this experiment (Figure 3-5D), levels of cell death observed under glucose-deprived conditions and thioglucose treatment were comparable. These results suggested that 2-DG might be metabolized beyond the 2-DG-6-phosphate conversion. A literature search revealed a study that discussed the catabolism of 2-DG via the pentose phosphate pathway (PPP) under certain conditions (Zabos et al., 1978). It is important to point out that Noxa had been initially identified as an adult T cell leukemia-derived phorbol 12-myristate-13-acetate (PMA) response gene; and tumor promoting phorbol esters are known to impart resistance to 2-DG in hematopoietic cells. The Zabos et al. study had demonstrated that PMA (or TPA) enhanced the conversion of 2-DG6-P into an analog of R5P, suggesting flux through the PPP. Figure 3-6 briefly delineates the path of glucose and the suggested path of 2-DG utilization to illustrate the argument. The continued production of NADPH, reductive molecules that help protect against cellular oxidative stress, was likely to be the underlying reason for partial resistance that we observed in N5 and N11 cells treated with 2-DG. These results suggested to us that higher levels of Noxa were preferentially facilitating the diversion of glucose to the pentose phosphate pathway and the production of nucleotides for cell proliferation.

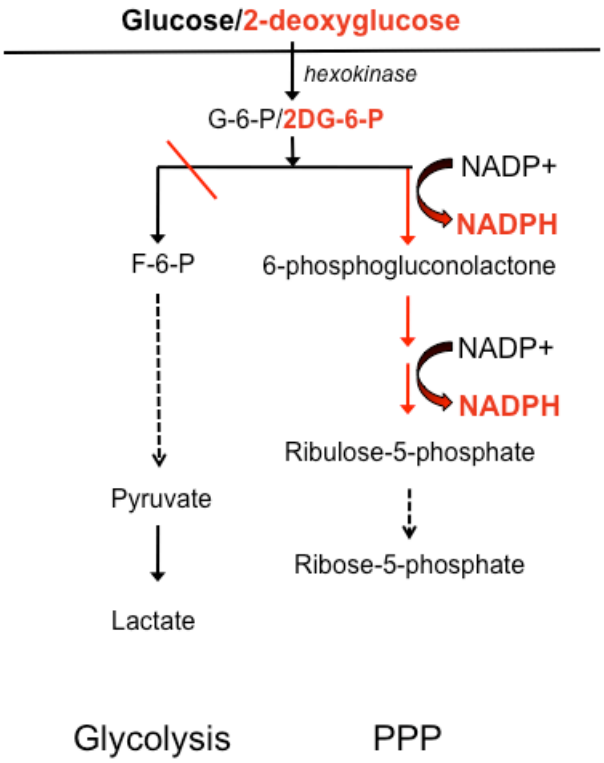


Figure 3-6. Initial metabolism of glucose and 2-deoxyglucose. Glucose enters the cell and is phosphorylated by hexokinase to become glucose-6-phosphate (G-6-P). It can be converted to fructose-6-phosphate (F-6-P) and further metabolized through glycolysis to pyruvate and lactate. G-G-P can also be acted upon by glucose-6-phosphate dehydrogenase and processed through the pentose phosphate pathway (PPP) to generate NADPH and ribose-5-phosphate (R5P). 2-deoxyglucose (red) may be taken up by the cell and phosphorylated by hexokinase. It cannot be further utilized in the glycolytic pathway, but it can be metabolized to some extent through the PPP.

Conclusions

Here we show that constitutively expressed human Noxa is phosphorylated on S13 by the kinase Cdk5 in dividing hematopoietic cells in the presence of glucose. The phosphorylation occurs in proliferating cancer cells as well as primary activated T cells and is, consequently, physiologically relevant. This phosphorylation serves two functions—first, it suppresses Noxa's proapoptotic function while targeting it to cytosolic protein complexes, and second, it appears to promote a novel role for the protein in glucose metabolism. We propose that Noxa stimulates glucose consumption and may enhance glucose turnover via the pentose phosphate pathway rather than through

aerobic glycolysis. Increased utilization of glucose in activated T cells is essential for their proliferation, survival, and effector function and the requirement for Noxa and Mcl-1_L during this period of expansion and high-energy utilization again suggests a role for the Noxa/Mcl-1_L axis in glucose metabolism. The model in Figure 3-7 illustrates how Noxa could play dual roles in proliferating hematopoietic cells and cancers in response to fluctuating glucose levels.

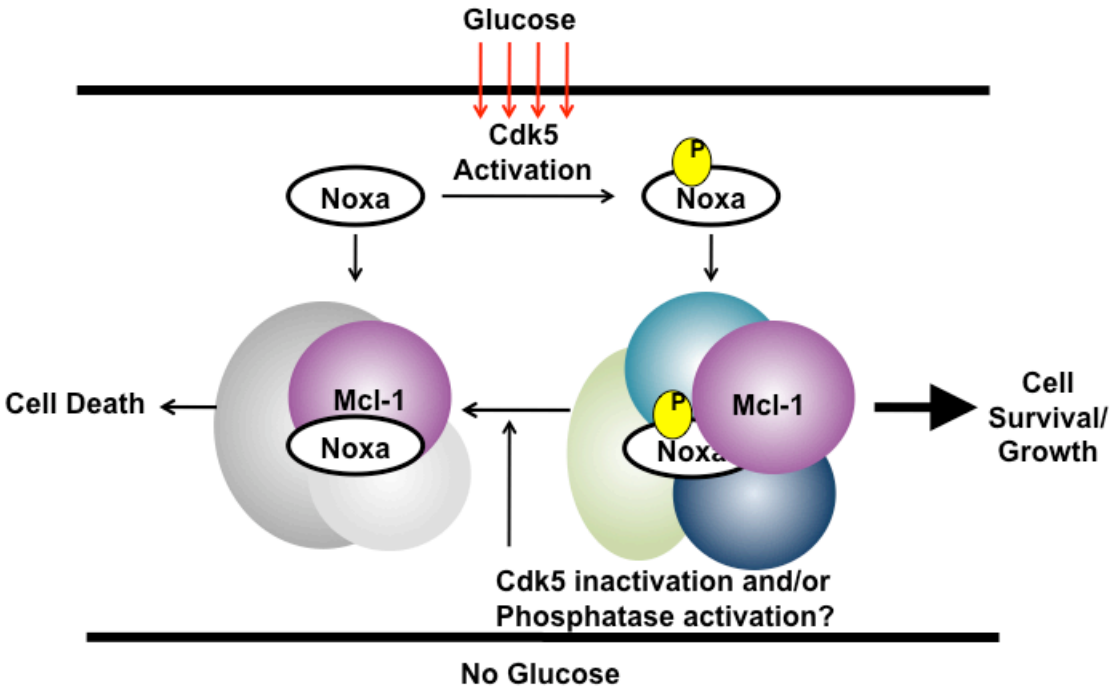


Figure 3-7. Proposed model of Noxa's dual function. Noxa associates with Mcl-1_L to promote cell death. Noxa is phosphorylated by active Cdk5 in the presence of glucose. This modification targets Noxa to a multi-protein complex that confers cell survival and it is proposed to be important for cell growth. Cdk5 inactivation and/or the activation of a phosphatase in response to glucose-deprivation dephosphorylates Noxa and makes it accessible to immunoprecipitation, which correlates with cell death.

The following two Chapters begin to address some important questions arising from the studies described in Chapters 2 and 3 i.e. If Noxa does indeed facilitate reprogramming of glucose breakdown via the pentose phosphate pathway, how does its phosphorylation and its interactions with Mcl-1 regulate this metabolic role? What is the nature and composition of the protein complex that engulfs phosphorylated Noxa? Does it regulate metabolism in proliferating cells?

Chapter 4

Noxa contributes to an altered metabolic phenotype

Introduction

We showed in the previous chapter that Noxa facilitated glucose uptake and proposed that it increased glucose turnover through the PPP. There were two possible interpretations for the growth-promoting phenotype of modified Noxa (1) the protein played a distinct metabolic role that was different from the canonical tumor-suppressor role exerted through binding interactions with Mcl-1, or (2) the phosphorylation prevented Noxa from carrying out its BH3-only tumor-suppressor function which allowed Mcl-1 to exert a robust growth-promoting, anti-apoptotic phenotype. To begin to distinguish between the two possibilities we designed approaches that would allow us to identify specific alterations in the metabolic pathways that were due to Noxa over-expression and modification in T-ALL cells. In this chapter we describe measurements of reductive molecules, such as NADPH, and of reactive oxygen species as indicators of the direction of glucose flux. We also describe early attempts to determine metabolite flux in cells labeled with ^{13}C isotopomers, using GC-MS based tracer analyses (in collaboration with the Metabolomics Core at the Mayo Clinic in Rochester, MN) (Le et al., 2012).

Results

NADPH/NADP ratio fluctuations in Noxa overexpressers indicate higher oxidative stress

We had observed resistance to cell death in Noxa over-expressing cells treated with 2-DG. A literature search into 2-DG processing had revealed that the glucose analog was metabolized in part via the PPP, suggesting that the delayed cell death we observed was due to the production of NADPH early in this pathway. We hypothesized that Noxa overexpressing cells preferentially diverted glucose into the PPP. First, we looked at levels of glucose-6-phosphate dehydrogenase (G6PDH), the initial rate-limiting enzyme that catalyzes the conversion of glucose-6-phosphate into 6-phosphoglucono-δ-lactone and observed no difference in levels between Noxa overexpressing and knockdown cell lines (data not shown). We also measured G6PDH activity using a kit, but the results were inconclusive. We then shifted our focus to NADPH, the reductive molecule that is an important by-product of the pentose phosphate shunt. The PPP comprises oxidative and nonoxidative reactions; two molecules of NADPH are generated in the oxidative

segment for each glucose molecule that enters the pathway. One of the primary functions of the PPP is to generate NADPH; therefore, if Noxa were diverting glucose through the pentose phosphate shunt we predicted a higher NADPH/NADP+ ratio in these cells. We checked the levels of these molecules in Jurkat parental and the Noxa overexpressing, cell lines. The cells were cultured in standard conditions or deprived of glucose (Figure 4-1A). The ratio of NADPH to NADP+ dropped in both parental and N5 cell lines but the drop was greater in Noxa overexpressing cells. In a related experiment glucose or 2-DG were re-added to 2 h glucose-deprived parental and N11 cell lines. The rapid drop in NADPH levels following 2 h of withdrawal and the dramatic reversal to higher NADPH/NADP+ ratios in N11 cells compared with controls within 1 h after addition of glucose or 2-DG was further indication that high Noxa levels favor PPP (Figure 4-1B).

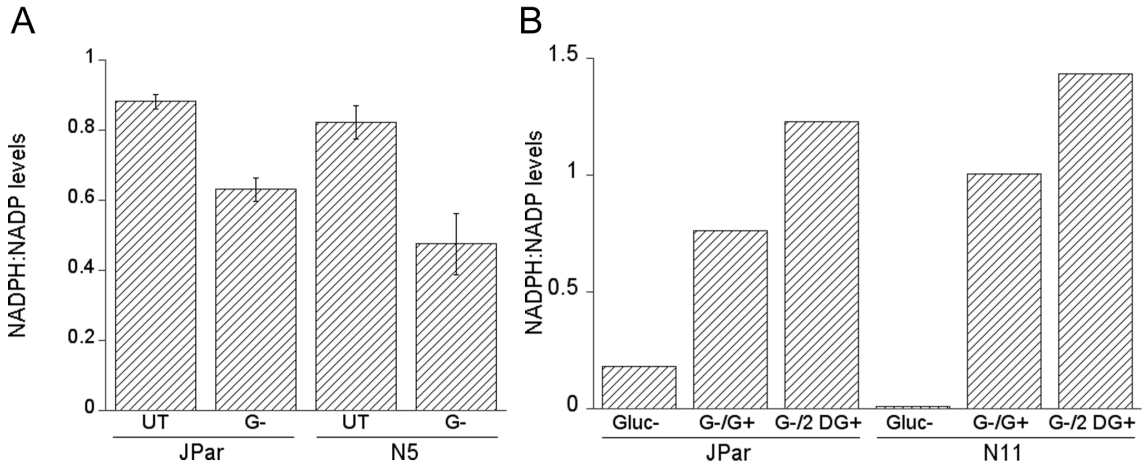


Figure 4-1. NADPH/NADP+ levels fluctuate in response to glucose manipulation. A. Jurkat parental (JPar) and Noxa overexpressing (N5) cells were cultured in standard media (UT) or deprived of glucose (G-) for 3 h. B. JPar and another Noxa overexpressing cell line (N11) were deprived of glucose for 3 h before adding back 25 mM glucose (G+) or 50 μM 2-deoxyglucose (2 DG+) for 1 h. NADPH and NADP+ levels were measured by a fluorescent plate reader from samples prepared using kits purchased from Cell Technology, Inc.

Noxa overexpressing cells show higher oxidative stress measured as reactive oxygen species (ROS)

NADPH is important for anabolic reactions, for nucleotide, protein, and lipid biosynthesis. Proliferating cells must make these macromolecules to grow and divide. In addition, NADPH helps to maintain redox homeostasis and protect against the toxicity brought on

by reactive oxygen species (ROS) generated as a result of the greater energetic demands of high proliferative activity (Stanton, 2012). We have consistently observed a higher level of spontaneous cell death (10-15%) in Noxa overexpressing cells compared to parental cell lines. We showed in a prior chapter that Noxa overexpressing cells secreted high levels of lactate, indicating greater metabolic activity. We compared ROS activity of unperturbed parental Jurkats with Noxa overexpressing cells using H2-DCFDA (Figure 4-2A). N5 cells had twice as much ROS activity, which we speculate may contribute to the spontaneous apoptosis observed in the cell lines. We then subjected the cells, along with the knockdown Noxa cell line, Nsh7, to glucose-deprivation for 3 h, added back glucose and determined the effect on ROS activity (Figure 4-2B). Again, we detected higher levels of ROS in the N5 cells. The Noxa knockdown had intermediate levels (compare UT bars). Glucose-deprivation resulted in a reduction of ROS activity, which may be attributable to the withdrawal of an energetic source resulting in a slower metabolism. The addition of glucose even for 1 h following deprivation restored ROS activity to UT levels, suggesting a return to metabolic equilibrium.

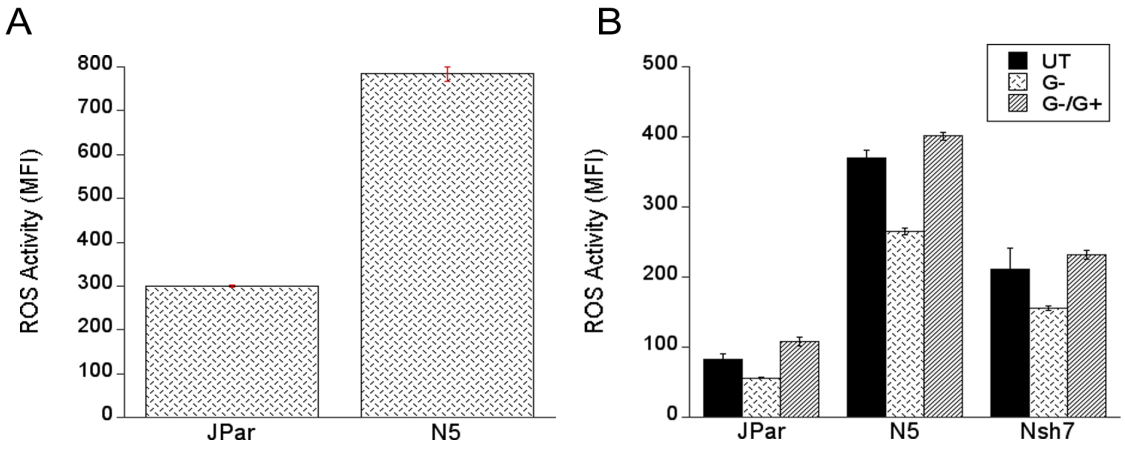


Figure 4-2. ROS activity is increased in Noxa overexpressing cells. A. ROS activity (10 μM H2-DCFDA) was measured in Jurkat parental (JPar) and Noxa overexpressing (N5) cell lines that were cultured in standard RPMI 1640. B. JPar, N5, and a Noxa knockdown cell line (Nsh7) were cultured in standard media (UT) or deprived of glucose for 2 h (G-) and re-fed 10 mM glucose for 1 h (G-/G+). ROS activity was measured by flow cytometry.

Noxa overexpressing cells are dependent upon glucose as well as glutamine

We had thus far shown that Noxa overexpressing cells were highly dependent upon glucose. However, the glycolytic rate of the Noxa overexpressing cells was low

compared to parental cells, despite the high levels of lactate detected in the medium. We sought to reconcile these observations by determining the requirement for the nonessential amino acid, glutamine. Recent studies show that certain cancer cells are addicted to glutamine as much as glucose (DeBerardinis and Cheng, 2010). Glutamine contributes to macromolecular synthesis, ATP generation, and antioxidant production in the form of glutathione (DeBerardinis and Cheng, 2010). We reasoned that in the Noxa overexpressing cells, glucose was diverted primarily to the PPP and glutamine was metabolized as a source of carbon for ATP generation, and for amino acids and TCA cycle intermediates, some of which could be converted to lactate. We measured viability of cells cultured in the absence of either glucose or glutamine or both (Figure 4-3). As expected, we observed accelerated levels of cell death in response to glucose deprivation in the Noxa overexpressing cells. Loss in viability from glutamine withdrawal was more pronounced in Noxa overexpressers cells compared to parental cells, although the cells were less dependent on glutamine than on glucose. These results suggest that Noxa overexpressing cells may utilize glutamine for anaplerosis, to replenish some of the mitochondrial TCA intermediates, although their primary source of fuel is glucose.

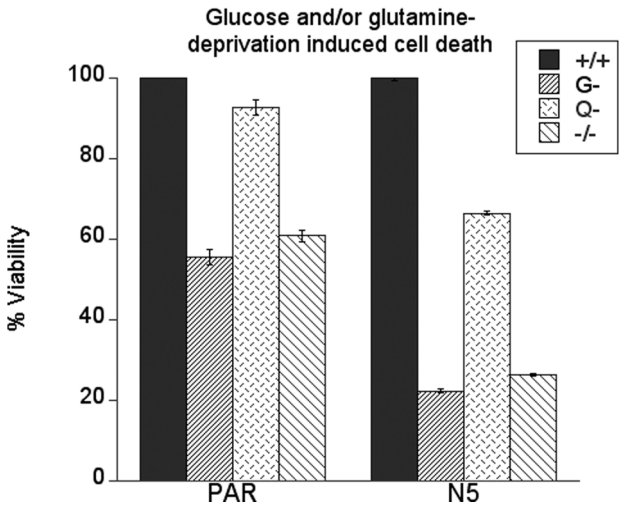


Figure 4-3. Noxa overexpressing cells are dependent upon glucose and glutamine. Jurkat Parental (PAR) and Noxa overexpressing cells (N5) were cultured in RPMI 1640 medium with 10 mM D-glucose and 2 mM L-glutamine (+/+), 2 mM L-glutamine (G-), 10 mM D-glucose (Q-), or without glucose and glutamine (-/-) for 36 h. The cells were stained with Annexin-V-FITC (Ann-V) and PI and the viability was measured by flow cytometry. Viable populations were gated upon Ann-V and PI- negative cells and standardized to untreated (+/+) conditions.

Identifying metabolic alterations using targeted metabolomics

We had hypothesized that Noxa overexpression preferentially diverts glucose to the PPP. To further investigate this, we collaborated with the metabolomics translational technology core at the Mayo Clinic (Rochester, MN) to perform targeted metabolomics. First, we replaced unlabeled glucose with the sugar labeled with ^{13}C at carbons 1 and 2 ($^{13}C_{1,2}$-Glc) and we measured the enrichment of ^{13}C in downstream metabolites by GC-MS (Le et al., 2012). Figure 4-4A shows the path of movement of the two carbons that would help to distinguish between lactate derived from glucose processed via the PPP from that generated early and rapidly by glycolysis. Glucose is a six-carbon sugar that is converted into two molecules of pyruvate. When taken up, glucose is phosphorylated by hexokinase and converted to glucose-6-phosphate (G6P). G6P may be isomerized to become fructose-6-phosphate and proceed through the canonical glycolytic pathway to produce the 3-carbon metabolites, pyruvate and lactate, labeled at two positions. The PPP rate-limiting enzyme G6PDH may also act upon G6P. Subsequent reactions result in a loss of the first carbon as CO_2. The five carbon intermediate of the PPP, ribose-5-phosphate (R5P) may re-enter glycolysis and proceed to generate pyruvate and lactate, which will retain a single ^{13}C label.

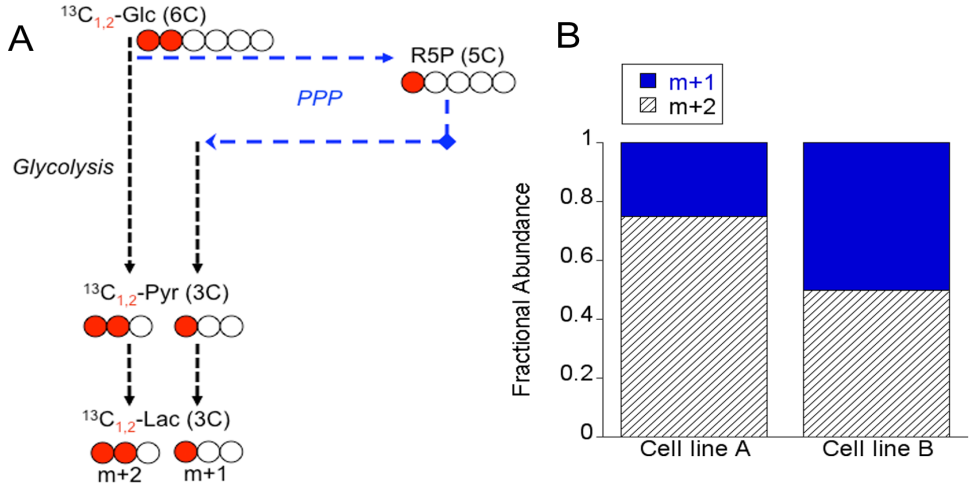


Figure 4-4. Scheme of $^{13}C_{1,2}$-Glc carbon flow through the glycolytic and the pentose phosphate pathway (PPP). A. Cells are cultured with labeled glucose at the first two carbon positions ($^{13}C_{1,2}$-Glc). Glucose may be metabolized to pyruvate (Pyr) via glycolysis and retain its two labeled carbons or it may be shunted to the PPP and lose a labeled carbon before entering the glycolytic pathway to yield one labeled lactate (Lac). B. Stacked columns showing the distribution of labeled lactate (m+1, m+2) from mass spectrometric analysis. The m+2 lactate yield from cell line A indicates more activity through glycolysis than cell line B, which has less m+2 and more m+1, suggesting glucose was diverted to the PPP.

Figure 4-4B illustrates a hypothetical readout of this method by comparing flux through the PPP and glycolysis between two cell lines, A and B. Lactate from cell line A is derived mainly from glycolysis; whereas lactate from cell line B is derived equally from both pathways. We also took advantage of uniformly labeled glutamine (13-U-glutamine), in which the five carbons were labeled with ^{13}C. If glutamine were a source for lactate, we would observe enrichment of ^{13}C in measured lactate from cells treated with 13-U-glutamine.

We compared Jurkat parental and Noxa overexpressing cells in the targeted metabolomics studies. Preliminary studies on lactate derived from ^{13}C-glucose-labeled cells using this approach did not allow us to distinguish between early glucose metabolism through the PPP or through glycolysis and were, therefore, inconclusive (data not shown). However, analysis of TCA cycle intermediates clearly show greater incorporation of labeled glucose (Figure 4-5A, sum of m1 to m6) in Jurkat parental cells compared to the N5 cells. This reduction in labeled glucose supports the previous studies showing a lower glycolytic rate in the latter. The data in Figure 4-5A show that the glucose taken up by the N5 cells is being utilized to a lesser extent in the TCA cycle, suggesting it may be diverted to other reactions/pathways that favor growth before it enters the TCA cycle as pyruvate. Further studies will be carried out using this approach as we continue to refine methods for labeling cells and preparing extracts. We also cultured cells in uniformly labeled U-13-glutamine and looked at the enrichment in lactate. We observe that N5 cells have five times more glutamine derived lactate than parental cells (data not shown). When we compare the abundance of labeled carbons from glutamine in TCA cycle intermediates (Figure 4-5B), we see an increase in Noxa overexpressing cells (see m4, m5 and m6), indicating increased utilization of glutamine for anaplerosis. This complements the results shown in Figure 4-5A, in that glutamine compensates to some degree in the N5 cells for the decrease in glucose utilization in the TCA cycle. Interestingly, we observed in the case of citrate, an almost two-fold increase in m5 labeling (blue) in Noxa overexpressing cells (25%) compared to parental (14%). This suggests Noxa overexpressing cells are using the reverse reaction, reductive carboxylation, to generate citrate. Citrate is a key metabolite for growing cells because of its support of lipid biosynthesis. Altogether, these results strengthen our working

hypothesis that Noxa overexpression alters the metabolic phenotype, reorganizing glucose and glutamine flux to support anabolic growth.

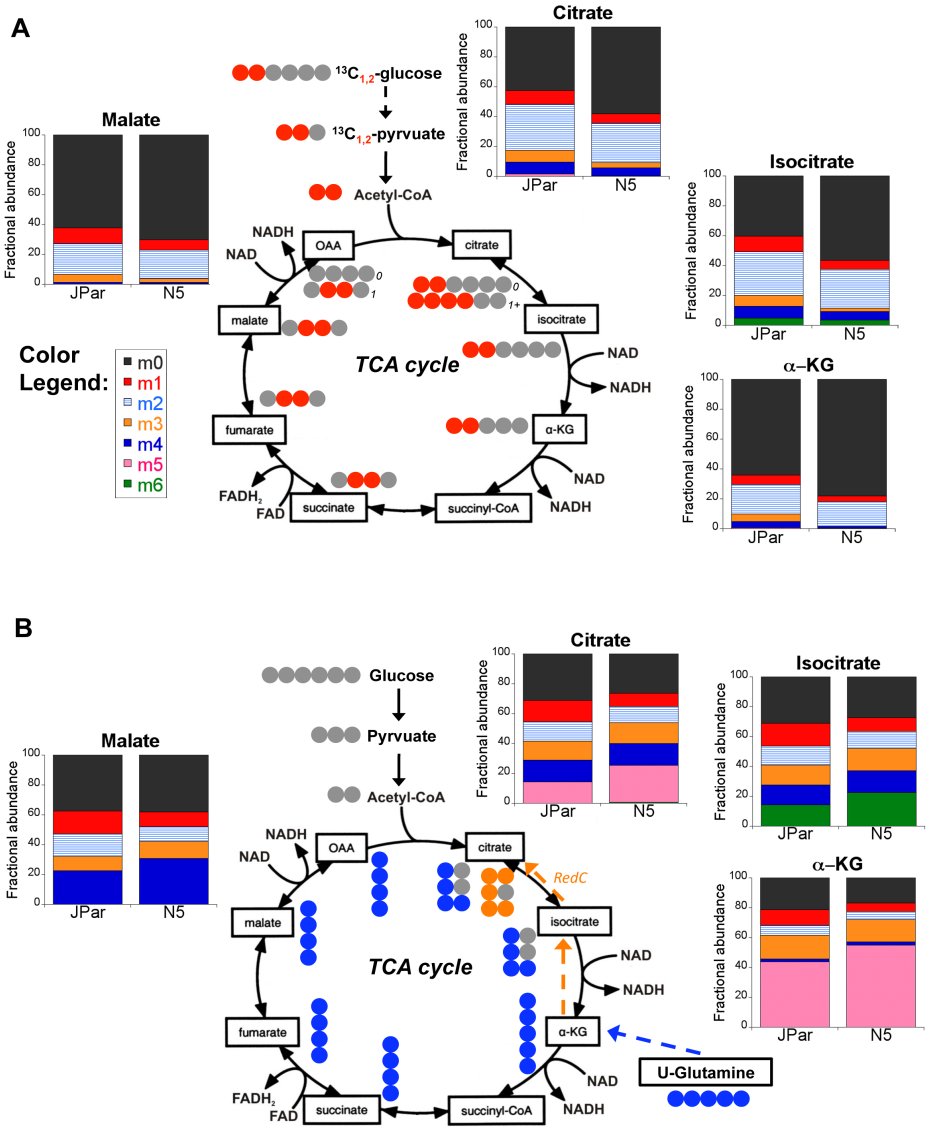


Figure 4-5A. Tracer analysis of glucose and glutamine in the TCA cycle of Jurkat parental (JPar) and Noxa overexpressing (N5) cells. A. Cells were cultured in $^{13}C_{1,2}$-glucose for 24 h. GC-MS data of enrichment of labeled TCA cycle intermediates, citrate, isocitrate, alpha-ketoglutarate (α-KG), and malate were graphed as a stacked column. M0 is unlabeled metabolite, m1, etc indicates the number of labeled carbons detected in the metabolite. The inset schematic traces the flow of carbons (red) derived from labeled glucose as it enters the TCA cycle (0) and begins a second turn (1). This illustrates the abundance of differently labeled fractions. B. JPar and N5 cells were cultured in 13-U-glutamine for 24 h. Labeled TCA cycle intermediates were graphed as a stacked column. The inset schematic traces the flow of carbons (blue) derived from labeled glutamine as it enters the TCA cycle (lower right) and goes through one cycle. The orange dashed arrows represent reductive carboxylation, which yields a five-carbon citrate.

Conclusions

In this chapter we set out to test our hypothesis that Noxa overexpression in T leukemia cells was causing them to reprogram their metabolism to favor anabolic pathways. We observe that Noxa overexpressing cells show greater dependence on both glucose and glutamine. ROS activity measurements suggest that these cells are under greater oxidative stress, a read-out for higher glucose utilization and higher proliferation. The sensitivity of NADPH/NADP ratio measurements to glucose levels in the Noxa high cells offer further confirmation. Early studies using targeted metabolomics indicate that a smaller proportion of the glucose taken up by Noxa overexpressing cells is utilized in the TCA cycle for oxidative phosphorylation compared with parental Jurkat cells, and that glutamine may be diverted in these cells to replenish TCA cycle intermediates and to generate metabolites for lipid biosynthesis.

Chapter 5

Noxa is part of a glucose-responsive signaling complex

Introduction

Studies described in the previous three chapters showed that the novel pro-survival role observed for Noxa in human hematopoietic cells was intimately connected to its modification by phosphorylation which, in turn, was dependent upon glucose availability. Although our data suggested that the phosphorylated state of over-expressed Noxa was influencing the rate and/or pathway of glucose flux it was difficult to conclude that this was a direct effect on metabolism or merely a consequence of its inability to activate apoptosis. Thus, it was important to examine the binding interactions, if any, between pS13 Noxa and Mcl-1_L. The observation that phosphorylated Noxa was inaccessible to immunoprecipitating antibodies in untreated Jurkat leukemia and proliferating primary T-cells also needed further investigation. Immunofluorescence and confocal microscopy had suggested the possibility of phospho Noxa being sequestered in a cytosolic protein complex in unperturbed leukemia cells (Lowman et al., 2010). What was the composition of this particle? And what was its function? Did it prevent Noxa from binding Mcl-1 and activating apoptosis? Was this particle, like phospho Noxa, regulated by glucose?

Our studies indicate that Noxa is a component of glucose-responsive signaling complexes in T-ALL and primary proliferating cells. We describe here our enrichment and purification scheme as well as some initial characterization of the multi-protein particles.

Results

Noxa and Mcl-1 associate in unperturbed proliferating cells

We had shown that Noxa was not immunoprecipitated from extracts of Jurkat and stimulated primary cells grown in glucose-rich media (Figure 3-1and 3-2), but that it was robustly precipitated following glucose deprivation. We show this again in Figure 5-1A and Figure 5-1B. However, we also look at the ability of Mcl-1 to co-precipitate Noxa in these experiments. It must be noted that Noxa promotes cell death by interaction with Mcl-1 through its BH3 domain. This interaction is believed to facilitate the degradation of Mcl-1. As expected, we detected a decrease in Mcl-1_L levels over time as the cells were deprived of glucose. We checked for the ability of Mcl-1_L Ab to immunoprecipitate

endogenous Noxa from the untreated and glucose-deprived lysates. Remarkably, Mcl-1 pulled down Noxa in otherwise healthy proliferating cells (Figure 5-1A and 5-1B). We also observed that in primary lymphocytes Mcl-1 pulls Noxa down from dying roscovitine-treated cells as well as from unstressed, stimulated cells (Figure 5-1C). Notably, the Noxa in cells with higher viability (center two lanes) was phosphorylated while Noxa in the rosco-treated cells was non-phosphorylated. The observation that Mcl-1 interacted with phospho-Noxa was intriguing and novel and we wanted to confirm this possibility *in vitro* through direct binding assays.

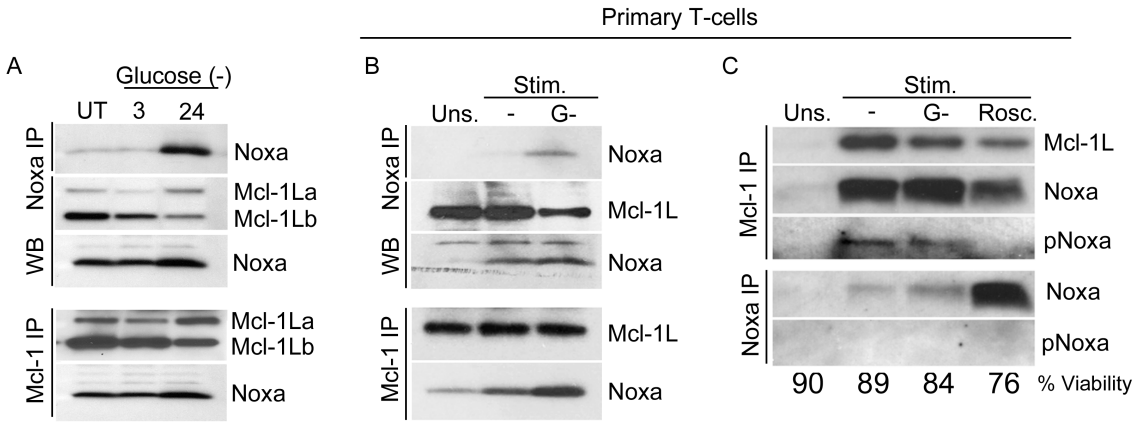


Figure 5-1. Noxa and Mcl-1 associate in proliferating cells of hematopoietic origin. A. Jurkat cells were cultured in standard media with 10 mM glucose or deprived of glucose for the indicated times, 3 and 24 h. RIPA lysates were made and used in immunoprecipitation reactions with anti-Noxa or anti-Mcl-1 polyclonal antibodies, or simply used to check for Noxa and Mcl-1_L protein levels (WB). B and C. Human primary lymphocytes (CD4+ and CD8+) were isolated and left unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies for 24 h. The stimulated cells were split into different conditions: untreated (-), deprived of glucose (G-) or treated with roscovitine (Rosc in C). Lysates made with RIPA were used for immunoprecipitating Noxa or Mcl-1 and for Western blotting for Mcl-1_L, Noxa, and pNoxa. In C, viability was measured by exclusion of Annexin V-FITC and Propidium Iodide staining on a flow cytometer.

Phosphorylated Noxa and Mcl-1 do not interact in vitro

Since we observed pNoxa precipitating with Mcl-1_L in primary lymphocytes, we asked whether we could detect the interaction in an *in vitro* assay using Noxa peptide that was phosphorylated in the presence of recombinant Cdk5. We incubated the pNoxa or Noxa peptides with *in vitro* translated (IVT) Mcl-1_L and proceeded to carry out an IP/Western using Mcl-1_L Ab for the IP. Results show that pNoxa does not bind to Mcl-1_L *in vitro* (Figure 5-2). The pull-down observed in proliferating primary T-cells may be a

consequence of an indirect association of Noxa with Mcl-1 rather than a direct interaction via its BH3-domain. Thus, it is possible that phospho Noxa and Mcl-1_L are both components of a common multi-protein macromolecular complex in which they do not interact directly unless Noxa is dephosphorylated.

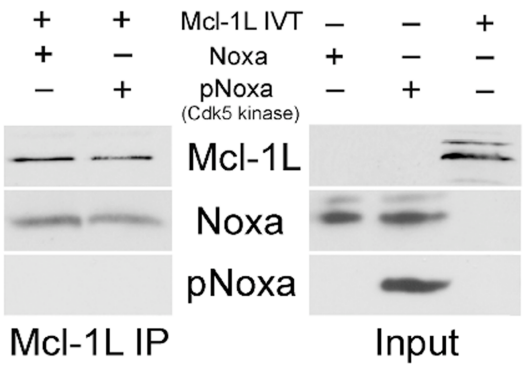
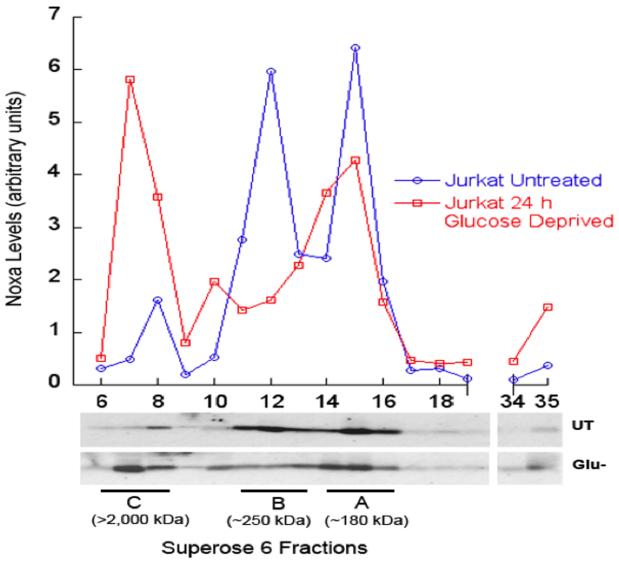


Figure 5-2. Phosphorylated Noxa and Mcl-1 do not bind *in vitro*. Noxa peptide was phosphorylated with active Cdk5 in an *in vitro* kinase assay and mixed with *in vitro* translated (IVT) Mcl-1. IVT Mcl-1_L was incubated for 1 h with 500 ng Noxa peptide (from an *in vitro* kinase assay with or without recombinant Cdk5). The Mcl-1: Noxa complex was immunoprecipitated with anti-Mcl-1 pAbs and precipitates were resolved by SDS-PAGE followed by Western blot analysis using antibodies against Mcl-1, Noxa, and phospho (p) Noxa. One ng of Noxa peptide (+/- Cdk5) and one-half of the IVT Mcl-1_L were used as input controls.

Noxa is a component of cytosolic multi-protein complexes

We utilized gel filtration chromatography to isolate Noxa-containing protein particles from cytosolic extracts of Jurkat cells. Cell extracts were applied to a fast protein liquid chromatography (FPLC) Superose 6 column to fractionate the mixture of proteins by size. We showed that in standard media (untreated cells, UT) Noxa fractionated into two complexes and when deprived of glucose, resolved into three distinct complexes (Figure 5-3).

Figure 5-3. Superose 6 profile of Jurkat cells cultured with or without glucose. Noxa fractionates in different high molecular weight complexes in the presence and absence of glucose (24 h). TCA-precipitated proteins from Superose 6 column fractions of cytosolic lysates of unperturbed and glucose-deprived Jurkat cells were immunoblotted for Noxa. Blots were densitometrically scanned and the values plotted as shown. Noxa can be immunoprecipitated from complex C (fraction 6-8) in apoptotic cells but not from complexes A (fraction 14-16) and B (fraction 11-13) in untreated cells.



These results strongly suggested that Noxa was a constituent of multi-protein complexes. We then proceeded to further enrich and purify this complex with an improved purification scheme (Figure 5-4).

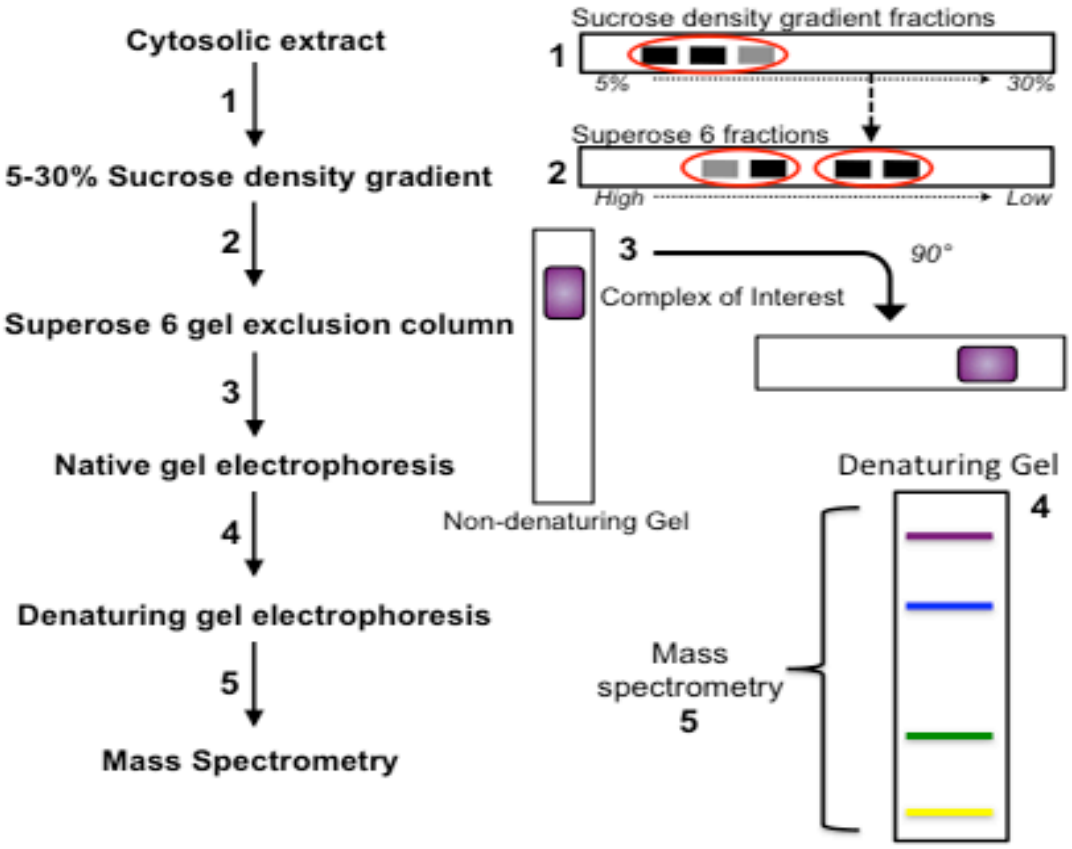


Figure 5-4. Schematic of the purification process to identify constituents in a Noxa-containing particle. Cytosolic cellular extract was made in Dignam buffer A prior to loading onto a 5-30% sucrose density gradient and centrifuged for 24 h. Twelve fractions were collected with the initial elution of proteins suspended in the lowest sucrose percentage (5%) and increasing with latter fractions (30%). A small volume was used to make TCA preps and subjected to SDS-PAGE, transferred to membrane, and immunoblotted for Noxa. The appropriate Noxa-containing fractions were pooled (red circle), dialyzed to remove sucrose, and concentrated to enrich for Noxa. The concentrated extract was loaded onto a Superose 6 column and 1-mL fractions were collected at 0.1 mL/min. High molecular weight complexes eluted in early fractions and the molecular size of the complexes diminished over time. A small volume was used to make TCA preps and subjected to SDS-PAGE to identify Noxa-containing fractions. These fractions were pooled and concentrated, then run on a non-denaturing gel. The lane was cut out and subjected to denaturing conditions. The complex was broken up and stained with Sypro-Ruby red and sent for mass spectrometric analysis.

For the scaled-up purification, cell extracts were first resolved over a 5-30% sucrose density gradient (SDG). We selected Noxa-containing fractions by performing Western blot analysis on TCA-precipitated fractions (Figure 5-5A). Pooled Noxa-containing fractions from six such gradients were dialyzed and concentrated 10-20 fold prior to loading onto a Superose 6 column. Again, Noxa containing fractions were determined by Western blot analysis using a small volume of the collected fractions (Figure 5-5B). The 2-step protocol allowed greater enrichment and higher resolution of complexes A and B. Short and long exposures of the western blots of TCA-precipitated fractions demonstrate the comparative abundance of the complexes. The apoptotic complex C is only weakly detected in the longer exposure since the extracts were from healthy proliferating cells. Relevant fractions containing the two smaller particles were pooled, concentrated and resolved in duplicate by native PAGE (second dimension).

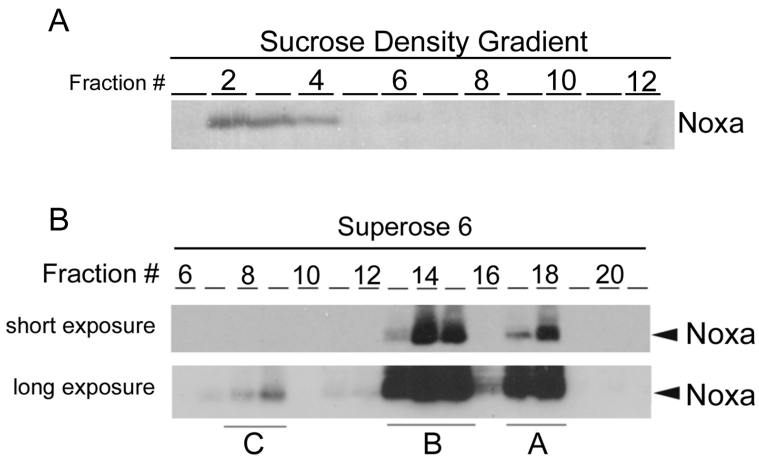


Figure 5-5. Purification of a Noxa-containing particle using cytosolic Jurkat extract. A. Jurkat cells were maintained in a 37°C incubator (5% CO_2) and grown to sufficient volumes for large-scale analysis. Cytosolic extract was made using Dignam buffer A and loaded onto a 5-30% sucrose density gradient and spun for 24 h. Fractions were collected and a small volume used for TCA-preps. The preps were subjected to SDS-PAGE and immunoblot analysis with anti-Noxa monoclonal antibody. Fractions 2-4 were pooled, dialyzed with buffer A for 48 h (buffer change every 24 h) to remove the sucrose, and concentrated 20-fold. B. Lysate was loaded onto a Superose 6 column and 21 fractions were collected at 0.1 mL/min. A portion of each fraction was used for TCA-preps and subjected to SDS-PAGE and immunoblot analysis using anti-Noxa monoclonal antibody. A short exposure shows the precipitation of two distinct Noxa containing fractions (B= fractions 13-15, A= fractions 17-18). A longer exposure reveals a larger Noxa containing complex (C= fractions 7-9).

Each complex was localized by Western blotting one of the gels. A native gel slice containing the complex was excised and placed horizontally as a stack above a denaturing gel and electrophoretically resolved in a third dimension. The larger of the two complexes crudely resolved into 5 (Sypro Ruby Red) stained bands and the smaller complex into 4 single bands, not including Noxa (not shown). Mass Spectrometric (MS)

analysis of each single vertical gel slice after processing was carried out at the Center for Mass Spectrometry and Proteomics at the University of Minnesota. The proteins identified with 4-12% coverage and > 95% confidence are shown in Table 5-1. Signal to noise ratio due to common contaminants was addressed during optimization. The diversity of the components was a surprise, although the presence of Mcl-1_L in the complex was not (we showed earlier that Mcl-1_L and Noxa co-associated not only in apoptotic cells, but also in proliferating primary T and T-ALL cells). The lists reveal the identified components in two Noxa-containing particles (complex A and B), which correspond with the two Noxa peaks that were crudely separated using untreated Jurkat cells in Figure 5-3. The detected proteins included the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in both complexes. The larger complex (B) also contained the phosphatase, PHLPP2; elongation factor 1 gamma (EF1-γ), and a subunit of the proteasome (PSM-α3 or HC8). The smaller complex (A) comprised another glycolytic enzyme, phosphoglycerate kinase 1 (PGK1) and a synthetase/carboxylase involved in purine biosynthesis, ADE2. In addition, we confirmed the composition of these complexes by immunoprecipitating Mcl-1_L from Jurkat cytosolic extract, subjecting it to native gel electrophoresis and mass spectrometric analysis (data not shown). All of the proteins purified without the use of antibody and listed in Table 5-1 were validated through this method.

Characterization of the Noxa-containing complexes - validation of the MS data

Noxa containing fractions enriched through SDG centrifugation (Figure 5-6A) was subjected to gel filtration, as described earlier. TCA-precipitated fractions were analyzed by immunoblot analysis for proteins in complex A and B (Figure 5-6B). PHLPP2, a component of complex B, was observed in earlier fractions, correlating with a higher molecular weight complex. Conversely, PGK1, a component of complex A, fractionated in later fractions corresponding to a smaller molecular weight particle.

Complex A	Complex B
Phosphoribosylaminoimidazole Carboxylase (ADE2)	Pleckstrin Homology domain Leucine rich repeat Protein Phosphatase 2 (PHLPP2)
Phosphoglycerate kinase-1 (PGK1)	Elongation factor 1-gamma (EF1-γ)
	Proteasome a 3 subunit 1 (HC8)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Myeloid cell leukemia-1 (Mcl-1_L)	Myeloid cell leukemia-1 (Mcl-1_L)
Noxa	Noxa

Table 5-1. Mass spectrometric identification of the components of Noxa-containing multi-protein complexes purified from the cytosol of Jurkat cells. The enrichment for a multi-protein Noxa-containing complex yielded two particles of different molecular weights by mass spectrometry. The smaller complex (Complex A) contained an enzyme required for purine biosynthesis (ADE2), two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1), involved in the energy generation phase of glycolysis, and the two Bcl-2 family members, myeloid cell leukemia-1 (Mcl-1_L) and its partner, Noxa. The larger complex (Complex B) also contained GAPDH, Mcl-1, and Noxa. In addition, it contained the phosphatase, Pleckstrin Homology domain Leucine rich repeat Protein Phosphatase 2 (PHLPP2), the elongation factor 1-gamma (EF1-γ), and a subunit to the proteasome termed proteasome α 3 subunit 1/human component 8 (HC8).

More recently, we have eliminated the time-consuming SDG step in protocols designed to further characterize these complexes. We were also able to show that complex A and B fractionated with similar profiles in activated T-cells (Figure 5-7A). Additionally, we immunoprecipitate Mcl-1-associated molecules from individual fractions to process for Western blotting and further validate the components (Figure 5-7B) and their association with Mcl-1. GAPDH co-precipitates with Mcl-1_L in fractions 11/12, HC8 (PSM-α3) is pulled down in fractions 10-14, and an association between Mcl-1_L and Noxa emerges in fractions 15-17. These results suggest that the particles identified in Jurkat cells are likely to be very similar, but not identical, to those present in primary proliferating lymphocytes.

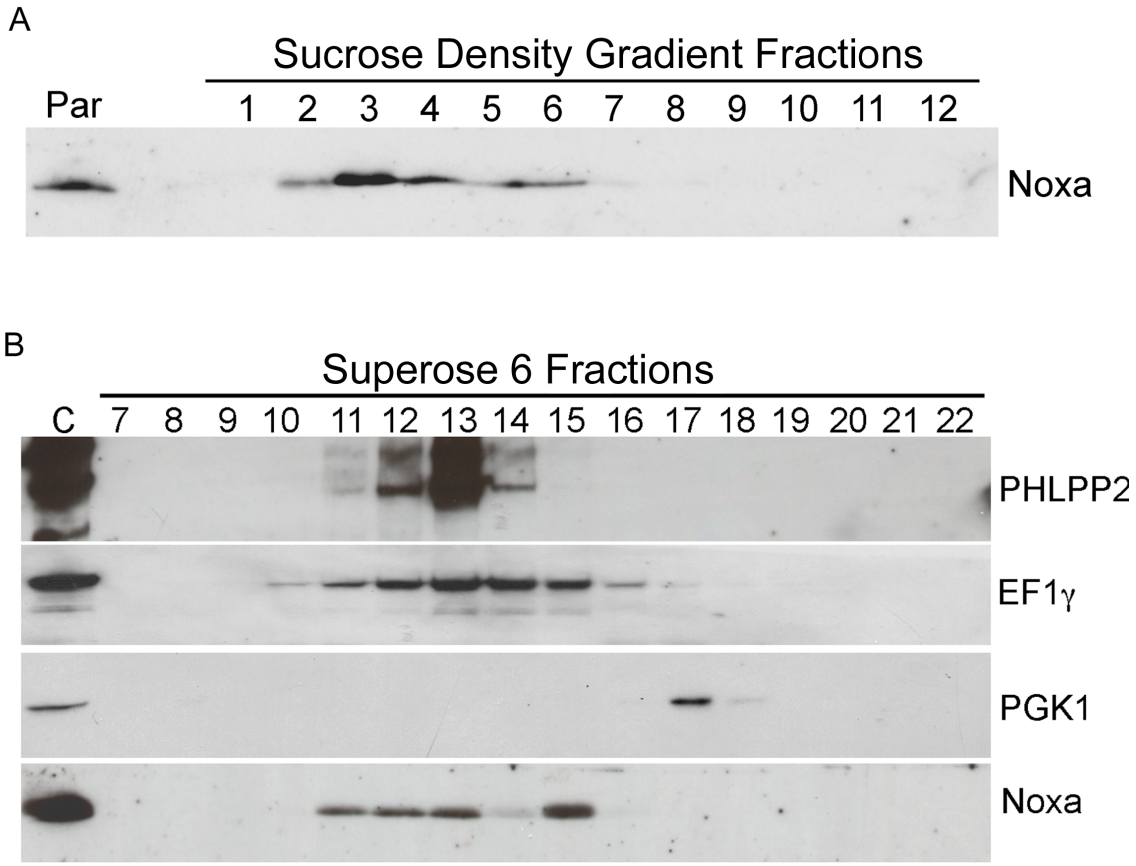


Figure 5-6. Western blot analysis of fractionated Jurkat extract purified by SDG and gel filtration. A. Unperturbed Jurkat cells were grown in standard conditions and made into lysate using Dignam buffer A (0.5% Igepal). The lysate was subjected to SDG centrifugation (5-30% sucrose) and eluted into 12 fractions. TCA preps were performed and run by SDS-PAGE, then immunoblotted for Noxa expression. An input of the original lysate (PAR) was used as a loading control. Fractions 2-6 were pooled, dialyzed, and concentrated prior to loading onto a Superose 6 column. B. Concentrated Noxa-enriched lysate was subjected to gel filtration at a rate of 0.1 mL/min. Twenty-two 1 mL fractions were collected and a tenth of the volume was used for TCA preps. An input control (C) of pre-loaded Superose 6 lysate was included on the SDS-PAGE. The gel was transferred to a membrane, blocked, and immunoblotted with antibodies against PHLPP2, EF1-γ, PGK1, and Noxa.

The Noxa DA BH3 domain point mutant does not bind to Mcl-1$_L$

We initially showed that Noxa associates with Mcl-1 in healthy proliferating cells (Figure 5-1B and 1C). Our studies had suggested that the two proteins did not directly bind to each other in the complex that assembled in proliferating cells. Therefore, we asked whether the BH3 domain was required for the formation of the complex. We re-expressed a BH3 domain point mutant Noxa DA (D32 mutated to A) in the Noxa knockdown cells (Nsh7 LV) to eliminate interference from endogenous Noxa. We also

transfected the Nsh7 LV cells with a pcDNA vector to serve as a control. We show by Western blot that Noxa is expressed in Jurkat parental, Noxa 5 (an overexpressing cell line), and Noxa DA cells, but not in Nsh7 or pcDNA transfected cells (Fig 5-8A). We further show that Mcl-1 immunoprecipitated Noxa in Jurkat parental cells, but not in the other Nsh7 LV-derived cell lines, including the line with the BH3 mutant, DA (Fig 5-8B). As expected, a Noxa antibody does not precipitate Noxa from Jurkat parental cells or from the knockdown lines. However, Noxa is robustly brought down in the Noxa DA cells. These results indicate that the non-binding mutant does not enter the complex and remains accessible and that Noxa DA does not interact with Mcl-1_L. Studies are ongoing to confirm this in FPLC fractionated extracts.

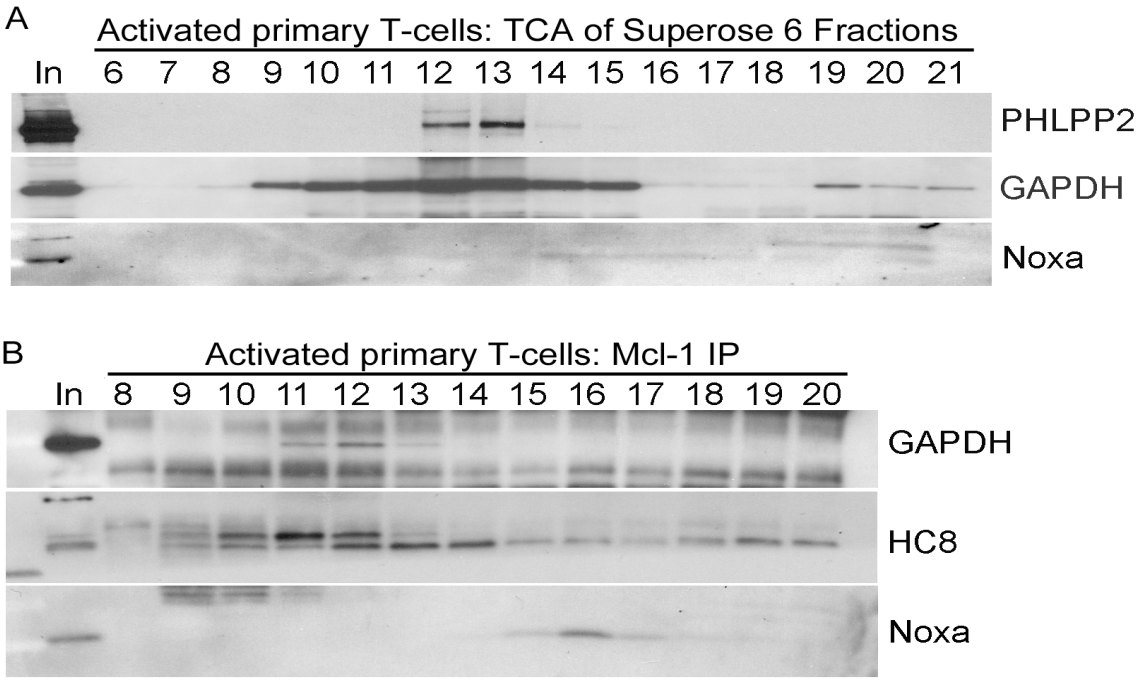


Figure 5-7. Components in the Noxa-containing complex fractionate and associate with Mcl-1 in activated primary T-cells. A) Primary T-cells were purified and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days. Cellular extract was made using the Dignam buffer A (0.5% Igepal) and loaded on to a Superose 6 column. Twenty-one 1 mL fractions were collected at a rate of 0.1 ml/min. A small volume (100 μL) was set aside for TCA preps and subjected to SDS-PAGE. The remaining volume was immunoprecipitated with anti-Mcl-1 pAb (B) overnight. The immunoprecipitated Mcl-1 complex was captured with Protein G Agarose beads and washed with NET-I buffer prior to separation by SDS-PAGE. Both gels were transferred to a membrane, blocked with 5% milk in TBS/0.1% Tween, and probed with antibodies against PHLPP2, GAPDH, HC8 and Noxa.

Noxa is not required for the formation of the complex

In order to continue our investigation of the contribution of phosphorylated Noxa to the assembly of these particles and, subsequently, to glucose metabolism, we asked whether the complexes could assemble in the absence of Noxa. Lysates from Noxa knockdown cells, described in previous chapters, were fractionated by gel filtration. Figure 5-9A shows that all of the proteins in complex B, except for Noxa (PHLPP2, EF1-γ, GAPDH, Mcl-1L, and HC8), were detected in expected fractions. The representative complex A protein, PGK1, was detected in later fractions, along with small amounts of GAPDH and Mcl-1. We immunoprecipitated Mcl-1L from fractions 8-19 to ascertain that these components still associated with Mcl-1. Although most of the GAPDH and HC8 emerged in fractions 8-21 and 9-15, respectively (Figure 5-9A), we detected a distinct population in Mcl-1 IPs of both proteins in higher molecular weight complexes (Figure 5-9B). It must be noted that we have detected GAPDH and HC8 in Mcl-1 complexes in a number of cancers of *epithelial* origin (Hanse and Kelekar, unpublished). Therefore, we hypothesize that the particles we identified in T cells are prototypes for glucose sensitive signaling complexes utilized by most cells to drive proliferation. The three proteins, Mcl-1_L, GAPDH, and hC8, may constitute the core of such a particle.

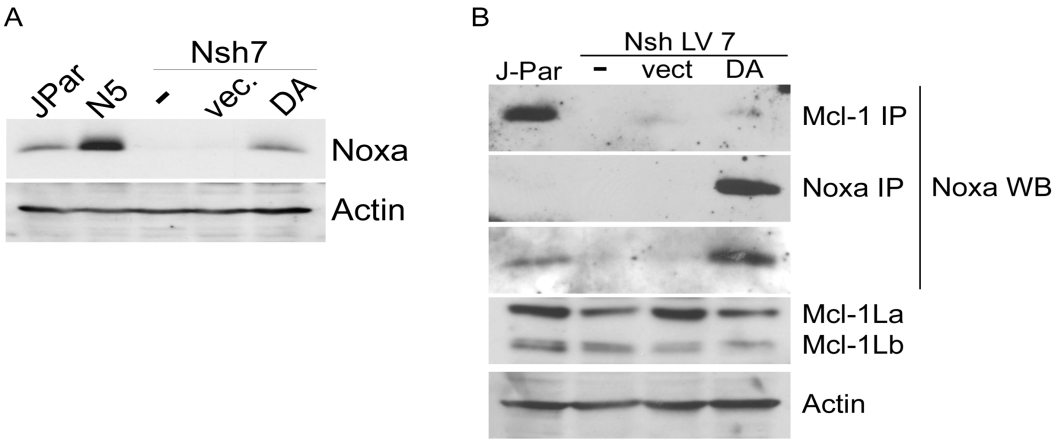


Figure 5-8. The Noxa binding mutant, Noxa DA, does not bind to Mcl-1. A. Noxa lentiviral knocked-down Jurkat cells were transfected with a pcDNA vector or a Noxa BH3 domain (DA) mutant in which the nucleotide sequence differed from WT, yet retained the correct WT amino acid sequence. Jurkat parental controls and a Noxa overexpressing Jurkat cell lysate was also made and run by SDS-PAGE and immunoblotted for Noxa and Actin expression. B. Jurkat Par and Nsh7 cell lines that were either not tampered with (-), transfected with pcDNA vector (vect) or the Noxa DA mutant (DA) were used to make RIPA lysate and subjected to either Mcl-1 or Noxa immunoprecipitation or simply loaded onto SDS-PAGE for Western blot analysis of Noxa, Mcl-1, and Actin expression.

The results described above also suggest that Noxa is not required for the assembly of the complex. Mcl-1 IPs using whole, *unfractionated* lysates of Noxa knockdown cells have confirmed the presence of all the originally identified proteins in IP/westerns (not shown). Given these observations, a possible role for Noxa within the complex is discussed later.

Conclusions

Noxa and its pro-survival binding partner, Mcl-1_L, interact in dying cells to facilitate cell death. We show for the first time that the two associate in proliferating Jurkat leukemia cells and stimulated primary lymphocytes, as well as in dying cells. In vitro studies showing phosphorylated Noxa does not interact with Mcl-1 suggest that the two proteins do not interact directly with each other within the multi-protein complex in healthy cells. However, purification of cellular extracts from both Jurkat leukemia and proliferating T-cells revealed Noxa precipitated with two large multi-protein particles. We identified the components of both complexes by mass spectrometry. We have optimized a protocol to purify the complexes and carried out some preliminary characterization to begin to understand their functional significance in glucose metabolism, cell proliferation and oxidative stress. We used Noxa knockdown cell lines and a non-binding Noxa mutant (Noxa DA) to show that Noxa is not essential for the assembly of the complex. Rather than affecting metabolism in any direct manner the function of Noxa in the complex may be to provide a platform that enables the cell to respond rapidly to an apoptotic signal such as glucose or nutrient deprivation. Studies in the Kelekar laboratory are pointing increasingly to a role for Mcl-1 as master regulator of this complex and of the glucose signaling response in collaboration with GAPDH. This will be discussed further in the final chapter.

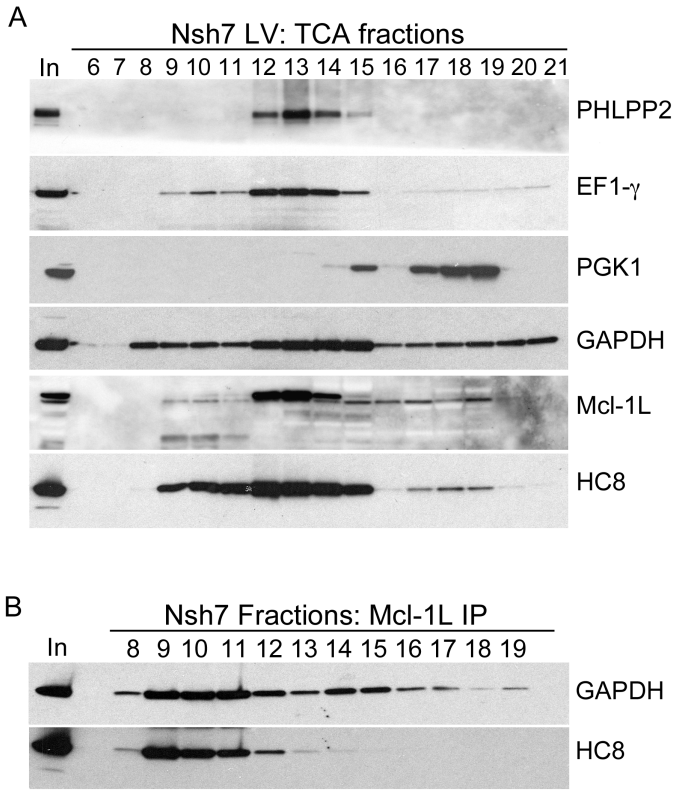


Figure 5-9. Western blot analysis of fractionated Noxa knockdown Jurkat cells (Nsh7) by gel filtration of TCA preps and Mcl-1 immunoprecipitation. Jurkat cells knocked down of Noxa using lentivirus (Nsh7 LV) were cultured in standard conditions prior to making cellular extract in Dignam buffer A (0.5% Igepal). The lysate was loaded onto a Superose 6 column and fractions were eluted at a rate of 0.1 mL/min. A. TCA preps were performed and subjected to SDS-PAGE for Western blot analysis of the following proteins: PHLPP2, EF1-γ, PGK1, GAPDH, Mcl-1, and HC8. Pre-loaded lysate was used as an input control (In). B. Fractions 8-19 was used for Mcl-1 immunoprecipitation. Complexes were captured using protein G agarose beads, washed in NET-I (0.05% Igepal) buffer, and resuspended in sample buffer. The samples were subjected to SDS-PAGE, transferred, blocked, and immunoblotted with anti-GAPDH and anti-HC8 antibodies.

Chapter 6
Discussion and Future directions

The studies described here offer a new and insightful look at human Noxa, a poorly understood member of the BH3-only subset of the Bcl-2 family. Most published studies on Noxa had focused on the murine protein, which is significantly structurally different from its human counterpart. We show that the smallest known Bcl-2 protein, is stably expressed, exquisitely regulated, and helps direct the fate of proliferating normal and malignant cells of hematopoietic origin. The post-translational modification of Noxa plays a significant role in regulating both cell division and death in these cells in response to glucose. We identified Cdk5 (referred to until recently as a 'neuronal' kinase) as the glucose-responsive Noxa kinase in lymphoid and myeloid cells. The high expression of Noxa and the availability for immediate activation as an apoptosis inducer makes it a highly attractive therapeutic target in leukemia. This study adds Noxa to the growing list of BH3-only proteins harboring functions in growth, survival and metabolism not intuitively attributed to this class of Bcl-2 proteins (Happo et al., 2012). In recent years, studies that have focused on the crosstalk between metabolism and apoptosis have revealed that these processes share many regulatory factors (Vander Heiden et al., 2009). A close relationship between cellular metabolism and cell death regulation in cancer cells ensures the cell's survival and continued proliferation. In elucidating dual and opposing roles for Noxa in glucose-sensitive signaling pathways, our studies offer proof for this concept (Lowman et al., 2010).

We conclude that the phosphorylation of Noxa targets it to multi-protein complexes. We have determined that Cdk5 is not a component of this complex, although we detect an interaction between the two proteins in cytosolic extracts. Studies to determine the effect of phosphorylation on Noxa structure and protein-protein interactions within the complex are ongoing. Preliminary evidence from a collaborative effort with Dr. D. Thomas and Dr. C. Karim in the BMBB department at the University of Minnesota points to a dramatic structural change in the phosphorylated protein that could have the ability to mask the BH3 domain. If phospho Noxa cannot directly interact with its canonical binding partner in the complex through its BH3 domain how does it remain within the complex? Studies are being directed at addressing this question. Current studies are also focusing on the functional significance of the novel Noxa/Mcl-1_L association observed in healthy proliferating cells. Although both proteins are induced and appear to be part of a multi-protein complex in activated lymphocytes and dividing leukemia cells, as stated earlier,

only the dephosphorylated form of Noxa interacts directly with Mcl-1_L. This increased expression correlates with the increased energy requirement of the expanding cell populations. The interaction, which requires an intact BH3 domain on Noxa, contributes to reducing the pool of activated lymphocytes following an immune response.

We have established a clear link between glucose deprivation and activation of the pro-death function of Noxa, and identified a growth-promoting role for the protein as well in leukemia cells and actively dividing primary thymocytes. Our results point to preferential glucose utilization in anabolic rather than catabolic pathways for the Noxa over-expressers, but this will have to be further investigated. Moreover, we have yet to interrogate the activity of significant glycolytic intermediates that have been shown to be crucial for biosynthesis in Noxa overexpressing cells. The low glycolytic rate observed in the Noxa overexpressing cells could be due to the immediate diversion of metabolites early in glycolysis. Additionally, we show that Noxa overexpressing cells are dependent upon glutamine. The breakdown of glutamine (glutaminolysis) is known to provide carbon and nitrogen sources, as well as energetic and antioxidant power to cancer and proliferating cells that primarily use glucose to generate biomass. Metabolite measurements using glucose and glutamine tracers show higher levels of lactate-derived glutamine in cells with higher levels of Noxa.

We observe that increased Noxa promotes increased ROS activity. Recent studies have shown that cancers have higher levels of oxidative stress as a result of deregulated metabolism and excess fuel uptake and ATP generation, which contributes to tumor malignancy (Parri and Chiarugi, 2012). Proliferating cells also tolerate higher oxidative stress. Oxidants may modulate proteins and damage DNA but the reductive anti-oxidant molecule NADPH can buffer its effects. It has been proposed that glycolytic enzymes often harbor the capacity to sense oxidative stress and divert glucose into pathways that would promote NADPH production (Anastasiou et al., 2011). The high NADPH/NADP ratios and their greater sensitivity to glucose levels support our hypothesis that a higher Noxa level drives glucose down pathways that generate NADPH, such as PPP.
We describe the purification of two large Noxa/Mcl-L containing multi-protein survival complexes from the cytosol of T leukemia cells and proliferating T cells. We have confirmed the protein composition of these complexes using two independent

approaches, and are currently working to further characterize them. The functional interactions within these complexes may shed light on how Noxa and glucose metabolism are regulated in dividing cells. We hypothesize that these complexes are glucose-responsive signaling particles and that complex B in particular enables metabolic flux via the PPP by inhibiting aerobic glycolysis. Our hypothesis is based on observation of a reversible ROS-responsive inactivating modification of the GAPDH residing in the complex and a putative, novel role for the elongation factor, EF-1γ as the ROS-sensitive 'modifier' of GAPDH (Eric Hanse, unpublished). Another component of complex B, the proteasome alpha 3-subunit isoform 1 (HC8) is a structural subunit of the eukaryotic 26S proteasome. The alpha subunits contribute to substrate selectivity and may be contact sites for regulatory complexes. HC8 may help to stabilize Mcl-1_L in actively proliferating cells or be one of the first proteasome components to associate with Mcl-1_L in glucose-deprived cells. We will investigate this further. Another important component is the okadaic acid-insensitive phosphatase PHLPP2, a member of the 'PP2C' class of phosphatases. It has two known targets to date, Akt and PKC (Gao et al., 2008). It terminates Akt signaling by dephosphorylation at T^{473} (Brognard et al., 2007). We hypothesize that PHLPP2 directly dephosphorylates Noxa in response to a stress signal such as glucose loss and early studies suggest that it interacts directly with phospho Noxa within the complex.

Our research has contributed in novel ways to the growing field of cancer cell metabolism and offered a better understanding of the metabolic requirements during T-cell activation and lymphocyte proliferation. The Kelekar laboratory is currently pursuing a number of questions that have emerged from the work described in this dissertation. For instance, these studies have the groundwork for the development of more effective drugs to target Noxa and activate its pro-apoptotic function in leukemia. In collaboration with medicinal chemists at the University of Minnesota we are designing highly specific inhibitors of Cdk5 that mimic the binding properties of specific inhibitor peptides of this kinase. We are also interested in elucidating glucose regulated signaling pathways upstream of Cdk5 in leukemia cells and studying the role of Cdk5 in T cell activation.

We have exploited the regulatory and structural differences between the murine and human Noxa protein to develop a conditional transgenic mouse expressing wild type

human Noxa as well as the non-phosphorylatable SA mutant in hematopoietic cells. This will be a crucial step towards studying the contribution of human Noxa to the growth and survival of hematopoietic cells in an in vivo setting and could ultimately lead to the design of therapies targeting the protein in leukemia.

Chapter 7
Materials & Methods

Reagents, Chemicals, Kits, and Antibodies

Antibodies (Ab) against Noxa (SC-56169, mAb and FL-54, pAb), Mcl-1_L (S-19, pAb and RC-13, mAb), Cdk5 (DC-17, mAb and C-8, pAb), EF1-γ (X5-P, mAb), PGK1/2 (H-300, pAb), 20S proteasome α3 (A-9: sc-166205)/HC8 mAb) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb against Actin was purchased from Calbiochem (Gibbstown, NJ) and mouse mAb against anti human-Noxa was purchased from IMGENEX (San Diego, CA). Rabbit pAb against PHLPP2 was purchased from Bethyl Laboratories, Inc (Montgomery, TX). The anti-pS13 Noxa pAb (rAk2, Western blotting) was developed by Dr. Ron Jemmerson (University of Minnesota). Cdk5 inhibitor (Roscovitine), MAPK inhibitor, and GSK3α/β inhibitor were all from Calbiochem (Gibbstown, NJ). Purified recombinant Cdk5 were from Upstate Biotech. Etoposide (ETOP) and Camptothecin (CPT) were from Sigma (St. Louis, MO). H2-DCFDA (D399) was purchased from Invitrogen (Grand Island, NY). Lactate and glucose were quantified using colorimetric reaction kits from BioAssay Systems (Hayward, CA) and NADPH/NADP levels were measured using a fluoro detection kit from Cell Technology, Inc (Mountain View, CA).

Cell culture maintenance of epithelial cell lines

A549 and H522 human lung cancer epithelial cell lines were grown in RPMI-1640 with 10% FBS, 100-units/mL penicillin/streptomycin, HEPES, sodium pyruvate, and L-glutamine. The cells were maintained in 5% CO_2 at 37°C and passaged when confluent. LN-CAP and C4-2 human prostate cancer cell lines were cultured in RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin antibiotics. DLD-1 cells were a gift from Tin Yin Gao (University of California at San Diego). Jurkat T-cell leukemia, Molt-4 T-cell leukemia, K562 erythroleukemia, and HL-60 promyelocytic cells were grown in RPMI-1640 with 10% FCS, 2 mM L-glutamine, 100-units/mL penicillin and 100-μg/mL streptomycin. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. If viability was >80%, cells were used in experiments; however, cells underwent gradient centrifugation with ficoll if viability was <80%.

Immunoprecipitation

For immunoprecipitation, cells were lysed in NET-I buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 0.5% IGEPAL) supplemented with protease and phosphatase

inhibitors (Calbiochem). Cell lysate was incubated with 1-5 μg/mL of antibody and complexes were captured with protein G Agarose beads (Invitrogen), immunoprecipitates were resolved by SDS-PAGE and processed for detection as described in the Western blot analysis section.

Western blot analysis

For Western blotting, cells were lysed in RIPA buffer (50 mM Tris-HCl, ph 7.5; 150 mM NaCl; 0.5% v/v sodium deoxycholate; 1% v/v Nonidet P-40; 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membrane blocking, washing, Ab incubations, and chemiluminescence reactions carried out using the ECL Plus kit (Amersham) or Super Signal West Femto kit (Pierce Biotech). Blots were stripped for reuse by washing for 30 minutes in TBS-T buffer (pH 2.5-3.0).

Plasmid constructs, RNA interference, and expression

The hNoxa wild type sequence and point mutants (S13A, S13E, D34A) were cloned into the pcDNA 3.1(+) vector (Invitrogen) by the lab prior to 2007. Carboxy terminal FLAG-tagged inserts were cloned into pGEX2T vector (GE Life Sciences) or into pcDNA 3.1(+) for recombinant protein production and transfection, respectively. For the Noxa shRNA constructs, sequence AACTTCCGGCAGAAACTTC, and a scrambled Noxa derivative sequence were cloned into BamH1 and EcoR1 sites of the pGSU6 vector (Gene Therapy Systems). Transfectants were selected in culture medium containing 1 mg/mL G418 (Cellgro). For the lentiviral knockdown lines, Noxa shRNA lentiviral particles containing four specific shRNA expression constructs (Santacruz Biotech and SBI) were transduced into Jurkat cells and selected for shRNA expression in 2 μγ/mL puromycin. Single cell clones were isolated by limit dilution analysis. For Cdk5 knockdown, the pGIPZ shRNAmir Cdk5 vectors and nonsilencing control were purchased from Open Biosystems (Cdk5 clone identification code V2LHS_62188). Selection of transfectants was carried out with 2 μg/mL puromycin.

Transient Transfection Assays

Electroporation of Jurkat or Molt-4 T-cell leukemia cells with pIRES2-EGFP vector (Clontech) alone or inserted with the hNoxa wild type or Noxa SA construct was carried

out with the following conditions: 975 μF, 250 mV, resistance set at infinity using a BioRad Electroporator. Transfection efficiency was monitored by flow cytometric analysis on the GFP fluorescence of ficolled cells. Jurkat cells were harvested at 16 and 24-hour intervals following separation of the cells into glucose +/- medium, and viability was determined by flow cytometric analysis of Annexin V-PE uptake on GFP+ cells.

Cell death assays

Exponentially growing hematopoietic cells were cultured in fresh medium before exposure to CPT (2 μM), ETOP (100 μM), glucose-deprived medium or hypoxia (2% O_2) for indicated time periods. Cell viability was measured by flow cytometric analysis of cells stained with Annexin V (Ann V)-FITC and Propidium iodide (PI) and analyzed using FlowJo Software (Tree Star, Inc). Ann V-PE-CY5 was used to detect apoptosis of EGFP expressing cells. Automated viable cell counts were also carried out by trypan blue dye uptake using a ViCell counter (Beckman Coulter).

Measurement of ROS activity

Exponentially growing hematopoietic cells were cultured in standard medium for indicated time periods. Cell viability was determined by trypan blue dye uptake using the ViCell counter. A cell concentration of 0.5 x 10^6 cells/mL was used for each triplicate/condition. The light sensitive ROS indicator, H2-DCFDA (Invitrogen) was added to the cells at a concentration of 10 μM (1:2000 dilution). Cells were incubated at 37°C for 20 minutes. One mL was processed and resuspended in FACS buffer (1% FBS and 0.1% NaN_3 in PBS) and immediately read by flow cytometry. Further data analysis was done using FlowJo Software (Tree Star, Inc).

***In vitro* kinase assays**

In vitro kinase assays with recombinant Cdk1 and Cdk5 (Upstate) were carried out according to the manufacturer's instructions. For *in vitro* assays of endogenous kinases, lysate protein (10 μg) harvested in Tonk's lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% IGEPAL, 25 mM β-glycerophosphate), supplemented with protease and phosphatase inhibitors, was incubated for 30 min at 30°C with kinase buffer (KB) (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ß -glycerophosphate), 10 μM cold ATP, 5 μCi [γ-^{32}P]-ATP and 250ng – 1 μg Noxa full length peptide per reaction. Assays were

attenuated with either 2.5 mM EDTA and used for immunoprecipitation, or with loading buffer for direct SDS-PAGE resolution. For cold kinase reactions the concentration of ATP was 0.1 mM. For in vitro assays of endogenous Cdk5, the kinase was captured with protein G agarose beads from pre-cleared lysates using an anti-Cdk5 Ab, washed in NET-I and KB, resuspended in KB before being added to the reaction mix.

Sucrose density gradient centrifugation

Cell extract prepared in Dignam lysis buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.05% IGEPAL, 0.5 mM DTT, pH 7.9) was loaded on to six tubes with a 5-30% sucrose density gradient (SDG). The tubes were centrifuged for 18-24 hrs at 25k rpm at 4°C. Twelve fractions from the six tubes were collected and pooled. 500-1000 μL of each fraction were subjected to trichloroacetic acid and acetone preps as well as Western blot analysis to identify which fractions contained Noxa.

Gel exclusion chromatography

Cell extract (0.5-1 mg protein) prepared in lysis buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.05% IGEPAL, 0.5 mM DTT, pH 7.9) was loaded on to a Superose 6 column (GE/Amersham) previously equilibrated with the lysis buffer A and eluted in the same buffer at a constant flow rate of 0.1 mL/min. Twenty-two to thirty-two fractions of 1 mL were collected. Proteins were precipitated using trichloroacetic acid and acetone, and Noxa was detected by Western blotting and quantified by densitometry. The following proteins were used as molecular weight standards: Blue Dextran (2,000 kD), Thyroglobulin (669 kD), Ferritin (474 kD), Catalase (232 kD), and Aldolase (160 kD).

Measurement of glycolysis

Glycolysis was measured by monitoring the conversion of 5-[^{3}H]-glucose to [^{3}H]$_2$O, as described previously (Liang et al., 1997; Vander Heiden et al., 2001). Briefly, 1 x 10^6 cells were washed and incubated in G-F medium for 30 min at 37°C. Cells were then pelleted, resuspended in 0.5 mL of Krebs buffer containing 10 mM glucose and spiked with 10 μCi of 5-[^{3}H]-glucose. Following incubation for 1 h at 37°C, triplicate 50-μl aliquots were transferred to uncapped thin-walled PCR tubes containing 50 μl of 0.2 N HCl, and each tube was transferred to a scintillation vial containing 0.5 ml of H_2O such that the liquids in

the vial and the tube did not mix. Vials were sealed and diffusion was allowed to occur for 36 h. The rate of glycolysis was calculated as described previously (Ashcroft et al., 1972) after determining the total amount of $[^3H]_2O$ in each sample taking into account appropriate 5-[^{3}H]-glucose- and $[^3H]_2O$-only controls.

Targeted metabolomics

More than 25 x10^6 exponentially growing hematopoietic cells were cultured in standard RPMI-1640 medium prior to incubation in different RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10 mM $^{13}C_{1,2}$-glucose, medium containing 10 mM D-glucose and 2 mM 13-U-glutamine, or in unlabeled RPMI-1640 medium with 2 mM L-glutamine and 10 mM D-glucose. After 24 h of incubation, the cells were centrifuged and one mL aliquots referred to as 'spent medium' were stored at -80°C. The remaining supernatant was discarded and the cell pellet washed in cold PBS. The cells were centrifuged at 1500 rpm for 5 min prior to resuspension in 400 μL cold methanol. The cells were immediately stored in -80°C and shipped to Mayo Clinic (Rochester, MN). Dr. Tumpa Dutta performed gas chromatography-mass spectrometric analyses on the provided samples.

Human Primary T cells- preparation and stimulation

Peripheral blood mononuclear cells were isolated by ACCUSPIN System-HISTOPAQUE- 1077 (Sigma-Aldrich) density gradient centrifugation. CD4+ and CD8+ T cells were co-purified by positive selection via the MACS system (Miltenyi Biotec). Sample purity, assessed by FACS on the LSRII (Becton Dickinson) was >88% CD3+ and >99% CD4/8+ T cells. Tissue culture plates were coated overnight at 4°C with PBS alone or with 1 μg/mL anti-CD3 (Muromonab, Orthoclone-OKT3) in PBS. For stimulation, T cells were resuspended in media with 5 μg/mL anti-CD28 (BD Biosciences), plated and incubated at 37°C in 5% CO_2 for 24 h. For glucose withdrawal experiments, stimulated cells and unstimulated controls were washed twice in either glucose-deficient or glucose-sufficient RPMI medium. Cells suspensions were then replenished with anti-CD28 and plated on fresh tissue culture plates pre-coated with anti-CD3. Cells were harvested processed into lysate after an additional 15-38 h.

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