REGULATED RECRUITMENT OF A TRANSCRIPTIONAL COREPRESSOR

by

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction:

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LIST OF ABBREVIATIONS

Oxidized nicotinamide adenine dinucleotide, (NAD^+); reduced NAD^+, (NADH); silent information regulator protein, Sir2; carboxyl-terminal binding protein, CtBP; fluorescence resonance energy transfer, FRET; adenosine triphosphate, ATP; acetyl-Coenzyme A, acetyl-CoA; TATA binding protein, (TBP); TBP associated factors, (TAFs); downstream promoter element, DPE; initiator, INR; preinitiation complex, (PIC); carboxyl-terminal domain, (CTD); nuclear hormone receptor-interacting protein, RIP140; estrogen response element, (ERE); cyclic AMP response element, (CRE); chromatin immunoprecipitation, ChIP; glucocorticoid response element (GRE); histone acetyltransferase, HAT; histone deacetylase, HDAC; nitric oxide, NO; nitrosonium ion, NO^+; sulfenic acid, SOH; sulfinic acid, SO_2H; sulphonic acid, SO_3H; protein tyrosine phosphatase 1B, PTP1B; hypoxia-inducible factor; MAPK kinase kinase, MEKK1; glyceraldehyde 3-phosphate dehydrogenase, GAPDH.

Abstract

In single-cell organisms, such as bacteria, metabolic control of transcription is a central mode of gene regulation. In multicellular organisms, with different organs having unique metabolic functions and demands, the connection between gene regulation and metabolism is significantly more complicated and less well understood. While the links between the two processes in higher species have remained more elusive, they are expected to exist.

Recently, the dependence of several transcriptional coregulators on important metabolic molecules has aroused the interest of many researchers in elucidating how gene expression may be under the control of the metabolic state of the cell in higher species. In this thesis, I explain how the metabolic state is composed of the energy state and the redox state by reviewing the components that contribute to each. The integral role of oxidized nicotinamide adenine dinucleotide (NAD^+) and reduced NAD^+ (NADH) in both components of metabolism, and the ability of silent information regulator protein (Sir2) and carboxyl-terminal binding protein (CtBP) to bind pyridine dinucleotides, highlights this redox pair as a potential link between metabolism and gene expression in higher species. The biological relevance of the pyridine dinucleotide binding events depends on the affinity between the binding partners and the concentration of the free pyridine dinucleotides in cells. I determined the affinity of CtBP for NAD^+ and NADH and presented a collection of data suggesting the relevant cellular concentrations of these pyridine dinucleotides. In summary, I provide strong support for NADH serving as an important signaling molecule linking gene regulation to the metabolic state of the cell through its interaction with CtBP.

Introduction

The regulation of gene expression by the oxidative state of the cell has been under investigation for over a decade. Many transcription factors and proteins involved in signaling cascades that activate gene expression appear to be regulated by the oxidative state of the cell through a variety of mechanisms [1, 2]. More recent advances in the study of gene expression have uncovered a new theme linking transcriptional coregulators to the metabolic state of the cell via their dependence on molecules central to cellular respiration [3, 4].

The metabolic state encompasses the energy state and the redox state. The energy state describes the availability of fuel sources (glucose, lipids) and useable energy (ATP) while the redox state describes the oxidative potential that reflects the balance of the molecules able to undergo oxidation/reduction reactions. The redox pair, NAD^+/NADH, tightly couples the redox state to the energy state by serving as the link between fuel sources and useable energy. Thus, the dependence of transcriptional corepressors on NAD^+ and NADH ideally suits these major regulatory proteins to sense both the energy and redox state of the cell.

Energy is the fundamental requirement for life. Every cell must have an energy supply, the ability to convert that energy to a useable form and the mechanism to remove waste products. In cellular respiration, the useable energy is ATP and the waste products are CO_2 and H_2O. The energy supply consists of higher molecular weight carbon sources such as carbohydrates and lipids. The process of converting the energy supply to useable energy is a highly regulated process that involves the transfer of reducing equivalents from the supply source to the final acceptor oxygen. Organisms have storage mechanisms

installed to avoid starvation should the energy supply become low. Just as overproduction of ATP can result in starvation due to a depleted energy supply, underproduction of ATP would halt the many cellular processes that require it. It is important for a cell to maintain a constant level of the useable ATP. Because essentially every cellular process requires ATP, it must be continuously replenished. When the supply just meets the demand, there is no need to store the supply and cellular processes can operate efficiently. The concentration of ATP in a cell is important for maintaining its own levels by acting at the level of phosphofructokinase. Increased levels of ATP inhibit glycolysis and stimulate gluconeogenesis and glycogen synthesis. Decreased ATP levels promote the catabolic processes to synthesize more ATP. When carbohydrate supplies are depleted, other sources are utilized including lipids and proteins. Lipids undergo beta-oxidation where $NADP^+$ is reduced to NADPH and the carbons exit as acetyl-CoA. Proteins are broken down to amino acids which are also converted to acetyl-CoA. Acetyl CoA can then enter the Kreb's cycle. Acetyl-CoA is perhaps the most central of all intermediary metabolic molecules bridging the major anabolic and catabolic processes. Similar to ATP, acetyl-CoA levels remain stable being situated in the center of essentially all synthetic and breakdown pathways. Thus, a significant decrease in ATP or acetyl-CoA would represent a state of severe starvation or metabolic deregulation. In the absence of sufficient amino acid and ATP levels, gene transcription that leads to protein synthesis would be a waste of the already depleted energy supplies. It is possible that a drop in acetyl-CoA and ATP levels during states of severe metabolic stress could inhibit gene expression through their requirement by transcriptional coactivators.

As mentioned earlier, the pyridine dinucleotides NAD^+ and NADH appear to be suited to serve as metabolic indicator molecules reflecting the cell's energy and oxidative state. The central question addressed by this thesis is whether gene expression is regulated by the metabolic state of the cell through the electron carrier redox pair NAD^+/NADH. The focus of this thesis considers the ability of the transcriptional corepressor CtBP to serve as a metabolic sensor. The report that NAD(H) enhances the interaction between CtBP and repressor proteins suggested that CtBP may modulate gene expression under the control of metabolic events [5]. The hypothesis that CtBP serves as a sensor of cellular metabolism has been controversial. The conflict surrounding the potential for CtBP to provide such an important link between gene expression and the metabolic state of the cell led to studies designed to measure nucleotide binding.

Transcription

Transcription is the synthesis of an RNA molecule from a DNA template molecule. The transcription process requires that RNA polymerase binds to DNA at promoter elements, the two strands must be unwound and the RNA synthesized based on the sequence of the DNA. RNA polymerase II is the enzyme the carries out the synthesis of an RNA polymer. Ribonucleoside triphosphates are used to extend a growing strand in a reaction that releases pyrophosphate from the newly added nucleotide. A major part of regulation is the binding of RNA polymerase II to the TATA core promoter of a gene [6]. The binding of RNA polymerase II to a gene's promoter is an orchestrated process involving many components of the RNA polymerase complex, including TATA binding protein (TBP) and many TBP associated factors (TAFs) [7, 8]. Important steps in the

transcription process include DNA melting, initiation and RNA elongation. Post-translational modifications of the RNA polymerase complex dictate binding partners and the complex's structural organization. Two other core promoter elements are the downstream promoter element (DPE) and the initiator (INR) [9]. These elements are short, highly conserved and found in nearly all protein-encoding genes.

The preinitiation complex (PIC) is composed of TFIID, TBP and TFIIA which then binds TFIIB. The TAFs and TBP unite with TFIIF, followed by TFIIE and TFIIH. RNA polymerase contains a carboxyl-terminal domain (CTD) that is an important site of regulation by phosphorylation [10]. TFIID/TBP recognizes the core promoter and recruits TFIIB. TFIIA stabilizes TFIID and promoter binding whereas TFIIF assists RNA polymerase II to reach the promoter. TFIIE recruits TFIIH and modulates TFIIH's helicase activity.

Other DNA elements important for proper recruitment of the PIC are the enhancer elements that facilitate activation of transcription. The enhancer elements are bound by a family of sequence-specific transcription factors that activate gene expression. Some examples of activators include SP-1, Oct and GATA-1 [11]. Enhancer regions may consist of several elements recognized by different activators. Opposing the regulation of enhancers are the silencers. Silencers are the DNA elements that are recognized by repressors to inhibit transcription of a gene [12]. Some DNA elements, such as the E box, can differentially regulate gene expression by the binding of either activators or repressors [13, 14].

Response elements are recognized by transcription factors that are activated by a particular ligand or stimulus. Response elements are usually within 1000 bp of the

transcriptional start site. Examples of response elements are the estrogen response element (ERE), cyclic AMP response element (CRE) and the glucocorticoid response element (GRE) [10].

A regulated recruitment of RNA polymerase is suggested by the many different DNA elements that are recognized by many different transcription factors. A question that has not been addressed is how these DNA elements promote recruitment of RNA polymerase II to a particular gene. The transcriptional coregulators serve to bridge the DNA-binding proteins to the PIC for the synthesis of RNA transcript. Further study is demonstrating that the function of transcriptional coregulators goes beyond serving as a bridge, or adaptor protein. Several coregulators have enzymatic activities and are involved in altering the structure of chromatin [15]. Before describing the various transcriptional coregulators, the structure of chromatin will be discussed to provide a better understanding of the obstacles faced by the RNA synthetic machinery.

Chromatin

Chromatin is the material in the nucleus of a cell that contains the genetic information required for the synthesis of proteins. Chromatin is packaged into units called chromosomes. Chromosomes undergo major structural changes throughout the cell cycle being highly condensed during metaphase and much less condensed during interphase. Cytologists have long observed that chromatin can be arranged in two structural configurations termed heterochromatin and euchromatin (Figure 1). Heterochromatin is the condensed form of chromatin and is more abundant in resting cells and is heterochromatin is transcriptionally inactive. Euchromatin is less condensed,

has a threadlike appearance and is considered to be transcriptionally active. Note in figure 1 that heterochromatin is disrupted near the nuclear pore to prevent blocking transport.

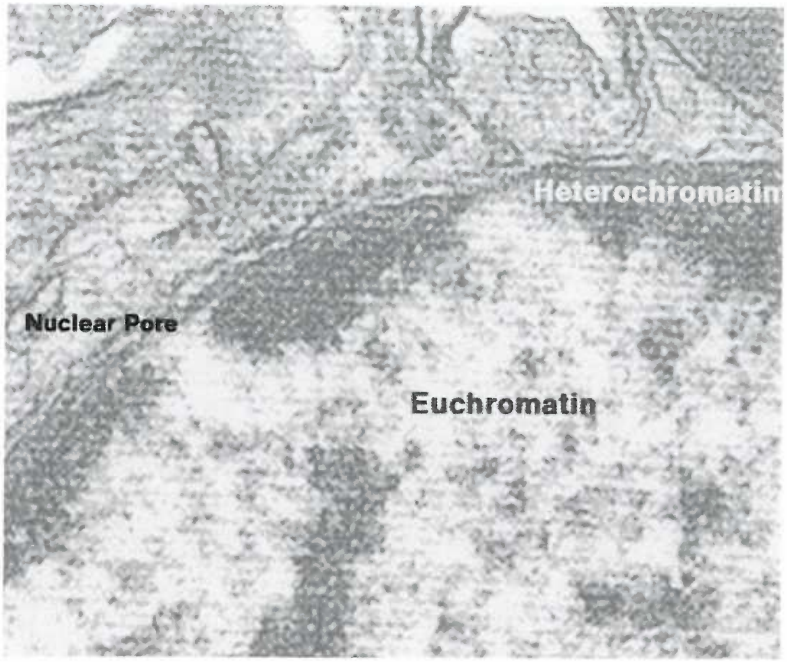

Figure 1. Taken from Molecular Biology of the Cell, Alberts, B. et al.

The length of DNA in a single human cell is 300,000 times longer than the diameter of the nucleus that contains it. In order to accommodate this apparent discrepancy between the length of DNA and the volume of the nucleus, DNA is organized into several structural layers [16]. The primary level of organization is the nucleosome [17]. The nucleosome is composed of DNA wrapped around a histone protein octamer containing two copies each of the core histones H2A, H2B, H3 and H4. The nucleosome core DNA is wrapped around the octamer 1.7 times, equivalent to 146 base pairs. The DNA between the nucleosomes is termed the linker DNA and is bound by the linker histone H1. Several nucleosomes in tandem appear as "beads on a string." The secondary level of organization is the coiling of nucleosome beads into a helical structure called the 30 nm

fiber present in both interphase and mitotic chromatin. The tertiary level of organization is the arrangement of the 30 nm fiber into loops, scaffolds and domains.

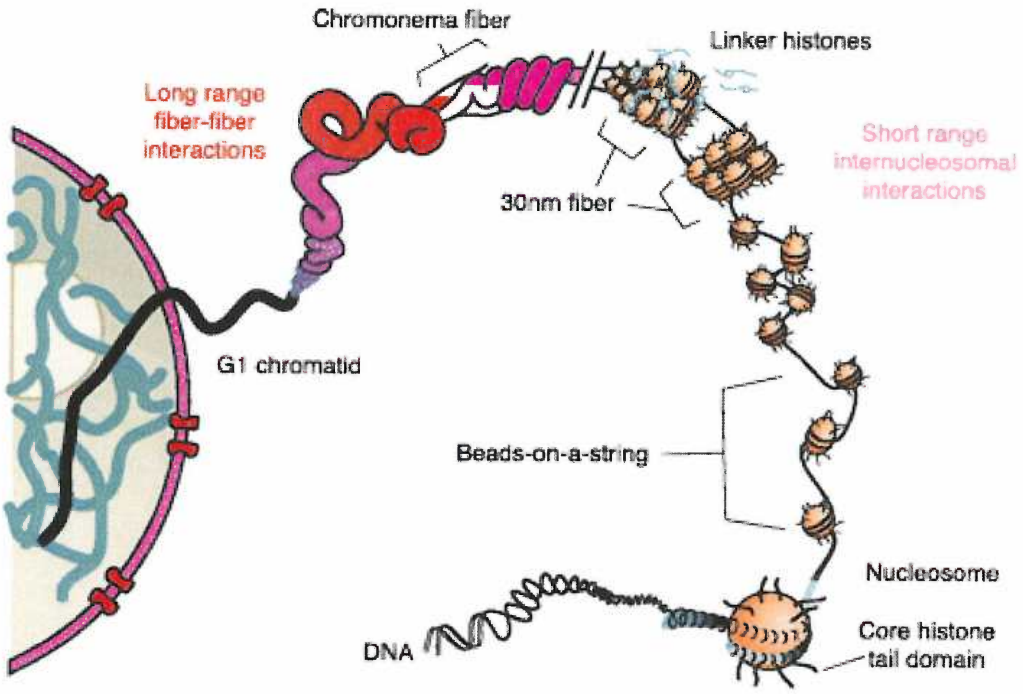

Figure 2. Taken from Johnstone R.W. Nature Reviews Drug Discovery 2002;Volume 1: 287-299.

Histone proteins are basic at neutral pH due to an abundance of lysine and arginine residues while DNA is negatively charged due to the phosphate backbone. These attracting charges are largely responsible for the DNA/histone interaction. The histone amino terminus tails are important for primary and higher levels of chromatin structure. Thus, the post-translational modification of the amino termini influences chromatin structure and gene expression [18]. There are several different types of post-translational modifications that occur on histone proteins. These modifications influence histone / DNA interactions, histone / nonhistone protein interactions and higher order chromatin structure such as the packaging of nucleosomes. Acetylation of histone lysine residues is

generally associated with gene activation [19, 20]. Acetylation neutralizes the charge of lysine weakening the interaction with the DNA phosphate backbone and relaxing the structure of chromatin and increasing access of transcription factors and machinery. Lysine acetylation is important for much more than disrupting DNA histone interactions, an acetylated lysine serves as a binding module recognized by proteins containing bromodomains [21]. The ability of a bromodomain to bind a particular acetylated Lys with a high degree of specificity is important for proper translation of the histone code.

Acetyl-CoA-Dependent Histone Acetylation

Histone acetylation promotes gene activation by altering the structure of chromatin towards a more relaxed state and by facilitating the recruitment of additional chromatin remodeling and transcription complexes. Histone acetyltransferases are the proteins that catalyze the acetylation reaction. There are several families of HATs based on sequence and function including the GNAT, MYST and CBP/p300 [22]. The HATs share a weak homology, most significant over the acetyl-CoA binding region. The crystal structures of several HAT family members revealed that the different HAT families are structurally similar despite the weak sequence homology [22]. Each HAT is also thought to carry out a similar catalytic mechanism where a glutamate residue serves as an active-site base to abstract a proton from the epsilon amino group of the lysine substrate. The deprotonated lysine of the substrate then serves as a nucleophile that reacts with the acetate carbonyl of acetyl-CoA to generate CoA-SH and acetylated (Lys) protein. HATs have been shown to acetylate non-histone proteins such as p53 and RIP140 to promote gene activation without directly affecting chromatin structure. Acetylation stimulates p53

transactivation by a mechanism that is still under dispute [23]. Acetylation relieves RIP140 repression by disrupting its interaction with a transcriptional corepressor [24].

A histone code based on sequential post-translational modifications of the amino termini has been proposed [25]. The detailed mapping of amino termini modifications by chromatin immunoprecipitation (ChIP) analysis during activation of human IFN-β has suggested how cells process the histone code based on the DNA code of the enhancer. The DNA code provides the information for the assembly of the higher-order three-dimensional transcripition factor / enhancer DNA complex termed the enhanceosome. The enhanceosome recruits the histone modifying proteins that print the histone code. Translation of the histone code involves the recruitment of transcription complexes to the enhanceosome and specifically acetylated histone aminotermini. Figure 3 depicts a model for the histone acetylation code for activation of the IFN-β gene in response to viral infection. Panel A represents a nucleosome with histone tails exposed for modifications based on the DNA code of the enhancer. Panel B represents the formed enhanceosome recruiting the HAT GCN5. In Panel C and D the histone code is printed. GCN5 acetylates H3 on Lys9 and H4 on Lys8. Phosphorylation of H3 on Ser 10 by an unidentified kinase is a prerequisite to acetylation of H3 on Lys14 by GCN5 shown in panel D. Translation of the code involves recruitment of the bromodomain containing complexes SWI/SNF and TFIID as well as the replacement of GCN5 with CBP.

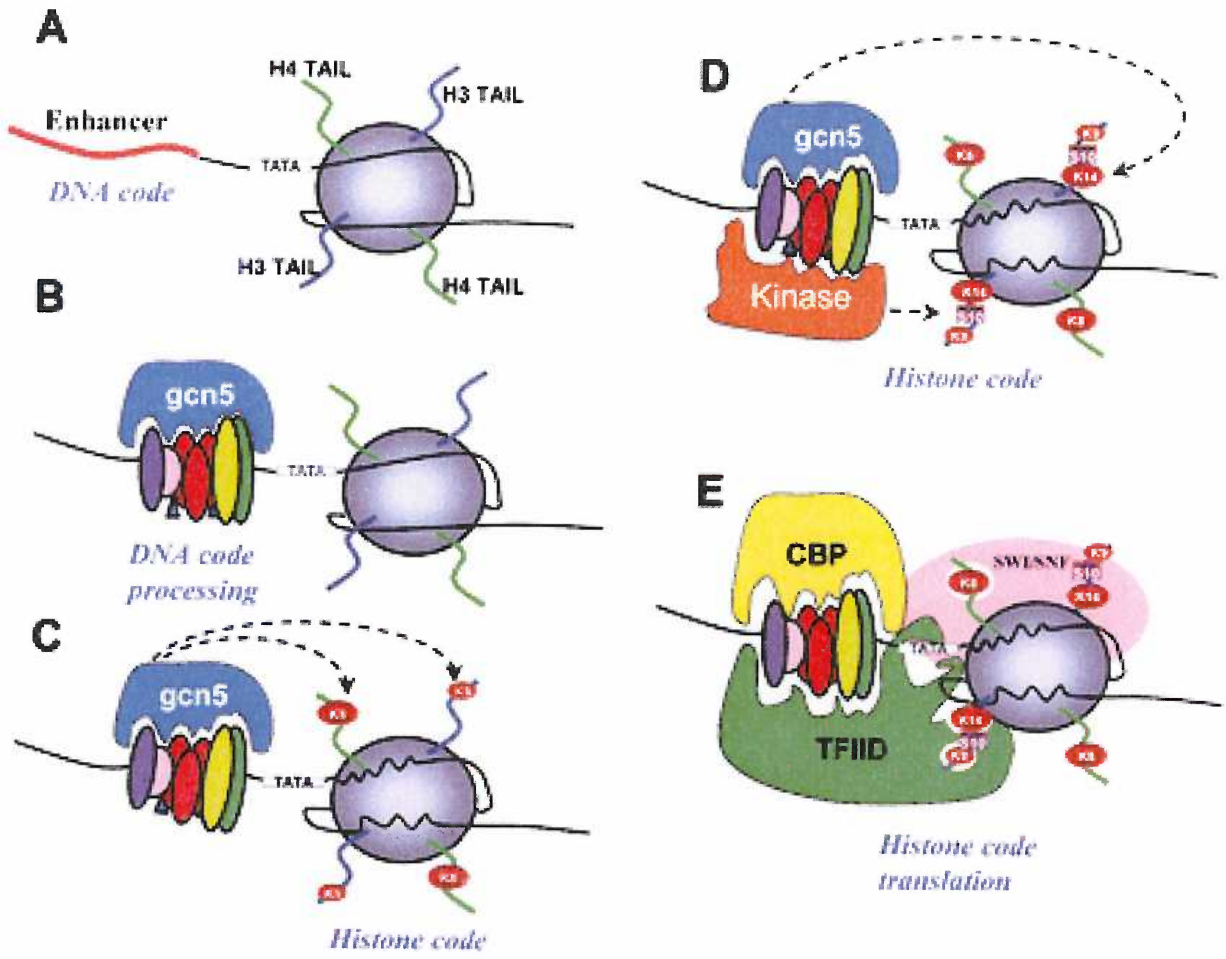

Figure 3. T. Agalioti, G. Chen, and D. Thanos Cell, Vol.111, 381-392 [25].

NAD$^+$-Dependent Protein Deacetylases

Histone deacetylases (HDACs) reverse the post-translational modifications of the HATs. There are two families of histone deacetylases. The first is composed of loosely related class I and II HDACs [26]. These enzymes serve a corepressor function and utilize a mechanism involving the hydrolysis of free acetate from lysine residues. The second family of histone deacetylases is the Sir2 family [4, 27]. The Sir2 family of histone deactylases require NAD$^+$ for deacetylation where the ADP-ribose moiety of NAD$^+$ serves as the acetate acceptor. Nicotinamide is a byproduct of the reaction and

serves as an effective inhibitor of SIR2 [28, 29]. The K_i of Sir2 for nicotinamide is similar to the cellular levels of nicotinamide suggesting that the inhibition may be relevant to Sir2's cellular functions. The regulation of Sir2 activity remains controversial and is discussed in much more detail in the Discussion chapter of this thesis.

ATP-Dependent Chromatin Remodeling Complexes

ATP-Dependent Chromatin Remodeling Complexes have been categorized into three families based on the identity of the catalytic ATPase subunit: the SWI2/SNF2 family, the ISWI family and the MI-2 family [15]. ATP-dependent chromatin remodeling proteins act in concert with other coregulators to assist in the rearrangement of chromatin towards a more or less folded state. These proteins use the energy of ATP hydrolysis to influence the higher order chromatin structure by acting on nucleosomal arrangement [30]. The activity that seems best characterized is nucleosome sliding by proteins like NURF [31]. It has been suggested that these ATP-dependent chromatin remodelers may have a DNA helicase activity important for unwinding DNA wrapped around the core histones. Two models proposed for the mechanism utilized by remodeling proteins are sliding and conformational change. Sliding involves changing the translational position of nucleosomes on DNA to expose new regions of DNA. Conformational change refers to altering the shape of the histone octamer and/or the DNA. Various assays have been developed to better understand the activities of the different remodeling families including altered restriction enzyme access and assembly of nucleosomes. The precise mechanism and alterations of the ATP remodeling proteins may be difficult to tease apart because these proteins are components of a larger complex that works within an even

larger machinery. The involvement of the ATPase activity in gene regulation is supported by the data demonstrating that the activity within ISWI and Mi-2 complexes is stimulated by nucleosome substrates [32, 33].

Metabolic State

NADH as an Indicator Molecule

Molecules central to cellular respiration reflect both the energy state and oxidative state of a cell. A basic understanding of the many factors that influence the levels of NADH is necessary to appreciate the significance of a transcriptional coregulator sensitive to NADH. NADH is in equilibrium with NAD^+ having a redox potential of –3.9 mV. NADH is a product of glycolysis and the Krebs cycle. NADH is also in equilibrium with NADPH, an important molecule for biosynthetic pathways. NADH is a substrate for oxidative phosphorylation where its reducing equivalents are transferred to molecular oxygen to yield water and provide the proton gradient required for ATP synthesis. Thus, NADH levels reflect levels of molecular oxygen and other oxygen species related to oxidative stress such as hydrogen peroxide, superoxide, hydroxyl radical and nitrogen oxide. The integral role of NADH in ATP synthesis, as a product of carbon breakdown and a substrate required for maintaining a proton gradient, demonstrates the ability of NADH to serve as an indicator of a cell's energy state. NADPH's role in the reduction of glutathione, by glutathione reductase, suggests another link between NADH and the cell's oxidative state. The reduction of glutathione is critical for maintaining protein cysteine residues in a reduced state. Oxidation of protein cysteine residues includes disulfide formation as well as the formation of other oxidized forms such as the sulfenic,

sulfonic and sulfinic acid derivatives [34]. Glutathione is able to reduce disulfide bonds and reverse the formation of sulfenic acids to free sulfhydrals. Oxidation to the sulfenic acid is now accepted as an important regulatory modification of several proteins containing critical cysteine residues such as caspases and protein tyrosine phosphatases. NADH is linked to the oxidation state of protein cysteine residues through the enzyme that monitors the NADH/NADPH equilibrium -- NADH transhydrogenase. Transition metals, particularly Fe, are also important components of a cell's oxidative state. The ability of Fe^{+2} to be oxidized by oxygen species and undergo fenton chemistry provides the link between the redox balance of transition metals and NADH.

Reactive oxygen species

Molecular oxygen is the ultimate recipient of reducing equilvalents generated from the catabolism of carbons sources. The electron configuration of molecular oxygen allows it to accept one electron forming the highly reactive superoxide radical that is rapidly dismutated without catalysis. Superoxide dismutase accelerates the dismutation 10^4 fold in a reaction converting superoxide to hydrogen peroxide and molecular oxygen at a rate approaching diffusion [35]. A two-electron reduction of molecular oxygen yields hydrogen peroxide. Hydrogen peroxide is more stable than superoxide and is thought to serve as a more selective signaling molecule. Reactive cysteine residues with lowered pK_a values are targets of hydrogen peroxide. Hydrogen peroxide is also reactive with reduced transition metals such as Fe^{+2} to produce hydroxyl radical and Fe^{+3}. The roles of reactive cysteine residues, hydrogen peroxide, and transition metal redox reactions are discussed later. A three-electron reduction of molecular oxygen yields water and a

hydroxyl ion while a four-electron reduction of oxygen results in the formation of two water molecules. In summary, superoxide and hydroxyl radicals are highly reactive and essentially oxidize the first molecule with which they collide resulting in an irreversible oxidation while hydrogen peroxide seems to act more specifically targeting protein thiols and reduced transition metals. The oxidation of thiols appears to be a reversible modification.

Reactive nitrogen species have important roles in redox chemistry and cellular signaling. Nitric oxide (NO) is synthesized by nitric oxide synthases from L-arginine in a reaction yielding the side product L-citrulline. The roles of reactive nitrogen species are still under debate. The ability of nitric oxide to activate guanylate cyclase by interacting with the ferrous heme of the enzyme was the first known physiological function of NO. The ability of NO to bind to ferric heme is thought to promote the nitrosation of biological nucleophiles. The chemistry of this modification involves transfer of an electron from the nitric oxide radical to the ferric heme forming ferrous heme and the electrophilic nitrosonium ion (NO^+). Nitrosation of reactive thiols is a modification similar to the oxidation of cysteine by hydrogen peroxide. Nitrosation of a cysteine residue can be reversed in a manner similar to the reduction of sulfphenic acids discussed below. Another method of nitrosating thiols is by the formation of N_2O_3 from the oxidation of NO with O_2. There are other proposed mechanisms for nitrosating thiols including the reaction of NO with superoxide to form peroxynitrite. The precise mechanism(s) involved in thiol nitrosation are still under investigation. Nevertheless, S-nitrosthiol formation is a physiologically occurring phenomenon connected to the redox

environment of the cell via reactivity with transition state metals, thiols and oxygen species.

There are several enzymes that function to rid cells of reactive oxygen species. The first, superoxide dismutase, was already mentioned. Three enzymes that remove hydrogen peroxide are catalase, glutathione peroxidase and thioredoxin peroxidase. Catalase carries out a dismutation reaction converting hydrogen peroxide to water and and oyygen. Glutathione peroxidases make up a family of selenium proteins that reduce hydrogen peroxide using glutathione to produce oxidized glutathione and water. Thioredoxin uses its own reactive cysteine residues to reduce hydrogen peroxide to produce a disulfide and water.

Redox Thiols

The sulfur of cysteine residues can be oxidized to a disulfide bond, a sulfenic acid (SOH), a sulfinic acid (SO_2H) or a sulfonic acid (SO_3H) [34]. The sulfinic and sulfonic acids are essentially irreversible modifications. At physiological pH most protein cysteine residues (pK_a = 8.3) are not susceptible to oxidation by reactive oxygen species. Some proteins contain cysteine residues that are unusually reactive due to their unique protein environment such as a cysteine coordinating a metal ligand or near a protein residue capable of reducing the cysteine's pK_a. Protein cysteines with a reduced pK_a are in the reactive thiolate (S-) form at cellular pH. The thiolate can react with hydrogen peroxide to form the sulfenic acid. The sulfenic acid can lead to an intramolecular disulfide or a disulfide with glutathione. A second glutathione molecule will regenerate the protein free thiol (SH) and yield oxidized glutathione (GSSG).

Two families of enzymes that are oxidized by reactive oxygen species are the protein tyrosine phosphatases and the caspases. Structural studies have provided a closer look at cysteine oxidation of protein tyrosine phosphatase 1B. Hydrogen peroxide is considered a second messenger required for full activation of protein tyrosine kinase signal transduction pathways [36]. One mechanism of activation is the transient inhibition of PTP1B by reversible oxidation of the catalytic cysteine that prevents protein dephosphorylation. The crystal structure revealed that the sulfenic acid is converted to a sulphenyl-amide species formed between the catalytic cysteine and the main chain nitrogen of an adjacent residue [37]. Further oxidation of this cysteine to the sulphinic and sulphonic acid derivatives was also observed in crystal structures of PTP1B [38].

Caspases are cysteine-aspartate proteases responsible for regulating inflammation and executing apoptosis. Similar to protein tyrosine phosphatases, caspases contain an unusually reactive catalytic cysteine residue that is susceptible to oxidation by hydrogen peroxide and other reactive oxygen species [39]. The sulphenic acid derivative renders caspases inactive and may result in the inhibition of apoptosis.

Oxidized proteins are reduced by one of several different enzymes using a reducing molecule such as glutathione or thioredoxin to carry out the reduction [40]. Glutathione is a tripeptide synthesized nonribosomally from glutamate by glutamylcysteine synthetase that forms intermolecular disulfides. The cellular concentrations of glutathione are high (1-11 mM). This is higher than most other cellular redox compounds suggesting that glutathione plays an important role in the redox state of a cell. NADPH is the primary source of electrons for reducing oxidized glutathione to the free sulphydral form in a reaction catalyzed by glutathione reductase.

Thioredoxin is particularly important for reducing cysteine disulfides of DNA-binding transcription factors. Thioredoxin is a protein that forms intramolecular disulfides. Cellular thioredoxin levels are much lower than those of glutathione (~1-10 μM vs. 1-11 mM, respectively). Similar to the glutathione system, NADPH is the main source of electrons for reducing the thioredoxin disulfide to the dithiol form in a reaction catalyzed by thioredoxin reductase.

As pointed out by Schafer and Buettner, the three redox systems $NADP^+$ / NADPH, GSSG/2GSH and TrxSS/Trx(SH)2 are not isolated systems [41]. Rather, they are thermodynamically connected to each other because NADPH provides both Trx and glutathione with reducing equivalents. The half-cell potentials of these linked redox couples influences the redox environment of cells.

Another enzyme that influences the redox balance of a cell is NADH transhydrogenase [42]. This protein couples the proton chemical gradient to the hydride transfer between NADH and $NADP^+$. The $[NADPH][NAD^+]/[NADP^+][NADH]$ ratio can be > 400 [43]. The NADPH produced is available for reduction of glutathione and biosynthesis of lipid molecules. The production of NADPH from NADH provides a direct connection between NADH and the NADPH/thiol systems.

Transition State Metals

A survey of cellular redox molecules should include a description of the involvement of transition metals such as iron and copper. These metals participate in one-electron transfers that are often important for their roles in enzyme catalysis. These metals also have a high affinity for O_2 making them ideal for O_2 transport and utilization

[44, 45]. The reactivity of transition metals towards oxygen species results in the formation of highly reactive species such as the production of the damaging hydroxyl radical from hydrogen peroxide in a reaction termed the fenton reaction [46]. Increased iron accessibility and production of reduced oxygen species such as hydrogen peroxide and superoxide shifts the cell to a prooxidant state [47]. One important consequence of a prooxidant state is DNA damage by reactive oxygen species, such as hydroxal redicals which oxidize DNA bases. Another result of fenton chemistry is the regulation of the oxygen sensor hypoxia-inducible factor (HIF-1). HIF-1 and several other transcription factors are sensitive to changes in the cellular redox state by a variety of mechanisms. The regulation of gene expression by oxygen, and oxidative stress will be dicussed in the next section.

Redox sensitive nuclear transcription regulators

Transcription factors and thiol / oxygen species

There are a number of bacterial transcription factors sensitive to alterations in the redox environment [48]. A brief discussion of SoxR and OxyR provides examples of how redox changes are sensed by a redox iron center and a redox sensitive cysteine. The genes regulated by these proteins agree with their classification as redox sensors. For example the SoxRS protein induces genes for antioxidant defense such as those encoding Mn-containing superoxide dismutase and a DNA repair endonuclease for oxidative damages [49]. SoxRS also induces genes of aerobic metabolism and resistance genes for antibiotics, organic solvents and heavy metals. The SoxRS regulon is activated by

exposure to agents that generate intracellular superoxide or by exposure to nitric oxide. Activation of SoxRS is thought to involve the protein's [2Fe-2S] center by oxidation or metal assembly.

The OxyR protein is responsive to hydrogen peroxide and induces at least eight genes of oxidative defense including catalase-hydroperoxidase and glutathione reductase [50]. The signal to activate OxyR is thought to be the oxidation of a cysteine that stimulates DNA binding and gene activation. Many of the genes regulated by OxyR are also regulated by the stationary phase/starvation response system programmed by the σ^{38} protein.

Eukaryotic DNA-binding transcription factors

The DNA-binding transcription factors, NF-κB, and AP-1 are examples of eukaryotic activator proteins influenced by the cell's redox environment [2]. NF-κB regulates an array of genes involved in the inflammatory response such as cytokines and interleukins. Activation of the NF-κB pathway provides an example of how redox regulation of a gene can occur at locations ranging from the cell's plasma membrane to the nucleus. Reactive oxygen species activate Ras which stimulates the phosphorylation of MAPK kinase kinase (MEKK1) and subsequent phosphorylation and release of IκBκ. Release of IκBκ is required for NF-κB nuclear translocation. Phosphorylation of the p65 subunit of NF-κB is required for activation and is also a target of the MEKK1 phosphorylation cascade. Reactive oxygen species stimulate the degradation of phosphorylated IκBκ. A reducing environment is required for NF-κB DNA binding. Oxidation of a cysteine in the DNA binding region prevents DNA binding. This cysteine

is thought to form an intermolecular disulfide that can be reversed by thioredoxin and Ref-1.

Activation of the NF-κB pathway by Ras provides an example of ROS acting at many cellular locations to alter gene regulation. ROS stimulate the phosphorylation of p38 and IκB kinase by MEKK1 as well as the phosphorylation of IκBκ. The phosphorylated IκBκ is then degraded in a manner also enhanced by ROS. Phosphorylation of IκBκ results in its release from NF-κB and subsequent degradation. NF-κB is able to enter the nucleus in the absence of IκBκ under reducing environments. A cysteine in the DNA-binding region of NF-κB must be in the reduced state for activation. Oxidation of this cysteine to an intermolecular disulfide prevents DNA-binding. The disulfide can be reversed by thioredoxin and Ref-1. NO has been reported to have many effects on NF-κB signaling that appear to both antagonize and synergize with those of ROS.

AP-1 is formed from heterodimers with c-fos and jun. The binding of AP-1 to DNA is favored under reducing conditions as a cysteine in the basic DNA-binding region of c-Fos and c-Jun confers the redox sensitivity. Activation of AP-1 involves ROS stimulated expression, and subsequent phosphorylation, of the two subunits c-fos and c-jun. The phosphorylation of the c-Fos and c-Jun is the result of MAP kinase family members. The inactivation of tyrosine and dual-specificity phosphatases by ROS is thought to enhance activation by preventing dephosphorylation and inactivation of ERK. Ref-1 is able to reduce AP-1 and is regenerated by thioredoxin.

Transcriptional coregulators and metabolic messenger molecules

The dependence of two classes of transcriptional coregulators on central metabolic molecules was described in the discussion of the HATs and the chromatin remodeling proteins which require acetyl-CoA and ATP respectively. While ATP and acetyl-CoA do not undergo oxidation or reduction chemistry, these two molecules are of major importance in metabolism. ATP is the energy source for essentially all cellular processes and the end product of cellular respiration. ATP synthesis is driven by a proton gradient generated by a process dependent on reducing equivalents provided by NADH produced during catabolism of carbon sources. Acetyl–CoA is perhaps the most central of all intermediary metabolites, bridging the major catabolic and anabolic processes to the Krebs cycle, where its two carbons are converted to the respiratory product, CO_2. A summary of cellular respiration is provided below (Fig. 4) and highlights the roles of acetyl-CoA, NAD^+, NADH and ATP.

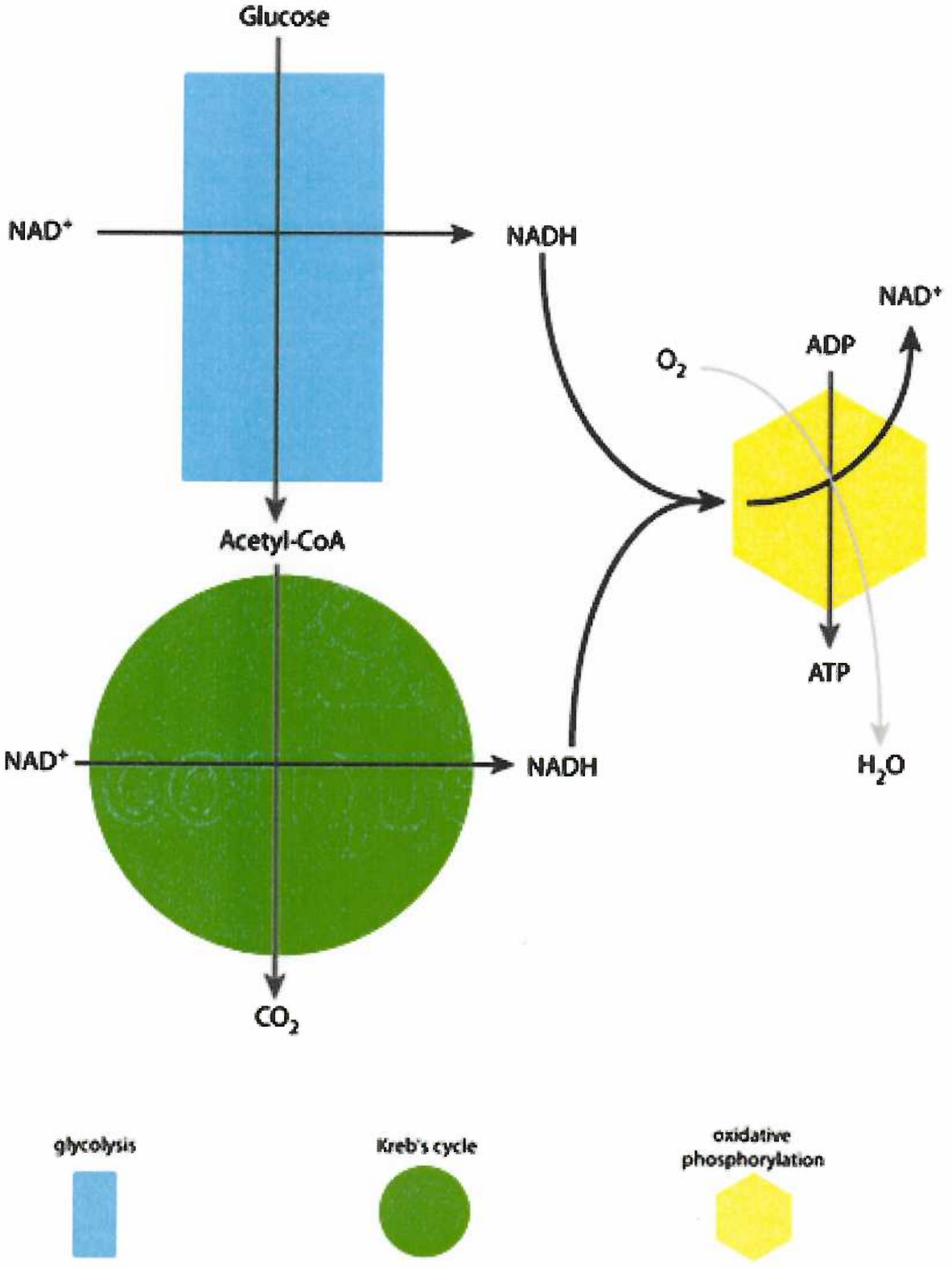

Figure 4. Cellular respiration.

Sir2 and NAD^+

The ability to sense the cellular redox state via thiol chemistry with oxygen species appears to be an attribute commonly seen with DNA-binding transcription factors. Transcriptional coregulators, on the other hand, are highly specialized in their form and function to be sensitive to molecules that appear to serve as metabolic messengers linking cellular respiration to gene regulation. Perhaps the best example of this connection is the NAD^+-dependence of the histone deacetylase, Sir2. Studies in yeast and other species suggest that the regulation of Sir2 by NAD^+ may be important for the affects of calorie restriction on life-span [51, 52]. The deacetylase activity of Sir2 is critical for Sir2's roles in gene repression and the maintanance of heterchromatin. The observation that one NAD^+ is consumed in the histone deacetylation reaction for every peptide lysine deacetylated led investigators to conclude that NAD^+ is involved in the chemistry of the reaction. Efforts to identify free acetate as a reaction product were unsuccessful and led to the identification of the novel reaction product O-acetyl-ADP ribose [53, 54]. The functional significance of O-acetyl-ADP ribose remains to be determined. Nevertheless, the NAD^+ dependence of Sir2 provides a connection between the metabolic environment and gene silencing.

The role of Sir2 in repressing muscle cell differentiation was shown to be regulated by the redox state of the progenitor cells [55]. First, Fulco et al established that Sir2's deacetylase activity was required for muscle cell differentiation. Small molecule inhibitors of Sir2 deacetylase activity and a catalytically inactive mutant of Sir2 (H355Y) each increased transcription of muscle-specific reporter constructs.

Additionally, overexpression of Sir2 resulted in a block in differentiation. A microarray analysis of cells expressing high levels of Sir2 demonstrated specific repression of muscle-cell differentiation genes. Fulco et al. then manipulated the redox state of the cell by the addition of pyruvate or lactate which causes an increase or decrease in the NAD^+/NADH respectively. The pyruvate/lactate ratio was used to determine that the NAD^+/NADH ratio decreases in cells undergoing differentiation in support of the hypothesis that NAD^+ levels influence Sir2 activity and muscle cell differentiation. The authors suggest that Sir2 is regulated by the NAD^+/NADH ratio based on the changes in the ratio due to different pyruvate/lactate treatments. The inability of Sir2 to bind NADH suggests that Sir2 is not influenced by changes in NADH directly.

An important question concerning the ability of NAD^+ to regulate the Sir2 proteins is the physiological concentration of free nuclear NAD^+. In yeast the total cellular NAD^+ concentration was determined to be 1.5 - 2 mM. A recent study in yeast demonstrated that Hst1, a homolog of Sir2, is responsible for regulating NAD^+ biosynthesis and for controlling cellular levels of NAD^+. The NAD^+-dependent deacetylase activity of Hst1 is required for the repressed transcription of de novo NAD^+ biosynthesis genes. Such a mechanism provides a feedback loop where a decrease in NAD^+ levels induces the expression of NAD^+ biosynthesis genes.

Sir2 serves as a metabolic sensor through its NAD^+ dependent deacetylase activity. It seems unlikely that the NAD^+/NADH regulates Sir2 activity given the weak affinity of Sir2 for NADH and the low nuclear concentrations of free NADH. It is also unlikely that the free nuclear NAD^+ levels change adequately to alter the activity of Sir2

under physiological conditions. The model presented by Bedalov et al. requires the lower affinity of Hst1 for NAD^+ relative to that of Hst2 and Sir2 and suggests that in yeast the NAD^+ dependence of Hst1 serves to maintain NAD^+ homeostasis and buffer Hst2 and Sir2 from sensing NAD^+ fluctuations that may result from noninvasive metabolic challenges [56]. This buffering affect of Hst1 would seem to prevent stimulation of Sir2/Hst2 by caloric restriction through repression of the NAD^+ biosynthesis genes, as well as by preventing inhibition of Sir2/Hst2 by a decrease in NAD^+ levels through induction of the NAD^+ biosynthesis genes. The sensitivity of Sir2 to nicotinamide, a product and potent inhibitor of the Sir2 deacetylation reaction, may be involved in the ability of Sir2 to serve as a metabolic sensor by direct inhibition of Sir2.

It has been proposed that the basis for life-span extension by calorie restriction in yeast is the activation of Sir2 by increased NAD^+ levels. If the free nuclear NAD^+ concentration and the relative affinities of the various Sir2 family members are similar to the values described by Bedalov [57], then Sir2/Hst2 would not be significantly activated by an increase in NAD^+ levels as they would be essentially saturated in NAD^+. The mechanism utilized by Sir2 to serve as a metabolic sensor remains a complex and interesting question that is addressed, in depth, in the Discussion of this thesis.

CtBP and NADH

The carboxyl terminal binding protein (CtBP) is another transcriptional corepressor proposed to be sensitive to metabolic events through its interaction with the reduced form of NAD^+, NADH. CtBP was first identified through its ability to interact with the carboxyl terminus of adenovirus E1A oncoprotein [58]. The residues in the

carboxyl terminus critical for the interaction were determined to be the PLDLS sequence [59].

CtBP shares high homology with dehydrogenase proteins. For example, the dehydrogenase domain of CtBP and 3-phosphoglycerate dehydrogenase are nearly 40% identical. A weak dehydrogenase activity has been detected for CtBP in the presence of pyruvate and NADH that went undetected in initial studies [60]. Mutation of the critical catalytic residue (H315Q) did not result in a loss of repression at endogenous promoters in mouse embryonic fibroblast cells from CtBP knockout mice suggesting that the dehydrogenase activity is not important for CtBP's corepressor function [61, 62]. It has been proposed that the nucleotide binding capabilities of CtBP are important for recruitment of CtBP to promoters. The first observation of nucleotide regulated binding was seen by Zhang et al. in the ability of Gst-E1A to interact with hCtBP1 [5]. Zhang et al. observed that NADH was capable of enhancing the CtBP/E1A interaction at much lower concentrations compared to NAD^+. Additional support was provided by limited proteolysis assays demonstrating an induced comformational change of CtBP at low nanomolar levels of NADH and at micromolar levels of NAD^+. Experiments in culture designed to increase NADH levels served to enhance repression of reporter genes that required NADH binding as suggested by the lack of stimulated repression by the nucleotide binding deficient CtBP mutant (G183A). The inability of other groups to detect the differential binding of NAD^+ and NADH to CtBP [60, 63] led to the studies central to this thesis. The differential binding of CtBP to NAD^+ and NADH is critical to CtBP's ability to serve as a redox sensor. Structural information provided by Kumar et al. [60] stimulated the studies described later in chapter 1 of this thesis. Structural

information provided by Nardini et al. [64] became available upon completion of the work described in chapter 1. A comparison of the structure of CtBP bound to NADH with CtBP bound to NAD^+, explains the NADH preference by revealing that several critical contacts to the nicotinamide moiety of NADH are closer with bound NADH compared to bound NAD^+. Thus, structural data suggests that CtBP prefers NADH relative to NAD^+.

The crystal structures of CtBP bound to NADH and NAD^+ help reveal how CtBP is able to distinguish between the reduced and oxidized forms of the nucleotide [60, 65]. The structural data reveal that CtBP takes advantage of the positive charge on NAD^+ to distinguish it from NADH. In addition, the nicotinamide carboxamide is rotated to a staggered position in the structure of CtBP bound to NADH while it is planar in the structure of CtBP bound to NAD^+. The distances between CtBP residues and NAD(H) suggest that NADH binds more tightly based on the difference in charge and configuration of the carboxamide (Fig.5). Arg 266 and His 315 are 4.18 and 6.24 Å from N1 of the nicotinamide ring of NAD^+, respectively. In the structure with bound NADH, these distances are reduced to 3.69 and 5.60 Å suggesting a repulsion between charged residues and NAD^+ plays a role in the NADH preference. The result of the carboxamide rotation appears to be stronger hydrogen bonds between CtBP and N7 of the amide based on the shorter interatomic distances.

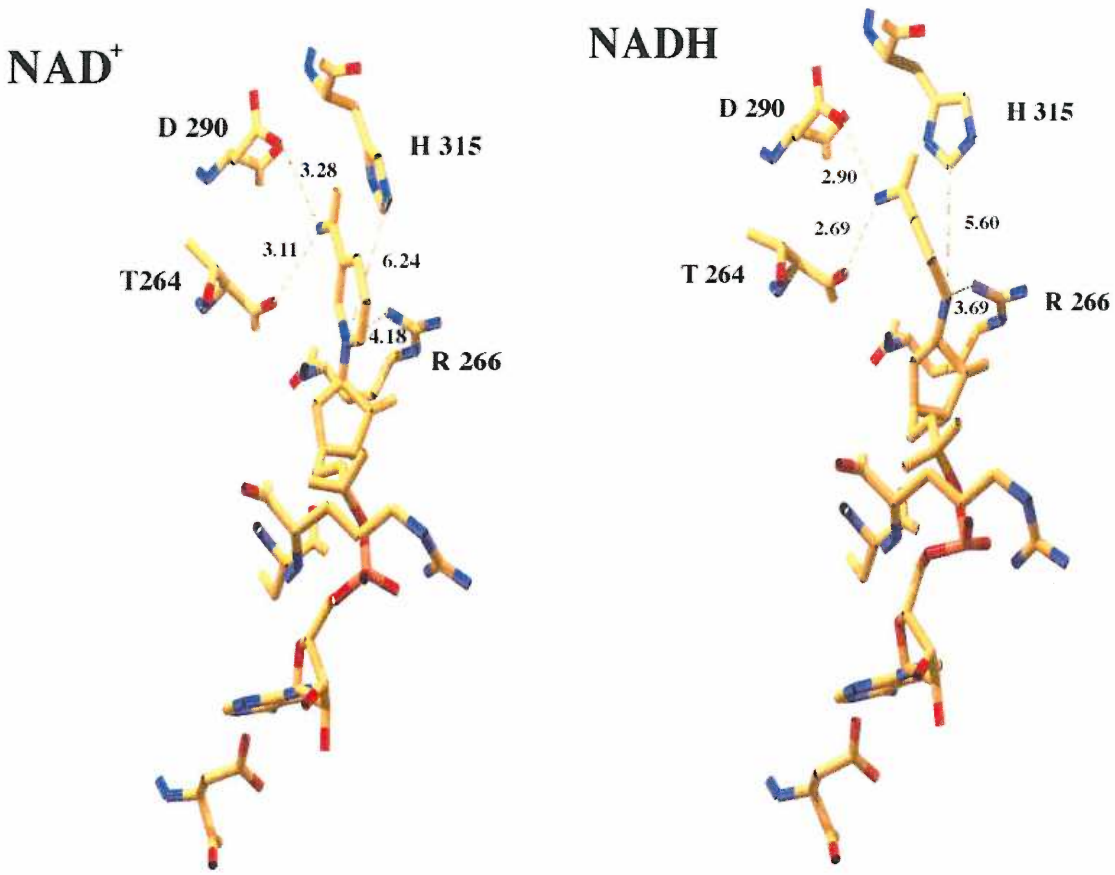

Figure 5. Structural data suggesting preference of CtBP for NADH over NAD^+, taken from [65] and [60], respectively. Contacts demonstrating higher affinity for the reduced nicotinamide are shown. These structures are deposited in the Protein Data Bank (accession numbers for CtBP/NAD+, 1MX3, ref. [60], and CtBP NADH, 1HL3 ref. [65]).

CtBP oligomerization

The studies by Zhang et al. strongly suggest that nucleotide binding enhances the interaction between CtBP and E1A [5]. This phenomenon has also been observed by others in Gst-pull down assays. Evidence that the enhanced binding is the consequence of a conformational change is supported by the limited proteolysis experiments

demonstrating that nucleotides are able to protect CtBP from proteolytic cleavage. The observations that nucleotides protect from limited proteolysis are not surprising given the immense knowledge collected over the past several decades related to dehydrogenase structure and function. In general, nucleotide binding to dehydrogenase proteins induces a conformational change involving the movement of a flexible loop over the active site. The nucleotide bound conformation is termed the closed state and favors catalysis by squeezing out water and optimizing the positions of catalytic and substrate binding residues.

Balasubramanian et al. [63] found that NAD(H) doubled the apparent molecular mass of CtBP, suggesting that its addition promoted dimerization or tetramerization (depending on whether the 65- to 70-kDA species is a monomer or dimer, respectively). Nardini et al. propose that nucleotide binding induces a conformational change that promotes CtBP dimerization, which is essential for corepressor activity. Both structures of CtBP are nucleotide bound, in the closed conformation and reveal CtBP dimers. The ability of CtBP to dimerize was reported by several groups prior to the publication of the crystal structures. Thus, CtBP is able to dimerize in a manner that appears to depend on nucleotide binding. It is possible, therefore, that this ability of NAD(H) to stimulate CtBP oligomerization could contribute to the enhanced binding of E1A and other transcriptional repressors.

Post-translational modifications of CtBP

Phosphorylation

The phosphorylation of CtBP, first described ten years ago, has recently been shown to be important for blocking gene repression. The first report by Barnes et al.

demonstrated that CtBP can be phosphorylated on Ser158 by p21-activated kinase 1 (Pak1) [66]. The phosphorylation of CtBP by Pak1 results in attenuation of CtBP corepressor activities in reporter and chomatin assays possibly by the redistribution of CtBP in response to signaling. The phosphorylation of CtBP by Pak1 is enhanced by NADH, but not by NAD^+. This suggests that there are differences in the position of Ser158, or in the Pak1 binding site of CtBP, depending on the oxidation state of the bound nucleotide. Such a difference in the conformation of CtBP was not observed in the limited proteolytic studies performed by Zhang et al. [5] and by Kumar et al. [60] which suggested that the reduced and oxidized forms of NAD^+ induced the same conformational change. Barnes et al. also observed that the NADH stimulated phosphorylation event completely inhibited the weak dehydrogenase activity of CtBP [66]. The relevance of this remains to be determined. Understanding the mechanism of inhibition may provide further insight on the role of NADH binding. For example, phosphorylation may affect CtBP oligomerization or nucleotide binding/release.

The second CtBP phosphorylation site also reportedly results in gene activation by a derepression mechanism involving the translocation of CtBP from the nucleus to the cytoplasm. Specifically, homeodomain interacting protein kinase 2 (HIPK2) promotes phosphorylation of CtBP on Ser422 which promotes apoptosis by downregulating CtBP through a degradative mechanism involving removal of CtBP from the nucleus to the proteosomal digestive pathway [67].

Sumoylation of CtBP

The conjugation of SUMO (small ubiquitin-related modifier) to target proteins has functional consequences such as targeting proteins to specific subcellular locations and for regulating the activity transcription factors. To date, sumoylation has not been shown to target proteins for degradation. The carboxyl-terminus of CtBP contains the amino acid sequence VKxE which matches sumoylation motif ΨKxE (Ψ is hydrophobic). Lin et al. found that sumoylation enhances nuclear localization of CtBP by either preventing nuclear export or increasing nuclear import [68]. The E3 ligases Lin et al. found to sumoylate CtBP1 include PIAS1 and PIASxβ. Binding of nNOS to the PDZ domain of CtBP was found to be mutually exclusive with sumoylation of Lys428. A model was proposed where sumoylation of CtBP promoted the corepressor activity of CtBP by preventing nuclear export by nNOS. Sumoylation did not affect binding to PxDLS determined with ZEB.

The ability of Polycomb Protein PC2 to stimulate the sumoylation of CtBP in PcG bodies led to the identification of PC2 as an E3 ligase [69]. Previously, PC2 had been shown to recruit CtBP to PcG bodies that are large protein complexes involved in the stable repression of gene expression. An E3 ligase functions as an adapter that brings E2 and substrate together and not through some intrinsic enzymatic activity as suggested by its name. Interestingly the PC2 contains a CtBP binding motif demonstrated to be critical for CtBP binding.

Chapter 1

Differential Binding of NAD^+ and NADH Allows the Transcriptional Corepressor C-Terminal Binding Protein to Serve as a Metabolic Sensor

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Abstract

Carboxyl-terminal binding protein (CtBP) is a transcriptional corepressor originally identified through its ability to interact with adenovirus E1A. The finding that CtBP-E1A interactions were regulated by the nicotinamide adeninine dinucleotides NAD^+ and NADH raised the possibility that CtBP could serve as a nuclear redox sensor. This model requires differential binding affinities of NAD^+ and NADH, which has been controversial. The structure of CtBP determined by x-ray diffraction revealed a tryptophan residue adjacent to the proposed nicotinamide adenine dinucleotide binding site. We find that this tryptophan residue shows strong fluorescence energy transfer to bound NADH. In this report, we take advantage of these findings to measure the dissociation constants for CtBP with NADH and NAD^+. The affinity of NADH was determined using fluorescence resonance energy transfer (FRET). The binding of NADH to protein is associated with an enhanced intensity of NADH fluorescence and its maximum blue-shifted. NAD^+ affinity was estimated by measuring the loss of the fluorescence blue shift as NADH dissociates upon addition of NAD^+. Our studies show a greater than 100-fold higher affinity of NADH than NAD^+, consistent with the proposed function of CtBP as a nuclear redox sensor. Moreover, the concentrations of NADH and NAD^+ required for half-maximal binding are approximately the same as their concentrations in the nuclear compartment. These findings support the possibility that changes in nuclear nicotinamide adenine dinucleotides could regulate the functions of CtBP in cell differentiation, development, or transformation.

Introduction

An emerging theme in gene regulation is the dependence of transcriptional coregulators on molecules linked to cellular respiration. Acetyl-CoA is required by the histone acetyltransferase (HAT) coactivators [70] and is perhaps the most central of all intermediary metabolites, bridging the major catabolic and anabolic processes to the Kreb's cycle where its two carbons are converted to the respiratory product, CO_2. ATP is important for essentially all cellular processes requiring energy, including the chromatin remodeling proteins involved in modifying nucleosomal structure [71]. The pervasive involvement of acetyl-CoA and ATP in cellular processes generally obscures recognition of their specific contributions to transcriptional regulation, however. In addition, the relatively small changes in acetyl-CoA and ATP that occur during metabolism may not be suitable for regulating the activities of the relevant enzymes.

The breakdown of carbon sources is also associated with the reduction of the nicotinamide adenine dinucleotide NAD^+ to NADH. NADH serves as an electron carrier that transports reducing equivalents to the electron transport chain where ATP is synthesized. The synthesis of ATP involves oxidative phosphorylation, wherein NADH is oxidized to NAD^+ and molecular oxygen is reduced to water. These roles of NAD^+ and NADH provide additional, albeit somewhat indirect, connections between energy homeostasis and gene regulation. A more direct link was recognized when it became apparent that nicotinamide adenine dinucleotides were capable of interacting with certain enzymes involved in transcriptional silencing. Perhaps the best example of this regulation is the NAD^+-dependence of the histone deacetylase, Sir2 [52, 72]. The

deacetylase activity of Sir2 and related proteins has been shown to contribute importantly to gene repression and the maintenance of heterochromatin [4]. Recent data indicate that the regulation of Sir2 by NAD^+ may explain the interaction between dietary factors and longevity in yeast and other species [51].

The vast majority of the cellular nicotinamide adenine dinucleotide is bound to protein and, consequently, cannot play a regulatory role in controlling transcription factors involved in gene regulation. Presumably, only the free pool of nicotinamide adenine dinucleotides can subserve these functions and, in most instances, the relevant free pool is in the nuclear compartment. The concentration of free NAD^+, at least in the cytoplasm, greatly exceeds that of NADH. Because unbound nicotinamide adenine dinucleotides should pass freely through nuclear pores, this difference in the levels of free NAD^+ and NADH should pertain to the nucleus as well. Furthermore, because the free cytoplasmic (and presumably nuclear) NAD^+/NADH ratio is approximately 700:1 [73], conversion of NAD^+ to NADH causes a much larger relative change in the NADH level. This implies that the levels of free NADH are likely to more accurately reflect metabolic events than the levels of NAD^+. Thus, the ability of a transcriptional regulator to sense levels of free nuclear NADH rather than NAD^+ would seem to provide a more sensitive mechanism for linking metabolic events to transcription. Relatively little is known about the functions of NADH in the nucleus, however.

Two nuclear transcription factors have been proposed to be regulated by NADH. The first, NPAS2, is a DNA-binding protein involved in the maintenance of mammalian

circadian rhythms. Studies by Rutter, et al [74] suggested that the binding of the NPAS2:BMAL heterodimer to control elements in target genes was regulated by NADH and NADPH. The concentrations of NADH and NADPH required for stimulating binding were in the millimolar range, however, levels that are considerably higher than the concentrations of the free nucleotides. Thus, whether NPAS2 is sensitive to physiologically relevant levels of NADH or NADPH remains to be determined.

The second NADH-regulated factor is the carboxyl-terminal binding protein, CtBP. CtBP was named for its ability to bind to the carboxyl-terminus of the adenovirus E1A oncoprotein [59]. Like other E1A-binding proteins, CtBP interacts with a wide variety of cellular factors, including several transcriptional repressors. Thus, CtBP has been categorized as a transcriptional corepressor. Studies in *Drosophila* as well as mammalian systems have shown that the corepressor function of CtBP is critical for cell differentiation, transformation, and development [75]. Precisely how CtBP mediates gene repression has not been entirely resolved, however. Several reports have indicated that CtBP binds to histone deacetylases [76-78], but the importance of this interaction is controversial. Other proteins shown to interact with CtBP include co-REST, histone methyltransferases, and a polyamine oxidase [79]. Interactions between CtBP and the polycomb repressors have also been reported, but CtBP was not a component of the polycomb complex isolated by Levine, et al [80] A recent study showed that the association of CtBP with the polycomb protein PC2 led to its sumoylation [60], but how this modification alters CtBP function is unknown. Finally, the high homology between CtBP and 3-phosphoglycerate dehydrogenase led to the suggestion that an oxido-

reductase activity might also contribute to its gene repression effects [60]. The observation that this activity is dispensable in the regulation of endogenous target genes in CtBP-knockout mouse embryo fibroblasts casts doubt on this hypothesis, however [62].

Zhang, et al. [5] proposed that NAD^+ and NADH regulate CtBP binding to certain DNA-binding transcriptional repressors, thereby targeting its corepressor functions to particular gene promoters. Although the ability of nicotinamide adenine dinucleotides to stimulate CtBP binding to E1A and other proteins has been confirmed by several laboratories [60, 63], the differential efficacy of NAD^+ and NADH is controversial. Zhang, et al. [5] showed that NADH was two-to-three orders of magnitude more effective than NAD^+ in stimulating CtBP binding and proposed that this differential effect might link CtBP-mediated repression to the redox state of the nuclear compartment. In support of this model, they determined that the concentration of free nuclear NADH was about 100 nM, similar to that required for half-maximal CtBP-E1A binding. The free nuclear NAD^+ concentration cannot be measured directly but was estimated to be about 70 uM, also approximately the half-maximal level for stimulating CtBP binding. In contrast, however, Kumar, et al. [60] and Balasubramanian et al. [63] found that NAD^+ and NADH were equally effective in stimulating CtBP binding. These results would not be consistent with metabolic regulation of CtBP function. While Balasubramanian et al did not examine levels of NAD^+ and NADH that were low enough to discern a differential effect, Kumar et al tested a full range of nicotinamide adenine dinucleotide concentrations. Moreover, they concluded on the basis of the CtBP crystal structure that

the single proton difference between NAD^+ and NADH could not result in a two-to-three order of magnitude difference in binding.

Because the potential for CtBP to serve as a redox sensor depends upon its differential affinity for NAD^+ and NADH, we decided to investigate these binding events directly. The structure of CtBP determined by Kumar, et al. [63] showed that one of the three tryptophan residues in the protein lies within four angstroms of the nicotinamide in NAD^+. Thus, we predicted that we could directly measure the binding of NADH by measuring the transfer of energy from the adjacent tryptophan. We showed using single tryptophan mutants that tryptophan 318 is the major contributor to the fluorescence resonance energy transfer (FRET) to NADH. NAD^+ affinity was estimated by competition assays. Binding of NADH to protein enhances the intensity of the NADH fluorescence and shifts the fluorescence peak to a shorter wavelength (blue shift). We estimated the NAD^+ affinity by measuring its ability to displace NADH, as measured by the decrease in NADH fluorescence and blue shift. Our studies show that the K_d for NADH is significantly lower than that for NAD^+ and support the idea that CtBP is indeed suited to serve as a metabolic sensor through its ability to interact differentially with reduced and oxidized nicotinamide adenine dinucleotides.

Materials and Methods

Plasmids. The plasmid pRcCMV-hCtBP1 was a generous gift from Dr. G. Chinnadurai (St. Louis University Health Sciences Center). CtBP was cloned using PCR into pET24 in frame with the C-terminal His tag. Tryptophan to phenylalanine mutations

were generated using the Quikchange (Novagen) method after subcloning the CtBP cDNA into pBluescript II KS. All CtBP vectors were confirmed by DNA sequencing.

Proteins. His-tagged CtBP proteins were expressed in BL21(DE3) and purified by Ni-NTA affinity (Qiagen). Cultures (1L) were grown at room temperature to an optical density of 0.4 – 0.6, induced with IPTG (100 mg/ml) for 4-6 hours, pelleted by centrifugation (20 min, 5K rpm) and resuspended in 30 ml buffer A: 25 mM Hepes / KOH (pH7.6), 50 mM KCl, 10 mM BME and 15% glycerol. Cells were lysed twiced using a French press under a constant pressure of 11,000 psi. Cell debris was removed by centrifugation (25 min, 16K rpm) and the supernatant was slowly added to a Ni-NTA column (5 ml slurry) equilibrated with buffer A. The column was then washed sequentially with 150 ml buffer A, 100 ml pyruvate (0.25 mM), a glycerol gradient (15 - 40%, 250 ml), 50 ml buffer A, a KCl gradient (50 – 350 mM, 250 ml), and 50 ml buffer A. The column was then treated with a 50 ml ATP (1 mM) and $MgCl_2$ (1 mM) wash followed by 50 ml imidazole (30 mM). The protein was eluted with an imidazole gradient (0-250 mM, 200 ml). Fractions were analyzed by SDS-PAGE and silver staining. Fractions of highest purity were pooled, concentrated in a centricon 30 matrix, and dialyzed in 4L of buffer B: 25 mM Hepes / KOH (pH7.2), 50 mM KCl, 5 mM DTT and 10% glycerol. Purified protein was aliquoted and stored in –80°C. Protein concentration was determined by the Bradford method using a BSA standard and confirmed by amino acid analysis (Texas A&M University Protein Chemistry Laboratory).

Fluorescence Assays. Fluorescence studies were carried out using a Photon Technologies QM-1 steady state fluorescence spectrophotometer. NADH was excited directly at 340 nm or through FRET by exciting tryptophan at 285 nm. Emission spectra were collected from 300 to 500 nm. Titrations of NADH and NAD^+ were performed in 1ml volumes, at constant temperature (25°C) and stirring with excitation / emission slit widths of 2 nm and 3 nm, respectively. Assays were performed in 10 mM Tris pH 8.0 using 300-600 nM CtBP. NADH and NAD^+ were obtained from Sigma-Aldrich in preweighed vials. The increase in fluorescence at 425 nm (excitation 285 nm) was used to follow NADH binding. The displacement of NADH (100 – 500 nM) by NAD^+, observed as a decrease in fluorescence at 410 nm (excitation 340 nm), was used to measure NAD^+ binding. Binding constants determined at various protein concentrations were in agreement. Inner filtering effects were determined to be negligible at the NADH concentrations used (≤ 500 nM) by measuring the absorbance of NADH at the excitation wavelength (285 nm).

Data analysis. The data was analyzed using the computer program KaleidaGraph (Abelbeck Software). The fluorescent signal ΔF was plotted against the concentration of free NADH. Due to the high affinity of CtBP for NADH, the protein concentration must be considered when determining the free NADH concentration. This was done using the relationships in equations 1a-c below. The K_d was determined using equation 2 (rectangular hyperbola) to fit the binding data.

$$\Delta F / \Delta F_{max} = [CtBP_{bound}] / [CtBP_{total}] \qquad \text{Eq. 1a}$$

$$[NADH_{bound}] = [CtBP_{bound}] \qquad \text{Eq. 1b}$$

$$[NADH_{total}] = [NADH_{free}] + [NADH_{bound}] \qquad \text{Eq. 1c}$$

$$\Delta F = \Delta F_{max}\ [NAD(H)_{free}] / K_d + [NAD(H)_{free}] \qquad \text{Eq. 2}$$

The binding of NAD^+ was measured by monitoring the displacement of NADH as a decrease in fluorescence at 410 nm. A plot of the change in fluorescence versus the NAD^+ concentration was fit with equation 2 to determine the $K_{d(NAD^+, app)}$. The $K_{d(NAD^+)}$ was calculated using equation 3, where NAD^+ and NADH compete for the same site.

$$K_{d(NAD^+, app)} = K_{d(NAD^+)} (1 + [NADH] / K_{d(NADH)}) \qquad \text{Eq. 3}$$

Results

Crystal structure of CtBP reveals the potential for fluorescence resonance energy transfer. The hypothesis that CtBP serves as a nuclear redox sensor relies on the observation that NADH is more effective than NAD^+ in stimulating its binding to transcriptional repressors. A significant difference in the efficacy of NAD^+ and NADH was observed by Zhang, et al. [5] but was not seen by other investigators in subsequent studies [60, 63]. The CtBP in these studies was expressed using different techniques, however, and the proteins were purified to varying degrees. Additionally, the methods used, GST-pulldown and protease protection assays, are somewhat indirect and have limitations in their sensitivity. To examine the binding of NAD^+ and NADH more directly, we performed fluorescence measurements on highly purified bacterially-expressed protein. Purification of CtBP was monitored by silver staining and the

concentrations of the protein, determined by Bradford assay, were confirmed by amino acid analysis. Occasional CtBP preparations contained various amounts of contaminating NADH that could be detected by its fluorescence at 425 nm after excitation at 340 nm (Fig. 1, red trace). This residual NADH could be detected despite extensive dialysis, suggestive of an extremely low off-rate. Addition of saturating amounts of NADH (Fig. 1 blue trace) revealed that approximately 10% of the binding sites for NADH were occupied before addition of exogenous ligand in this particular CtBP preparation. We hypothesize that varying amounts of contaminating NADH might account in part for the inconsistency in the binding assays among various laboratories. To overcome this problem, we treated the purified protein preparations with pyruvate, which has been determined to be a substrate for the CtBP dehydrogenase activity [60]. Although pyruvate is a relatively poor substrate, its addition caused a release of the bound NADH, presumably after conversion to NAD^+. NAD^+ binds to CtBP at relatively low affinity (see below) and can be removed by dialysis. The addition of pyruvate (Fig. 1, black trace) caused a complete loss of NADH fluorescence. Only samples that were free of contaminating NADH were utilized for binding studies.

As described by Kumar, et al. [60] the crystal structure of CtBP bound to NAD^+ supports the prediction that CtBP is structurally similar to 3-phosphoglycerate dehydrogenase. An additional feature revealed by the structure was the location of a tryptophan residue adjacent to the nicotinamide moiety of NAD^+. This tryptophan (W318) is ideal for detecting fluorescence resonance energy transfer (FRET) because the nicotinamide portion of NAD^+ is within four angstroms (Fig. 2). FRET requires that the

donor and acceptor fluorophores are within the Förster radius, determined to be 25 Å for tryptophan to NADH energy transfer [81]. NADH absorbs at 340 nm (the emission maximum of tryptophan), thus excitation of the tryptophan at 285 nm would be expected to cause fluorescence from NADH at ~425 nm.

NADH Binding Constant Determined by FRET. When CtBP was excited at 285 nm, a typical tryptophan fluorescence spectrum was observed (Fig. 3a, black trace). Titration of NADH resulted in the loss of fluorescence at 340 nm and an increase in fluorescence at 425 nm, demonstrating the transfer of resonance energy from an excited tryptophan to the bound NADH.

There are three tryptophan residues in CtBP: W145, W318 and W362. To determine the contribution of each tryptophan to the FRET signal, we generated proteins containing single tryptophan residues by mutating the two of the three tryptophans to phenylalanine. Presumably, mutation of tryptophan to phenylalanine will cause only minimal perturbations of CtBP structure. A blue shift (see below) of NADH fluorescence was observed with each single tryptophan mutant suggesting that the ability to bind NADH was retained. We measured the contribution of each tryptophan to the FRET signal by exciting each mutant at 285 nm (Fig. 3b). Of the three mutants, only W318 demonstrated substantial FRET upon the addition of NADH (Fig. 3c). This result agrees with the structural information suggesting that W318 is in position to excite the bound NADH (Fig. 2). A relatively small decrease in 340 nm fluorescence was observed when NADH was added to the W145 and W362 mutants. This may be the result of a

conformational change induced by NADH binding and likely contributes to the quenching of wild type CtBP at 340 nm in the presence of NADH.

To avoid potential artifacts caused by the phenylalanine substitutions, we performed the NADH binding experiments using wild type CtBP. The increase in fluorescence at 425 nm (ΔF) was measured at various NADH concentrations. The concentration of free NADH at each titration point was calculated using the relationships described in the materials and methods. A plot of ΔF versus the free NADH was then fit using the standard binding equation for a rectangular hyperbola (and gave a K_d of 66 ± 14 nM (Fig. 4)). This affinity is consistent with the levels of NADH found to stimulate the CtBP-E1A interaction and the concentration of free NADH found in the nucleus under basal conditions.

Estimation of the affinity of NAD^+ by measurement of the NADH blue shift. NAD^+ does not fluoresce, therefore its binding to CtBP cannot be measured directly by fluorescence techniques. Theoretically, one could monitor the ability of NAD^+ to compete for the NADH binding site by measuring a loss in FRET signal, but the absorbance spectrum of NAD^+ overlaps to some degree with that of tryptophan. Because high concentrations of NAD^+ are required for competition, the inner filtering effect of NAD^+ complicates approaches that depend upon excitation at 285 nm. To estimate the affinity of NAD^+, we took advantage of the change that occurs in NADH fluorescence upon binding to protein. Free NADH excited at 340 nm has an emission maximum at 455 nm (Fig. 5a). Addition of CtBP causes a dramatic increase in NADH fluorescence as

well as a shift in the emission maximum to 425 nm. This change, termed a blue shift, occurs when a fluorescent molecule enters a more hydrophobic environment [82]. Because this measurement relies on absorption at 340 nm and not 285 nm, inner filtering is less problematic. By measuring the disappearance of the NADH blue shift as a decrease in fluorescence intensity, we were able to monitor NAD^+ binding and determine its dissociation constant (Fig. 5b). The emission maximum of displaced NADH at saturating NAD^+ concentrations is identical to that of free NADH (Fig. 5a), indicating that the binding of NAD^+ and NADH is mutually exclusive and that the loss of fluorescence at 425 nm is due to removal of bound NADH and not to quenching. Equation 3 was used to calculate the K_d for NAD^+ to be 8–11 μM. The apparent K_d of CtBP for NAD^+ is a function of the K_d for the competing substrate, NADH. At an NADH concentration of 100 nM, the apparent K_d for NAD^+ was 16 ± 1 μM (Fig. 5c).

Discussion

Studies of the histone deacetylase Sir2 first uncovered the connection between the nicotimamide adenine dinucleotides and gene regulation. In the case of Sir2, the related nucleotides NADH, $NADP^+$, and NADPH, cannot replace NAD^+ as an essential cofactor for enzymatic activity [4]. The finding that NAD^+ regulates Sir2 function has led to a far-reaching series of studies linking nutrition, gene silencing, and longevity in a variety of species. Presumably, nuclear NAD^+ is predominantly bound to protein and only the free fraction would be expected to be relevant for the regulation of Sir2 activity. It is difficult to quantitate this small pool of free nuclear NAD^+ directly, but estimates have suggested that the concentration is in the 10-100 μM range [5]. Interestingly, this range

corresponds to measurements of the K_M of Sir2 and the related Hst proteins for NAD^+ [54]. Alterations in the energy status of the cell are associated with changes in the ratio of free NAD^+ to NADH, however, raising the possibility that NADH levels could be equally important for controlling some transcriptional events. Indeed, it is probably inappropriate to view a protein with a single binding site as detecting a ratio of two ligands; although the binding of one ligand can compete with that of another, the binding events are technically two independent events. Thus, NAD^+ and NADH can be considered to be two interdependent ligands capable of recognizing a single binding site. Because the free NADH pool is so much smaller, metabolic events that alter the NAD^+/NADH ratio cause a much larger change in the levels of free NADH than NAD^+. Thus, NADH can be considered to provide a more sensitive reflection of alterations in cellular energy status.

Unlike NAD^+, NADH can be detected directly in various cellular compartments by measurement of its fluorescence. Quantitation of the fraction of nuclear NADH that is not bound to protein can be determined using fluorescence lifetime measurements but requires a few assumptions (including an estimation of the ratio of NADH to NADPH, since these two molecules cannot be distinguished by their fluorescence properties). With these caveats, Zhang, et al proposed that the concentration of free nuclear NADH is approximately 100 nM [5]. Interestingly, this value is very close to the level of NADH reported to stimulate half-maximal binding of CtBP to E1A. Because other investigators could not confirm these findings, however, we attempted to measure NADH binding to CtBP directly. Although it does not necessarily hold that the affinities of CtBP for NAD^+

and NADH will correspond exactly to the concentrations required for the effects on E1A binding, a difference in affinity for the two ligands would be expected.

Our FRET studies indicate that NADH has an affinity of approximately 66 nM for CtBP. This value is surprisingly close to the concentration of free nuclear NADH determined using two photon fluorescence microscopy and fluorescence lifetime measurements. Thus, we predict that alterations in the free NADH concentration could regulate NADH-driven changes in CtBP conformation. How the free nuclear NADH concentration changes *in vivo* in response to hypoxia or other metabolic stresses has not been determined, however. It is likely that the nuclear levels of free NADH would follow those in the cytoplasm, which vary under a variety of physiological conditions (see below). Determination of the affinity of NAD^+ is somewhat more problematic because the use of the NADH blue shift and its competition by NAD^+ is less established than FRET as a quantitative measure. Consequently, our determination that half-maximal NAD^+ binding occurs at a concentration of 8-11 μM must be considered an estimate. Additionally, because the concentration of free nuclear NAD^+ cannot be determined directly, it is difficult to compare this value with the calculated dissociation constant. Interestingly, however, the affinities of NADH and NAD^+ for CtBP are very reminiscent of those determined for 3-phosphoglycerate dehydrogenase over 40 years ago [83]. Because NAD^+ and NADH appear to cause similar changes in CtBP binding, albeit at different concentrations, it will be important to determine what fraction of CtBP is occupied by these ligands *in vivo*.

We suspect that the disparate results of various laboratories on the differential binding of CtBP to NAD^+ and NADH reflects the fact that NADH binds tightly and cannot easily be removed. It is important to account for protein concentration when determining the concentration of free ligand in binding experiments. Once the free NADH concentration is calculated and the contaminating NADH is removed, the higher affinity of NADH over NAD^+ is readily apparent. Although NAD^+ and NADH differ by only a single proton and two electrons, the structures of the nicotinamide moieties are vastly different. According to the literature, the nicotinamide of NAD^+ has a planar structure, while it is puckered in NADH [84]. This marked difference seemed likely to explain the differential binding of the two nucleotides and suggests that the nicotinamide moiety plays an important role in mediating NADH binding.

Balasubramanian et al. [63] found that NAD(H) doubled the apparent MW of CtBP, suggesting that its addition promoted dimerization or tetramerization (depending on whether the 65-70 kDA species is a monomer or dimer, respectively). It is possible, therefore, that this ability of NAD(H) to stimulate CtBP oligomerization could contribute to the enhanced binding of E1A and other transcriptional repressors. We determined the stoichiometry of NADH binding and found that each monomer subunit of CtBP bound one molecule of NADH (data not shown) using the fluorescence technique recently described [85] for determining the stoichiometry of nicotinamide adenine dinucleotide binding to 3-phosphoglycerate dehydrogenase. The 1:1 binding that we observed suggests that most of the CtBP used in our assay was properly folded and able to bind

nucleotide. The lack of cooperativity in nucleotide binding further suggests that each monomer subunit binds NADH independently.

The homology between CtBP and 3-phosphoglycerate dehydrogenase is striking but the biological significance of the CtBP dehydrogenase activity is unresolved. Three reports have demonstrated that CtBP has weak dehydogenase activity [60, 63, 79], as measured by the conversion of NADH to NAD^+, in the presence of pyruvate. The turnover rate of CtBP is 30,000-fold less than that of lactate dehydrogenase (unpublished data, [86], however, which is probably why it has been overlooked or ignored. The structure of CtBP, determined by Kumar, et al, also supports the idea that CtBP is a functional dehydrogenase. According to these investigators, the weak enzymatic activity reflects the likelihood that pyruvate is not the appropriate substrate. It is also possible that CtBP simply has very little dehydrogenase activity. This view is supported by the finding that the enzymatic activity, which can be blocked by mutation of a histidine residue essential for catalysis, is not required for repression of target genes in CtBP-knockout mouse embryo fibroblasts [62]. It remains possible that the dehydrogenase activity is required for some, as yet undescribed, CtBP function. One possibility, for example, would be that the dehydrogenase activity could contribute to the termination of repression. In this scenario, conversion of NADH to NAD^+ would allow the nucleotide to dissociate and thereby disrupt the interaction between CtBP and transcriptional repressor. This process would be somewhat reminiscent of the regulated binding events seen with the Ras family of GTPases, wherein the hydrolysis of GTP to GDP results in the release of downstream effectors. Regardless of its biological role, the dehydrogenase activity was

useful in our studies as it provided a method to convert the tightly bound NADH to NAD^+, which could then be removed by dialysis.

The regulation of CtBP by nuclear nicotinamide adenine dinucleotides places this factor in the category of molecules that link transcription to cellular metabolism and respiration. The importance of CtBP as a sensor of nuclear redox state *in vivo* has yet to be determined. Large changes in the free NAD^+/NADH ratio occur at birth [87], in response to ethanol [88], and in certain metabolic disorders such as diabetes [73]. Hypoxia also has a profound effect on free NADH levels, the relief of which may be responsible for the large change at parturition. Knockout of CtBP in MEF cells causes upregulation of genes involved in cell adhesion and apoptosis. It is possible, therefore, that increased levels of NADH could result in augmented CtBP function, which would be correlated with decreased cell adhesion and protection from apoptosis. These effects could be relevant in certain disease states, such as malignancy, where the relative hypoxia of cancer cells could contribute to metastasis and escape from mechanisms that lead to cell death. Alternatively, or in addition, the dramatic decrease in free NADH levels at birth could result in the relief of repression of CtBP-regulated genes. The relationship between these events, and the role of CtBP, will become more evident as we gain understanding of gene expression in cells where energy homeostasis is challenged.

Note added in Proof. Nardini et al. [64] recently determined the structural basis for the increased affinity of CtBP for NADH. They propose that nucleotide binding induces a conformational change that promotes dimerization, which is essential for corepressor activity.

Acknowledgements

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Figure 1. Removal of bound NADH with pyruvate.

Excitation of bacterially-expressed purified CtBP at 340 nm results in an emission with a peak at 425 nm. Saturating levels of NADH demonstrate fully bound CtBP. The addition of pyruvate abolished NADH fluorescence.

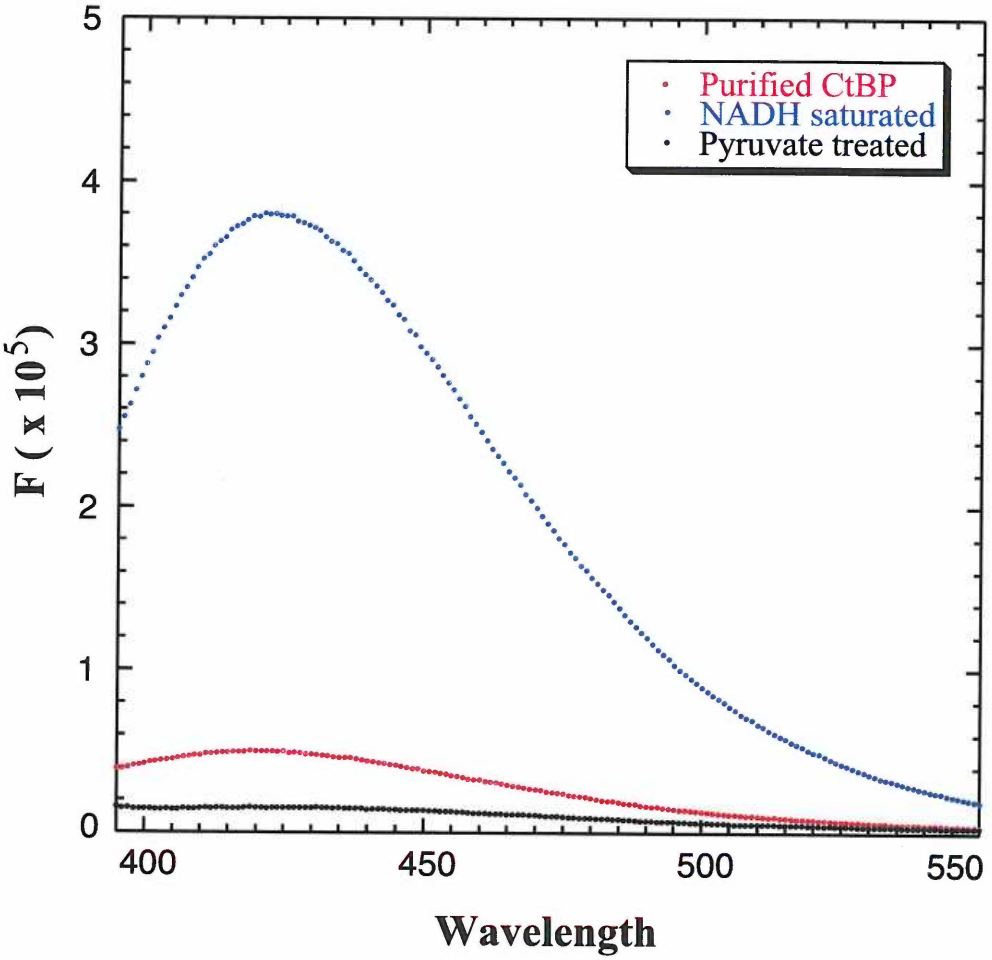

Purified CtBP
NADH saturated
Pyruvate treated
F (x 10^5)
Wavelength
0
1
2
3
4
5
400
450
500
550

Figure 2. NAD^+ binding site of CtBP, taken from Kumar et al [60].

Several contacts of CtBP residues and NAD^+ are shown. The distance between W318 and the nicotinamide moiety of NAD^+ is within the Förster distance observed for tryptophan to NADH energy transfer [81]. Protein Data Bank accession number 1MX3.

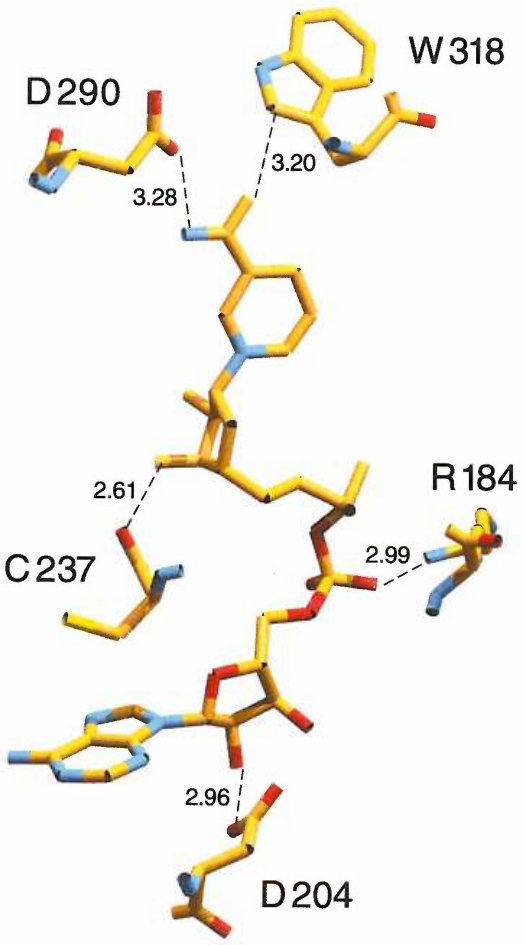

W318
D290
3.20
3.28
2.61
R184
C237
2.99
2.96
D204

Figure 3. FRET signal of CtBP.

a. The excitation of CtBP at 285 nm resulted in a typical tryptophan fluorescence emission with a peak at 340 nm. The titration of NADH resulted in a decrease of tryptophan fluorescence at 340 nm and an increase in NADH fluorescence at 425 nm.

b. Single tryptophan mutants were generated by mutating two of the three tryptophan residues to phenylalanines. The emission scan of each single tryptophan mutant excited at 285 nm is shown. Each mutant has a typical emission scan with a peak at 340 nm.

c. The addition of 200 nM NADH resulted in a strong energy transfer observed only with W318.

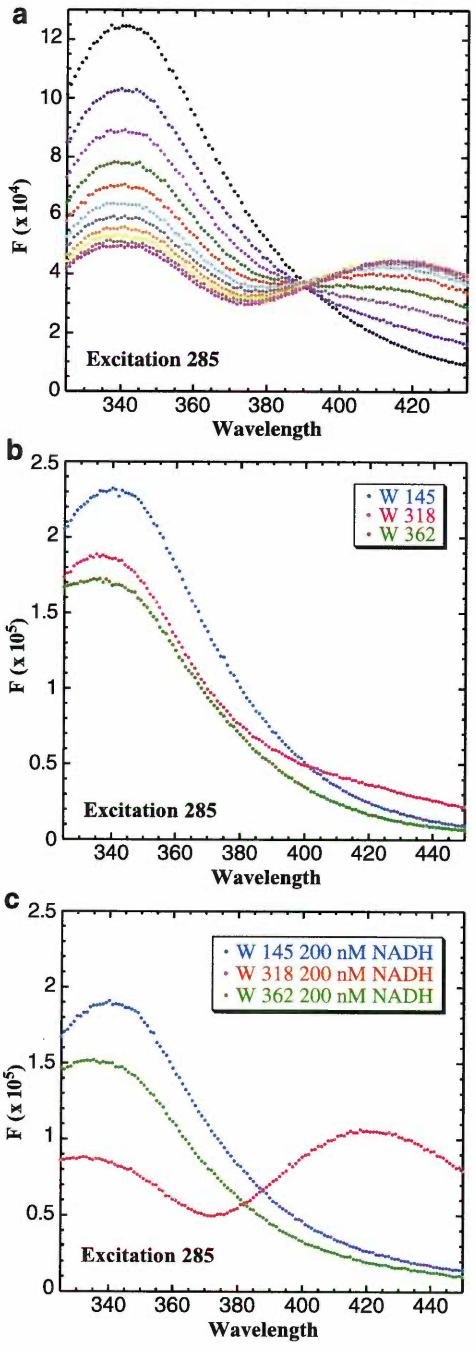

a
F (x 10^4)
Excitation 285
Wavelength
b
F (x 10^5)
W 145
W 318
W 362
Excitation 285
Wavelength
c
F (x 10^5)
W 145 200 nM NADH
W 318 200 nM NADH
W 362 200 nM NADH
Excitation 285
Wavelength

Figure 4. Plot of free NADH versus ΔF. Data fit with equation 2.

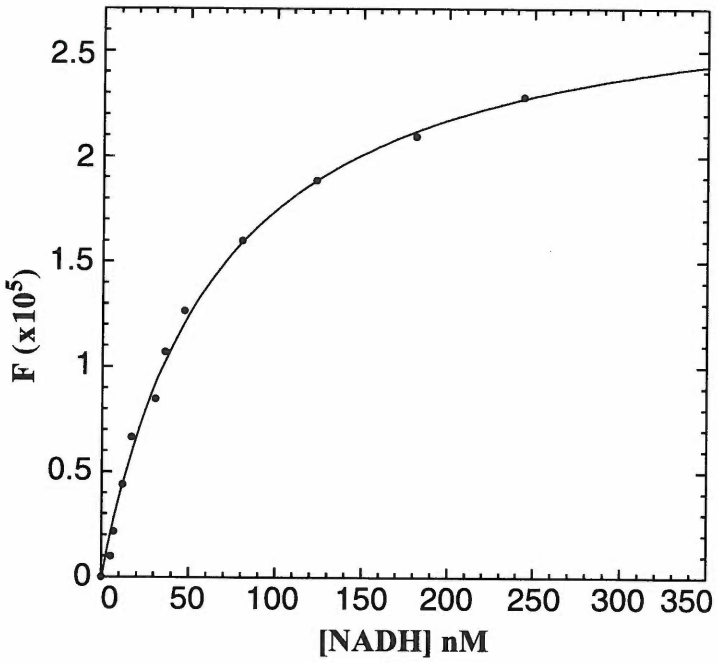
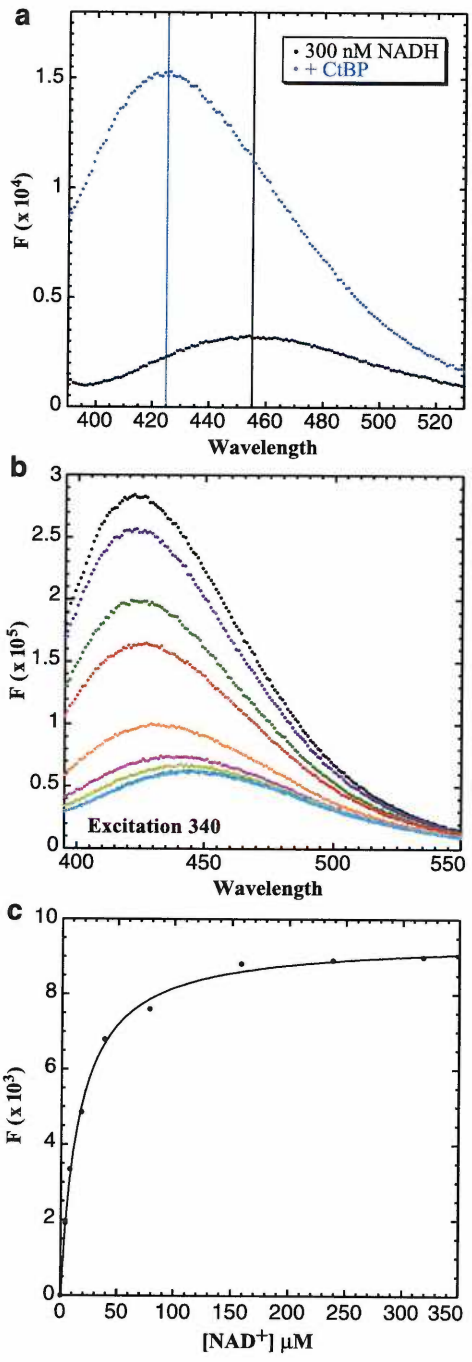

2.5
2
1.5
1
0.5
0
F (x10^5)
0
50
100
150
200
250
300
350
[NADH] nM

Figure 5. NAD^+ binding measured by NADH displacement.

a. Blue shift and enhanced fluorescence of bound NADH. Free NADH has a peak emission at 455 nm when excited at 340 nm. Addition of CtBP results in a shift of the emission peak to 425 nm and an increase in the quantum yield.

b. NAD^+ displacement of NADH. The emission scan of CtBP bound with NADH excited at 340 nm (black trace). The titration of NAD^+ resulted in a loss of NADH fluorescence. At saturating levels of NAD^+, an emission scan typical of free NADH was observed.

c. Plot of NAD^+ versus ΔF. Data fit with equation 2. See "Materials and Methods" for calculation of K_d.


a
300 nM NADH
+ CtBP
F (x 10⁴)
Wavelength
b
F (x 10⁵)
Excitation 340
Wavelength
c
F (x 10³)
[NAD⁺] µM

CHAPTER 2

Acetylation of nuclear hormone receptor interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP

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Abstract

CtBP (carboxyl-terminal binding protein) participates in regulating cellular development and differentiation by associating with a diverse array of transcriptional repressors. Most of these interactions occur through a consensus CtBP binding motif, PXDLS, in the repressor proteins. We previously showed that the CtBP binding motif in E1A is flanked by a Lys residue and suggested that acetylation of this residue by the p300/CBP-associated factor P/CAF disrupts the CtBP interaction. In this study, we show that the interaction between CtBP and the nuclear hormone receptor corepressor RIP140 is regulated similarly, in this case by p300/CBP itself. CtBP was shown to interact with RIP140 *in vitro* and *in vivo* through a sequence, PIDLSCK, in the amino-terminal third of the RIP140 protein. Acetylation of the Lys residue in this motif, demonstrated *in vivo* by using an acetylated RIP140-specific antibody, dramatically reduced CtBP binding. Mutation of the Lys residue to Gln resulted in a decrease in CtBP binding *in vivo* and a loss of transcriptional repression. We suggest that p300/CBP-mediated acetylation disrupts the RIP140-CtBP complex and derepresses nuclear hormone receptor-regulated genes. Disruption of repressor-CtBP interactions by acetylation may be general mode of gene activation.

Introduction

CtBP (carboxyl-terminal binding protein) was initially identified through its ability to interact with a five-residue motif, PLDLS, in the carboxyl-terminus of the adenoviral transforming protein E1A [59, 89]. Mutation of this motif increases the level of E1A-directed cellular transformation [58, 59], suggesting that the CtBP interaction normally serves to dampen E1A function. In keeping with this model, mutation of the CtBP-binding motif decreases the ability of E1A to repress transcription [89]. Like other transcriptional corepressors, CtBP does not interact with DNA directly but blocks gene expression when fused to a heterologous DNA binding domain [90]. Precisely how CtBP blocks transcription has not been resolved, however. Interactions have been demonstrated between CtBP and the class I and class II histone deacetylases (HDACs), as well as with other proteins believed to be involved in transcriptional silencing [91-93].

Like the better-characterized E1A-binding proteins CBP/p300 and Rb (retinoblastoma protein), CtBP has been shown to have critical functions in the regulation of cellular genes involved in growth and differentiation. Perhaps the most definitive demonstrations of these functions have been elucidated in *Drosophila*. These studies have shown that CtBP is essential for "short-range" repression, a process involved in the establishment of localized stripes, bands, and tissue-specific expression in the syncytial embryo [94]. Examples of CtBP-binding transcriptional repressors in *Drosophila* include snail, knirps, zhf-1, and kruppel [75, 95, 96]. In general, these factors each interact with CtBP through a motif, PXDLS, that is highly related to that in E1A. In mammalian cells, transcription factors that function through CtBP include BKLF (basic kruppel-like factor), ZEB (zinc finger, E box binding factor, a homolgue of zhf-1), Ikaros, and Net [97-100]. The effects of CtBP on gene expression are frequently cell- and context-dependent, however, suggesting that CtBP interactions with particular repressors may be

subject to additional levels of regulation [101]. We recently provided evidence that one such mode of regulation involves acetylation [102]. The CtBP binding motif in E1A is flanked by a Lys residue that we have shown is acetylated *in vitro* and *in vivo* by the p300/CBP-associated factor, P/CAF. Of note, acetylation of this residue was found to decrease the CtBP interaction. Thus, in the context of E1A, acetylation disrupts CtBP binding and leads to a loss of transcriptional repression. Whether this model pertains to other CtBP complexes is unknown.

In this paper, we examine the interaction of CtBP with the nuclear hormone receptor interacting protein, RIP140 [103]. We first identified RIP140 by searching a protein database for sequences containing an extended consensus CtBP-binding sequence, PXDLSXK/R. This sequence includes the core PXDLS motif in addition to the flanking basic residues (K/R) found in E1A and certain other known CtBP-binding repressors [89, 97, 104]. RIP140 was also identified as a CtBP-binding partner in a yeast two-hybrid screen.

RIP140 is perhaps the most enigmatic of the nuclear hormone receptor-interacting proteins. In the unliganded or antagonist-bound state, nuclear hormone receptors are bound by corepressor complexes containing NCoR/SMRT, Sin3, and one or more of the HDACs (reviewed in Ref. [105]). Upon ligand binding, nuclear receptors undergo a conformational change, resulting in displacement of the Sin3/ NCoR complex, followed by recruitment of ligand-dependent coactivators in the p160 family, p300/CBP, and P/CAF [106-110]. The intrinsic acetyltransferase activities of these coactivators are believed to modify the amino-terminal tails of nucleosomal histone proteins in a manner that facilitates transcription. In the sequential step model of transcriptional activation initially proposed by Roeder and coworkers, other coactivators, such as TRAP/ARC/DRIP, are then recruited to mediate RNA polymerase II-dependent

transcription (for review, see Ref. [111]). RIP140 does not appear to conform to the standard model because it associates with receptors in a ligand-dependent manner but is a corepressor rather than a coactivator [76, 112-115].

Because of its ligand-dependent association with the nuclear hormone receptors, RIP140 was initially classified as a transcriptional coactivator [103]. Subsequent studies refuted this role, however, and RIP140 is now generally acknowledged to function as a corepressor [113, 116]. Deletional analyses have identified three domains within RIP140 that are capable of inhibiting gene expression [115], but the mechanisms of repression remain unclear. Initially, it was proposed that RIP140 competed with the p160 family of coactivators for binding to the ligand-activated conformation of the receptor [76, 113]. More recent studies have suggested that HDACs might also contribute to repression. In support of this idea, a portion of the gene repression activity of RIP140 has been shown to be sensitive to trichostatin A (TSA), an HDAC inhibitor, and direct binding of RIP140 to the class I HDACs has been demonstrated [112, 117].

The studies in this paper indicate that the amino-terminal repression function of RIP140 is mediated by the corepressor CtBP. Of interest, the CtBP binding site in RIP140 is flanked by a Lys residue which, like the motif in E1A, is a potential site of acetylation. We demonstrate that RIP140 is acetylated at this site by CBP/p300 and that this modification abrogates the CtBP interaction, resulting in a loss of repression. These studies support the hypothesis that acetylation of transcriptional repressors may be a general mode of disrupting CtBP corepressor complexes.

Materials and Methods

Plasmids

pEFRIP140 [103] was a gift from F. Schaufele (University of California, San Franscisco). RIP140 (1-495), (623-951), and (977-1158) were cloned by PCR into pET23b (Novagen) or pGEXKG (Promega). The PIDLSCK motif in RIP140 is located between amino acids 440 and 446. The K446Q, PIDL->AIAL, and PIDL->AAAA mutations were constructed by the Quikchange™ method (Stratagene). His-tagged human CtBP1 (hCtBP1) was cloned by PCR into pET23b (Novagen). Rc/CMV-hCtBP1 was a gift from G. Chinnadurai (St. Louis University Health Sciences Center). Gal4-RIP140 (386-470) was cloned by PCR into a pcDNA3 vector lacking the SV40 origin. The VP16-hCtBP1 construct was subcloned into pcDNA3-VP16 (provided by R. Maurer, Oregon Health Sciences University). FLAG-hCtBP1-pcDNA3 was cloned by PCR into pcDNA3. LexA-hCtBP1 was cloned by PCR into pBTM116 [118]. The $(Gal4)_5$-E1b-luciferase vector was from M. Green (University of Massachusetts Medical Center). The $(Gal4)_5$-TK-luciferase vector and SV40-lacZ were gifts from L.-N. Wei (University of Minnesota Medical School). The Gal4-SV40-CAT vector and Gal4-ZEB (700-776) construct were gifts from D. Dean (Washington University School of Medicine). The $(ERE)_2$-pS2-CAT reporter gene was a gift from W. L. Kraus (Cornell University).

Protein purification

GST-RIP140 (1-495), (623-951), (977-1158) wild type and mutant constructs were expressed in bacteria and purified by glutathione sepharose affinity chromatography (Sigma). His-tagged hCtBP1 was expressed in bacteria and purified by Ni-NTA affinity chromatography (Qiagen). Full length FLAG-tagged p300 was expressed in Sf9 cells and purified using the M2 FLAG affinity matrix (Sigma).

Antibodies

The acetylated K446 RIP140 antibody (αAcK446) was generated against the peptide, PIDLSCac**K**HGTE. The antibody was affinity-purified against the acetylated peptide and cross-absorbed against the nonacetylated peptide (Research Genetics, Huntsville, AL). A commercially available RIP140 antibody (αRIP140) (Santa Cruz Biotechnology) was used for immunoprecipitation of endogenous RIP140. A monoclonal tetra-His antibody (Qiagen) was used to detect His-tagged hCtBP1. FLAG-tagged hCtBP1 was detected by using an antibody directed against the FLAG epitope (Upstate Biotechnology).

Yeast two-hybrid assay

L40 yeast cells expressing LexA-hCtBP1 were transformed with an E9.5 mouse VP16 fusion cDNA library [118] as previously described [119]. Yeast positive for histidine auxotrophy and ß-galactosidase production were identified and confirmed by a second round of transformations. LexA-lamin was used as a negative control.

***In vitro* acetylation assays**

Recombinant His-tagged RIP140 fragments or GST-RIP140 fragments were incubated with full length, baculovirus purified p300 for 30 minutes at 30 °C in a 30µl reaction containing acetylation buffer (10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM sodium butyrate, 1 mM DTT) and [^{3}H] acetyl coenzyme A (AcCoA; Amersham Pharmacia Biotech). Reactions were electrophoresed on a 10% polyacrylamide gel and analyzed by fluorography. For GST pull down assays, acetylation reactions were performed as described using unlabeled AcCoA (10 µM). Mock acetylation reactions were performed using 1 pmol p300 in the absence of 10 µM AcCoA or with AcCoA in the absence of p300.

GST pull down assays

Binding assays. GST or GST-RIP140 fragments were acetylated or mock acetylated prior to immobilization onto glutathione beads. Equimolar amounts of GST or GST fusion proteins were linked to glutathione beads (Amersham Pharmacia Biotech) in the presence of binding buffer (20 mM Hepes, pH 7.6, 300 mM KCl, 10% glycerol, 0.5% NP40, 1 mM DTT, 10 μM Na_3VO_4, 10 μM NaF, Complete™ protease inhibitors (Roche Molecular Biochemicals)) for 1 hour at room temperature. BSA was then added to a final concentration of 0.5% for 30 minutes at 4°C. The beads were washed once with binding buffer and incubated for 1 hour at room temperature with ^{35}S-labeled *in vitro* translated hCtBP1 (Promega) or recombinant his-tagged hCtBP1. The samples were washed 3 times with binding buffer, electrophoresed on a 10% polyacrylamide gel, and processed for autoradiography or western analysis.

Peptide competition assays. Recombinant His-tagged hCtBP1 (11.6 nmol) was preincubated with 20 μM or 40 μM of nonacetylated (SNCVPIDLSCKHGT) or acetylated (SNCVPIDLSCac**K**HGT) peptides in binding buffer for 30 minutes on ice and subsequently incubated with the immobilized GST or GST fusion proteins as described. After washing in binding buffer, the proteins were eluted with 5X SDS-PAGE buffer and electrophoresed on 10% polyacrylamide gels. The reactions were analyzed by western blotting using a tetra-His antibody (Qiagen).

Cell culture and transfection assays

Mammalian two-hybrid assays. HepG2 cells were cultured in DMEM (BRL) supplemented with 10% FBS (Hyclone). Transfections were performed with the Fugene reagent (Roche Molecular Biochemicals) as directed by the manufacturer. The cells were grown in 24-well plates and transfected with 100 ng reporter, 50 ng Gal4-RIP140 (386-470), and 50 ng VP16-hCtBP1. The total amount of DNA per well (500 ng) was kept

constant by the addition of an empty pcDNA3 vector. Luciferase assays were performed as described [102]. Each condition was assayed in triplicate a minimum of 3 times.

Repression assays. COS7 and HepG2 cells were cultured in DMEM supplemented with 10% FBS. Cells were cultured in 6-well plates and transfected using the Fugene reagent with 300 ng Gal4-SV40-CAT reporter, 20 ng SV40-lacZ, and 700 ng Gal4 fusion construct. The total concentration of DNA was maintained at 1.5 µg using empty pcDNA3 vector. Protein levels of the Gal4-RIP140 constructs were determined by immunoprecipitation followed by western analysis of cell extracts.

For studies of estrogen receptor-dependent transcription, HepG2 cells were cultured in phenol red free DMEM (BRL) supplemented with charcoal/dextran stripped FBS (Hyclone) for a minimum of two weeks before culturing in 24 well plates. Transfections of 100 ng $(ERE)_2$-pS2-CAT reporter, 20 ng SV40-lacZ, 100 ng full length pEFRIP140 wild type and mutants, and 100 ng pCMV-hCtBP1 were performed using the Fugene reagent. Cells were treated with 100 µM $CoCl_2$ for 16 hours and either 10 nM 17ß-estradiol or ethanol for 4-6 hours. The total DNA concentration was kept constant by addition of empty pcDNA3 vector. CAT ELISAs (Roche Molecular Biochemicals) were performed 48 hours after transfection as directed by the manufacturer. ß-galactosidase assays were performed using the Emerald II reagent (TROPIX™, PE Biosystems). Each condition was assayed in triplicate a minimum of 3 times.

***In vivo* acetylation assays**

COS7 and HepG2 cells were cultured in 10% FBS-supplemented DMEM and grown on 6 cm plates for transfection with 2.5 µg full length CBP. After 48 hours, the cells were washed with cold PBS and lysed by incubation on ice for 10 minutes in 500 µl lysis buffer (20 mM Hepes, pH7.6, 300 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.2 mM

$ZnAc_2$, 1% NP40, 1 mM DTT, 10 μM Na_3VO_4, 10 μM NaF, and Complete™ protease inhibitors). Cell debris was cleared by centrifugation at 14,000 rpm for 10 minutes at 4 °C. Approximately 0.8 μg polyclonal RIP140 antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Sigma) were bound to protein G sepharose beads (Amersham Pharmacia Biotech) and incubated with the cell lysates for 1 hour at 4 °C. The beads were washed with lysis buffer and eluted with 5X SDS PAGE loading buffer. The immunoprecipitates were subjected to western analysis using the affinity-purified acetylated K446 RIP140 antibody (αAcK446) or a commercially available RIP140 antibody.

Co-immunoprecipitation assays

COS7 cells were grown to 50% confluency on 100 mm plates and transfected with 4 μg FLAG-hCtBP1-pcDNA3. Cells were washed with cold PBS 48 hours post-transfection, collected in lysis buffer (PBS, 0.1% NP40, 1 mM DTT, 50 mM ß-glycerophosphate, 10 mM NaF, 10 μM Na_3VO_4, and Complete™ protease inhibitors), and sonicated. The cell lysates were then centrifuged, and the supernatants were added to an M2 FLAG affinity matrix (Sigma). Co-immunoprecipitations were performed for 1 hour at 4°C. Subsequently, the beads were washed 4 times in lysis buffer and eluted by boiling for 5 minutes in 5X SDS-PAGE loading buffer. After gel electrophoresis and transfer to PVDF membrane (Millipore), the immunoprecipitates were probed with an anti-RIP140 antibody (Santa Cruz Biotechnology).

Results

Identification of potential CtBP-interacting proteins

To determine whether acetylation might be a general mechanism of regulating CtBP interactions, we identified a family of CtBP binding partners using a yeast two-

hybrid screen. Approximately 68% of the 41 positive clones identified from a mouse embryonic day 9.5 cDNA library contained a sequence that was closely related to the PXDLS motif (Table 1). Of these, 75% contained a flanking Lys or Arg residue. Of the DNAs containing a PXDLS motif whose identities were known, 72% corresponded to known or suspected transcriptional repressors. Among this group were CtIP (CtBP-interacting protein), BKLF, and ZEB, which have previously been shown to repress transcription by binding to CtBP. The interaction of RIP140 with CtBP had not been described before.

Characterization of the RIP140-CtBP interaction

To identify the CtBP binding site in RIP140, we generated GST-RIP140 constructs spanning the three previously characterized repression domains (; Fig. 1). GST pull down assays verified that only the fragment containing the PXDLS motif, GST-RIP140 (1-495), was capable of interacting with CtBP (Fig. 2A). This fragment overlapped with the portion of RIP140 detected in the yeast two-hybrid assay. Mutation of the PXDLS motif completely abolished the interaction (Fig. 2B). Neither the portion of RIP140 containing the central cluster of LXXLL motifs, GST-RIP140 (623-951), nor the carboxyl-terminal portion, GST-RIP140 (977-1158), were capable of interacting with CtBP (Fig. 2A), suggesting that these domains may repress transcription through an alternate mechanism. Indeed, we showed that GST-RIP140 (977-1158) interacted with HDAC1 from HeLa nuclear extracts (data not shown).

To determine whether the full length RIP140 and CtBP proteins interact *in vivo*, we performed co-immunoprecipitation assays in COS7 cells, which contain low but detectable levels of RIP140. Cells were transiently transfected with FLAG-tagged hCtBP1 and lysates were incubated with an M2 FLAG affinity matrix. Immunoprecipitates containing FLAG-hCtBP1 were analyzed by western blotting using

an antibody directed against the amino-terminal portion of RIP140. These assays confirmed that endogenous RIP140 associates with CtBP *in vivo* (Fig. 3). Treatment of cells with estradiol did not affect the amount of CtBP that co-immunoprecipitated with RIP140 (data not shown).

RIP140 is acetylated in vitro and in vivo

Previous studies from our lab have demonstrated that acetylation of a Lys residue (Lys239) near the carboxyl-terminus of E1A regulates the interaction with CtBP [102]. The modified Lys residue in E1A is adjacent to the CtBP-binding site, an arrangement that is conserved in RIP140. We thus tested whether RIP140 could similarly become acetylated. Our previous studies showed that P/CAF and GCN5 were equally capable of acetylating E1A. To our surprise, neither enzyme was able to acetylate RIP140 *in vitro* (data not shown). In contrast, *in vitro* acetylation reactions using baculovirus expressed, full length mouse p300 and [^{3}H] AcCoA demonstrated that GST-RIP140 (1-495) is readily acetylated (Fig. 4A). Mutation of the Lys residue adjacent to the CtBP binding motif markedly attenuated acetylation (Fig. 4A). Although acetylation was not completely abolished, our results suggest that Lys446 is a major RIP140 acetylation site. Neither GST-RIP140 (623-951) nor GST-RIP140 (977-1158) could be acetylated by p300 (data not shown).

Commercially available antibodies do not recognize acetylated Lys residues in all contexts, and this appears to be the case for RIP140 (data not shown). Consequently, to determine whether acetylation of RIP140 at Lys446 occurs *in vivo*, we generated an antibody against a RIP140 peptide, PIDLSCac**K**HGTE, containing the acetylated Lys (αAcK446). After affinity-purification, the αAcK446 antibody recognized the acetylated form of RIP140 but did not detect RIP140 subjected to a mock acetylation reaction (Fig. 4B, top). Specificity of the αAcK446 antibody was also demonstrated by showing that it

did not recognize other acetylated proteins, such as p53 (data not shown). Under the conditions used, the αRIP140 and αAcK446 antibodies appear equally capable of recognizing their epitopes in the recombinant GST-RIP140 fusion proteins (Fig. 4B).

To determine whether RIP140 was acetylated *in vivo*, we transiently transfected COS7 and HepG2 cells, both of which contain endogenous RIP140, with a full length mouse CBP expression vector. The cell lysates were immunoprecipitated using an antibody that recognizes RIP140 (αRIP140) or IgG fractions from non-immunized rabbits. Western analysis of the immunoprecipitates demonstrates that in the absence of exogenous CBP, only low levels of endogenous acetylated RIP140 were detected (Fig. 4C, top panels). However, in the presence of exogenous CBP, RIP140 is readily acetylated *in vivo* in both cell types (compare Fig. 4C middle and bottom panels).

Acetylation of RIP140 abrogates its interaction with CtBP

To determine whether the Lys residue adjacent to the CtBP-binding site participates in the RIP140-CtBP interaction, we mutated this residue to Gln, which resembles acetylated Lys in that it is uncharged at neutral pH. Mutation of Lys446 to Gln abolished the interaction (Fig. 5A), confirming the importance of the Lys residue for CtBP interaction and suggesting that acetylation of this residue might also regulate CtBP binding. To test this hypothesis, GST-RIP140 (1-495) was acetylated or mock acetylated (treated in the absence of AcCoA) with p300 and incubated with *in vitro* translated hCtBP1. Acetylation abolished interaction of RIP140 with CtBP, while mock acetylation did not (Fig. 5A). These studies indicated that the ability of RIP140 to interact with CtBP was regulated by acetylation.

To confirm these results, we performed a series of peptide competition assays. Because only the non-acetylated form of RIP140 is able to interact with CtBP, the non-

acetylated peptide, but not the acetylated form, should be able to compete with GST-RIP140 (1-495) for CtBP binding. Peptide competition assays were performed using an acetylated RIP140 peptide (SNCVPIDLSCac**K**HGT) or a non-acetylated peptide (SNCVPIDLSCKHGT). Preincubation of the non-acetylated peptide completely blocked GST-RIP140 (1-495) binding to hCtBP1 while incubation with the acetylated peptide did not, even at the higher concentrations (Fig. 5B). These studies confirm the potent effects of RIP140 acetylation of CtBP binding.

It is not possible to manipulate the level of RIP140 acetylation very precisely *in vivo*, but evidence for the effect of Lys446 acetylation on CtBP binding can be obtained by examining the Lys446Gln mutant. Mammalian two-hybrid assays were performed using Gal4-RIP140 (386-470) and VP16-hCtBP1. Wild type Gal4-RIP140 is able to interact with VP16-hCtBP1 in HepG2 cells, a cell line that normally expresses RIP140, and activate the E1b or thymidine kinase (TK) promoters (Fig. 5C). Mutation of the PILD sequence in CtBP to AIAL completely blocked the interaction, while mutation of Lys446 to Gln reduced the interaction by 70-80%.

RIP140 represses transcription through its association with CtBP

Our studies suggest that one of the mechanisms by which RIP140 represses transcription is through its interaction with the corepressor, CtBP. Disrupting this interaction by acetylation or mutation of Lys446 should result in a loss of repression. To test this hypothesis, we analyzed the ability of wild type Gal4-RIP140 (386-470), which contains sequences spanning the CtBP interaction domain, as well as the Lys446Gln and AIAL mutants, to repress transcription of a Gal4-SV40-CAT reporter gene. As a control for these experiments, we analyzed Gal4-ZEB (700-776), a known CtBP-dependent transcriptional repressor [98]. In both COS7 and HepG2 cells, wild type Gal4-RIP140 (386-470) represses transcription to a similar level as Gal4-ZEB (700-776) (Fig. 6).

Alteration of the consensus CtBP interaction motif, either by mutation of Lys446 to Gln or alteration of the PIDL sequence to AIAL, restores transcription levels to those seen in the absence of repressor (Fig. 6). We conclude from these studies that modification of Lys446 contributes to the regulation of RIP140 repressor function.

RIP140 represses nuclear hormone receptor dependent transcription

We next examined the ability of full length RIP140 to repress transcription of an estrogen receptor-dependent reporter gene, $(ERE)_2$-pS2-CAT. This gene contains a duplicated estrogen response element (ERE) upstream from the pS2 promoter and has been used extensively to examine estrogen-responsive transcription [120]. In the absence of exogenous RIP140, CtBP did not significantly repress transcription (Fig. 7). However, in the presence of exogenous wild type RIP140 or a form in which Lys446 had been mutated to Arg, transcription was dramatically repressed. Mutation of Lys446 to Gln or alteration of the PIDL motif to AIAL severely attenuated RIP140-mediated transcription repression. These results support the conclusion that RIP140 represses transcription in large part through its association with CtBP.

Discussion

RIP140 associates in a ligand-dependent manner with a variety of nuclear hormone receptors, including the estrogen, glucocorticoid, retinoic acid, retinoid X, thyroid, and liver X receptors [76, 103, 113-115]. Unlike other proteins that interact with ligand-bound nuclear hormone receptors, RIP140 is a transcriptional corepressor. In this study, we have analyzed the regulation of RIP140 corepressor function by acetylation. Previous work from our lab has demonstrated that the E1A-CtBP interaction is similarly modulated by acetylation [102]. In the case of E1A, the acetylation activity of P/CAF was much more efficient than that of p300/CBP. With RIP140, p300/CBP was able to

mediate acetylation while P/CAF was completely ineffective. The common theme of both studies was that Lys acetylation regulated the binding of CtBP and, hence, repressor function. Our yeast two-hybrid assays suggest that this mechanism may be a fairly common mode of gene derepression, in that many of the putative CtBP binding sequences were flanked by a Lys residue (see Table 1, Group I). Whether the repressors in Group III can be acetylated and whether this modification blocks CtBP binding has not been addressed. In several instances, however, this residue is an Arg (shown in Group II), which is not a known target of the coactivator acetyltransferases. Indeed, we have shown in the context of E1A that a Lys to Arg substitution actually increases CtBP binding *in vivo* [102], although it is possible that this reflects a basal level of Lys acetylation rather than a true difference in the affinity of Lys- or Arg-containing proteins. Nonetheless, it appears that individual transcriptional repressors may be differentially responsive to coactivator acetyltransferases depending upon the identity of this single amino acid.

Why acetylation of the flanking Lys residue is so critical for CtBP interaction is unknown. Presumably, this issue will become clarified once the structure of one of the CtBP-binding repressors has been solved. Although such information is not yet available, clues to the nature of the CtBP interaction site may possibly be gleaned from other known structures. For example, the prohormone processing carboxypeptidase Kex1 contains a PXDLTXK sequence that might also be expected to bind CtBP. Indeed, we have shown that CtBP interacts with Kex1 in GST pull down assays (N. Vo, unpublished observations.) Structural analysis of Kex1 indicates that the motif is located on the surface of the protein and forms a pocket that is filled by the positively charged Lys residue [121]. Thus, neutralization of this positively charged Lys residue by acetylation would be predicted to perturb the CtBP-binding interface. In keeping with this model, we have shown that mutation of the Lys to Gln prevents CtBP binding. It is possible,

therefore, that the flanking Lys is ideally positioned to regulate CtBP binding. In contrast, the results of the two-hybrid assay suggest that the Lys is not essential for binding (see Group IV, where the flanking Lys is not present). We suggest, therefore, that although the Lys is not essential, its acetylation prevents the CtBP interaction.

Our model is somewhat reminiscent of that described for the coactivator, ACTR, which, when acetylated by p300, disrupts the coactivator complex and terminates transcription [100]. Unlike the situation with ACTR, however, acetylation of RIP140 leads to gene activation rather than repression. Coactivator histone acetyltransferases recruited by particular transcription factors are believed to modify specific nucleosomes in the vicinity of activated genes (reviewed in Ref. [122]). Recent studies demonstrate that histone modifications may occur globally as well [123]. The idea that the basal state of nucleosomes contains both acetylated and deacetylated histones suggests that chromatin can be "primed" for transcription. Thus, the ability of a transcription factor such as RIP140 to function as an activator or repressor may depend on the global acetylation state of a given gene. Alternatively, RIP140 could be acetylated locally through recruitment of p300/CBP. Whether nuclear hormone receptors can associate simultaneously with p300/CBP and RIP140 is unknown, however.

In conclusion, the acetylation state of CtBP binding proteins such as E1A and RIP140 directly modulates their ability to mediate transcriptional repression. In the unacetylated state, these proteins can act as transcriptional repressors through their interaction with CtBP but, in the acetylated state, the interaction is prevented. Although the mechanism by which CtBP represses transcription remains unknown, this corepressor has clearly been shown to be critical for the regulation of a large number of developmental and differentiation processes. Potentially, these activities can also be modulated by the acetylation state of the CtBP-interacting transcription factors. It is

widely accepted that the level of histone deacetylation is correlated with transcriptional repression while histone acetylation is correlated with transcriptional activation. Our studies suggest that disruption of repressor-corepressor complexes might be an equally plausible mechanism to explain how coactivator acetyltransferases activate selected genes.

Acknowledgements: We thank G. Chinnadurai, W.L. Kraus, and D. Dean for reagents, Hong Yao for help with the two-hybrid assays, and Q. Zhang for helpful comments. This work was supported by grants from the NIH

Figure 1. RIP140 contains a consensus CtBP binding site. The nine LXXLL motifs present in RIP140 are represented by the vertical bars. The fragment of RIP140 (amino acids 403-521) isolated from an E9.5 mouse embryo cDNA library is indicated by the gray bar. Fragments of RIP140 used in GST pull down assays are also depicted. Mutations in the consensus CtBP binding motif (amino acids 440-446) are indicated.

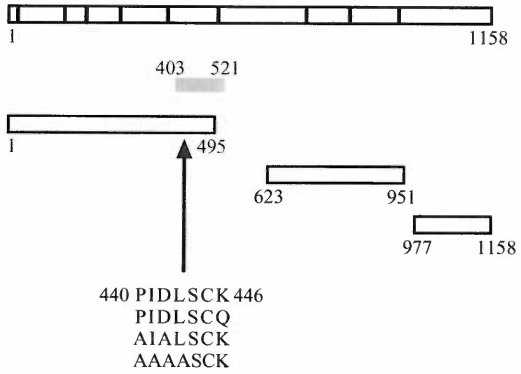

1
1158
403
521
1
495
623
951
977
1158
440 PIDLSCK 446
PIDLSCQ
AIALSCK
AAAASCK

Figure 2. RIP140 binds to hCtBP1 *in vitro*.

(A) Equimolar amounts of GST-RIP140 (1-495), GST-RIP140 (623-951), GST-RIP140 (977-1158), and GST alone were incubated with *in vitro* translated hCtBP1. The samples were electrophoresed on a 10% polyacrylamide gel and analyzed by autoradiography.

(B) Mutation of the consensus hCtBP1 binding motif abolishes the RIP140-hCtBP1 interaction. Equimolar concentrations of GST-RIP140 (1-495) wild type (WT), PIDL->AIAL, PIDL->AAAA, and GST alone were incubated with recombinant, His-tagged hCtBP1. The samples were electrophoresed on a 10% polyacrylamide gel and analyzed by western blotting using a monoclonal His-tag antibody.

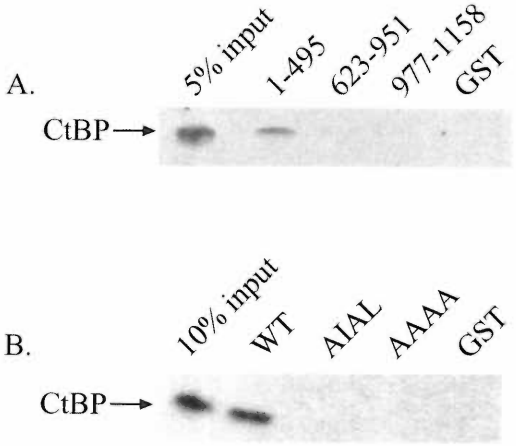

A.
5% input
1-495
623-951
977-1158
GST
CtBP
B.
10% input
WT
AIAL
AAAA
GST
CtBP

Figure 3. RIP140 interacts with hCtBP1 *in vivo*. COS7 cells were transiently transfected (+) or mock transfected (-) with FLAG-hCtBP1. Immunoprecipitations were performed using an M2 FLAG affinity matrix. Western analyses were performed using a polyclonal RIP140 (left panel) or FLAG (right panel) antibody, as indicated at the top of the figure.

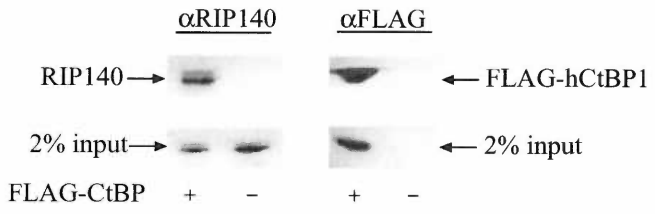

αRIP140
αFLAG
RIP140
FLAG-hCtBP1
2% input
2% input
FLAG-CtBP
+
–
+
–

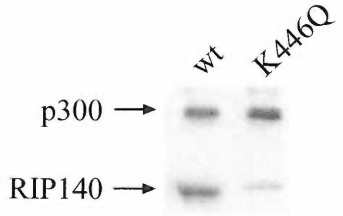
Figure 4. RIP140 is acetylated at lysine 446.

(A) 11 nmol of recombinant, GST-RIP140 (1-495) wild type or K446Q mutant were incubated with full length, baculovirus purified p300 (1 pmol) in the presence of [^{3}H] acetyl CoA. Reactions were analyzed by SDS-PAGE and visualized by fluorography.

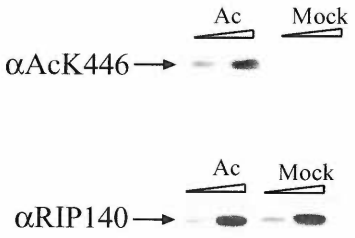
(B) Recombinant GST-RIP140 (1-495), 25 ng and 300 ng, was acetylated or mock acetylated with p300. Samples were electrophoresed on a 10% polyacrylamide gel and subjected to western analysis using an antibody directed against acetylated (top) or unacetylated (bottom) RIP140.

(C) In the top panels, endogenous RIP140 was immunoprecipitated from COS7 and HepG2 cells with a RIP140 antibody and subjected to western analysis using the acetylated K446 RIP140 antibody. Middle panels show cells transfected with full length CBP before immunoprecipitation. Total RIP140 protein immunoprecipitated from these cells are shown in the bottom panels.

A.


wt
K446Q
p300
RIP140

B.


Ac
Mock
αAcK446
Ac
Mock
αRIP140

C.

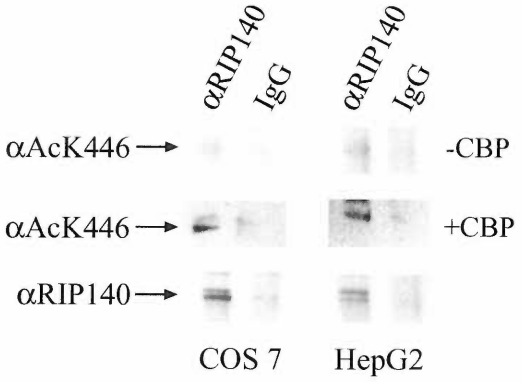

αRIP140
IgG
αRIP140
IgG
αAcK446
-CBP
αAcK446
+CBP
αRIP140
COS 7
HepG2

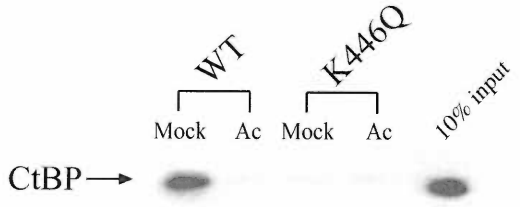
Figure 5. Acetylation of RIP140 disrupts its interaction with hCtBP1.

(A) Mutation or acetylation of K446 disrupts the interaction of RIP140 with hCtBP1. GST pull-down assays were performed using acetylated or mock acetylated GST-RIP140 (1-495) WT or K446Q. Samples were incubated with *in vitro* translated hCtBP1, electrophoresed on a polyacrylamide gel, and analyzed by autoradiography.

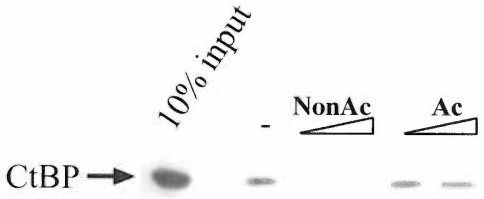
(B) Peptide competition assays. GST-RIP140 (1-495) was incubated with recombinant, His-tagged hCtBP1 (11.6 nmol) in the presence or absence of 20 μM or 40 μM nonacetylated (NonAc) or acetylated (Ac) peptides. Bound hCtBP1 was analyzed by western blotting using a His-tag antibody. (–) designates binding in the absence of peptide competitor.

(C) K446Q mutation attenuates the RIP140-hCtBP1 interaction in a mammalian two-hybrid assay. Gal4-RIP140 (386-470) and VP16-hCtBP1 were cotransfected with either $(Gal4)_5$-E1b-luc or $(Gal4)_5$-TK-luc reporter constructs into HepG2 cells. Luciferase activity was measured 48 hours post-transfection.

A.


WT
K446Q
Mock
Ac
Mock
Ac
10% input
CtBP

B.


10% input
-
NonAc
Ac
CtBP

C.

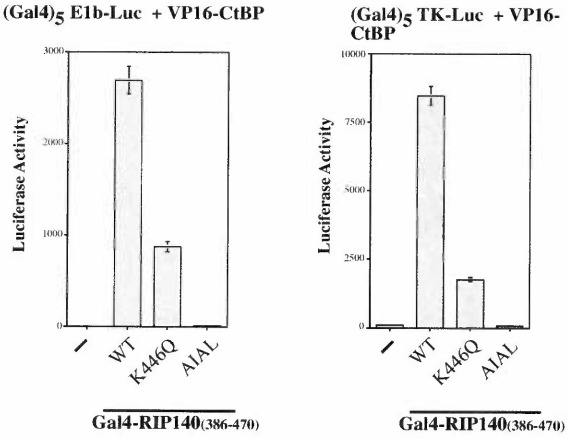

(Gal4)5 E1b-Luc + VP16-CtBP
Luciferase Activity
3000
2000
1000
0
-
WT
K446Q
AIAL
Gal4-RIP140(386-470)
(Gal4)5 TK-Luc + VP16-CtBP
Luciferase Activity
10000
7500
5000
2500
0
-
WT
K446Q
AIAL
Gal4-RIP140(386-470)

Figure 6. RIP140 represses transcription by binding to hCtBP1. Gal4-RIP140 fusion protein containing the CtBP interaction motif was analyzed for its ability to represss a Gal4-SV40-CAT reporter. Wild type, K446Q, or the AIAL mutants were compared to Gal4-ZEB (700-776). COS7 and HepG2 cells were transiently transfected with 300 ng reporter, 700 ng Gal4 fusion constructs, and 20 ng SV40-lacZ. CAT protein produced in the absence of repressor (-) is indicated. The total amount of DNA was kept constant using empty pcDNA3 vector. Protein levels of the RIP140 constructs are shown in the insets. CAT protein production was measured 48 hours after transfection using an ELISA system (Roche Molecular Biochemicals) and values are normalized to ß-galactosidase activity.

Gal4-SV40-CAT

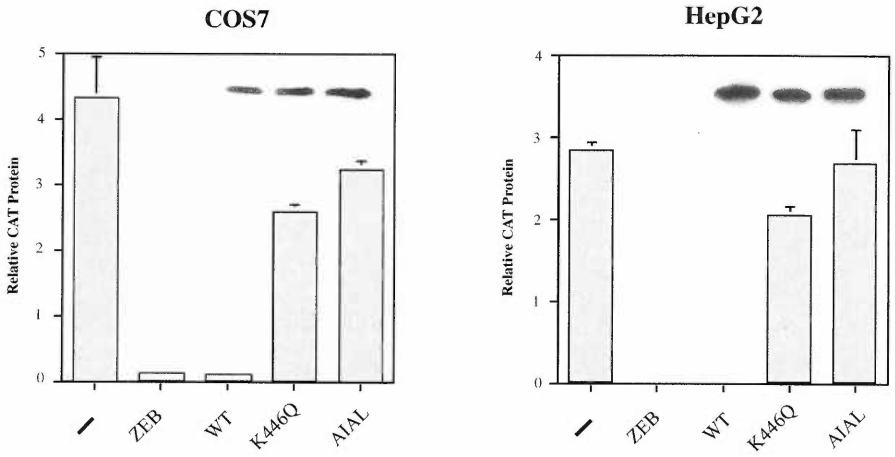


Figure 7. RIP140 represses estrogen receptor-dependent transcription. The ability of full length RIP140, wild type and mutants, to repress transcription of an $(ERE)_2$-pS2-CAT reporter gene was analyzed in HepG2 cells treated with 10 nM estradiol. Cells were transfected with 100 ng CAT reporter, 20 ng SV40-lacZ, 100 ng pEFRIP140 wild type and mutants, in the absence or presence of pCMV-hCtBP1. The total concentration of DNA was kept constant by addition of empty pcDNA3 vector. CAT protein production was measured 48 hours after transfection and values are normalized to ß-galactosidase activity.

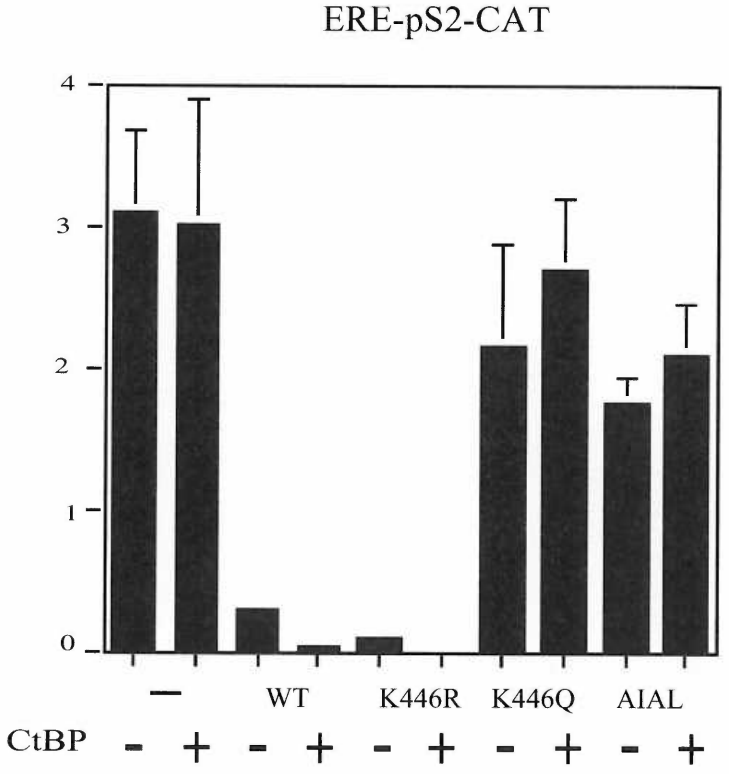

ERE-pS2-CAT
4
3
2
1
0
—
WT
K446R
K446Q
AIAL
CtBP - + - + - + - + - +

Table 1. CtBP-interacting proteins identified in a yeast two-hybrid assay are grouped into four categories, as indicated in bold letters. Proteins shown previously to interact with CtBP are indicated by asterisks. Two potential CtBP binding motifs in MyT1 and RIZ are listed. Thirteen clones did not contain an identifiable PXDLS motif.

Table 1. Yeast Two-Hybrid CtBP Interaction Proteins

GROUP	MOTIF								NAME
I	**P**	**X**	**D**	**L**	**S**	**X**	**K**		
	P	I	D	L	S	C	K		RIP140
	P	L	D	L	S	S	K		BCoR
	P	T	D	L	S	M	K		unknown
	P	L	D	L	S	A	K		unknown
	P	E	N	L	S	T	K		MyT-1
	P	Q	D	L	P	S	K		MyT-1
	V	L	D	L	S	T	K		unknown
	V	L	D	L	S	V	K		RP58
II	**P**	**X**	**D**	**L**	**S**	**X**	**R**		
	P	L	D	L	S	D	R		CtIP*
	P	L	D	L	S	M	R		unknown
	P	L	D	L	S	L	R		Zinc finger protein 219
	P	L	D	L	T	V	R		unknown
	P	L	N	L	S	S	R		SRp55-3
III	**P**	**X**	**D**	**L**	**S**	**X**	**X**		
	P	L	D	L	S	L	P	K	ZEB*
	P	V	D	L	T	V	N	K	BKLF*
	P	I	D	L	T	K	S	K	Teashirt2
	P	L	D	L	S	P	V	K	unknown
	P	M	D	L	S	T	V	K	RING 3, and unknown
	P	L	D	I	L	Q	S	K	Retinoic acid induced protein
	V	V	D	L	T	L	P	K	Ku70
	V	L	D	L	S	V	H	K	RIZ
IV	**P**	**X**	**D**	**L**	**S**	**X**	**X**	**X**	
	P	L	D	L	S	S	G	V	RIZ
	V	V	D	L	S	K	A	S	unknown
	P	L	D	L	S	C	G	S	unknown
	P	L	N	L	S	L	G	P	Zinc finger protein 217
	P	T	D	L	S	V	N	P	unknown
	V	I	D	L	T	I	E	S	Miz1
	P	L	N	L	R	I	P	S	NAB1

Discussion

CtBP is a transcriptional corepressor that is recruited to promoter elements of genes through its ability to interact with repressor proteins that either directly, or indirectly, bind DNA. Once recruited, CtBP represses gene expression by an unknown mechanism. The isolation of CtBP in a complex containing essential components for gene targeting and coordinated histone modifications [79] suggests that CtBP functions in concert with other corepressor activities. Although little progress has been made towards elucidating the mechanism utilized by CtBP to attenuate transcription, significant progress has been made towards understanding the regulation of CtBP recruitment to a gene's promoter. One important determinant of CtBP recruitment is the presence of a binding motif (PXDLS) on the repressor protein, first characterized in the carboxyl terminus of the adenoviral transforming protein E1A [58]. I performed a genetic screen to identify proteins that directly interact with CtBP using the LexA / VP16 yeast two-hybrid system[24]. The proteins expressed by several clones identified in the screen had been previously reported to interact with CtBP and contained a consensus CtBP binding motif (Table1). Additionally, the majority of expressed proteins contained sequences resembling the PXDLS motif and also contained flanking basic residues. Positive clones were classified into 4 groups based on the identity of the residues flanking the consensus site. Members of Group 1 contained the extended motif, PXDLSXK, which is also present in E1A. Studies with E1A demonstrated that acetylation of the flanking lysine residue blocked the interaction with CtBP [102]. The acetylation of a Group 1 protein, the nuclear receptor-interacting protein (RIP140), was found to block its interaction with CtBP and relieve repression mediated by CtBP [24] Members of Group 2 contain an

arginine residue instead of a lysine (PXDLSXR). Because arginine residues cannot be acetylated by HATs, an additional layer of regulation is provided where Group 1 proteins may be acetylated and Group 2 proteins are not. Group 3 consists of proteins that contain a lysine two residues after the PXDLS motif (PXDLSXXK.) The importance of acetylation at this site remains to be determined. Members of Group 4 contain the minimal motif and lack the flanking basic residues present in Groups 1, 2 and 3. Work by Vo et al. [102] and Zhang et al. [24] demonstrate two cases where acetylation regulates protein/protein interactions with CtBP. The two-hybrid screen emphasizes the importance of basic residues flanking the PXDLS motif and suggests that acetylation may be a general mechanism of regulating CtBP interactions.

The recruitment of CtBP to promoters is also influenced by pyridine dinucleotides. Zhang et al. observed that both NAD^+ and NADH were able to dramatically stimulate the interaction between CtBP and E1A [5]. According to this work, the stimulated E1A binding was observed at NADH levels >100-fold lower than observed with NAD^+. The physiological relevance of these observations depends on the concentration of these pyridine dinucleotides in cells. Because a large number of proteins bind pyridine dinucleotides, a large percentage of the total amount in cells is bound and unavailable to regulate CtBP. Furthermore, different cellular compartments may contain different levels of pyridine dinucleotides. For example, pyridine dinucleotides do not freely pass across the mitochondrial membranes. Rather, the malate/aspartate shuttle is required for transferring reducing equivalents from glycolysis in the cytoplasm across the mitochondrial membrane to the electron transport chain. Nuclear pores are large enough

to allow passage of small proteins and small molecules. It is likely that pyridine dinucleotides are able to freely diffuse between the nucleus and the cytoplasm.

It is important to consider the concentration of the free (unbound) nuclear forms of each dinucleotide when evaluating their potential roles in stimulating the recruitment of CtBP to promoters. The transcriptional corepressor, Sir2, contains an NAD^+-dependent protein deacetylase activity that is thought to link calorie restriction to life extension[52, 72]. A great deal of interest and work has been invested in finding the signal that regulates Sir2. The prime candidates for the signal are NAD^+, its precursor (nicotinamide), and metabolic product (NADH). Thus, the Sir2 literature provides information related to the cellular concentrations and metabolism of pyridine dinucleotides. Studies analyzing the physiological consequences of the pyridine dinucleotide binding properties of Sir2 and CtBP can be difficult to interpret because it is often unclear whether the NAD^+/NADH levels being considered are the total or free levels.

The metabolic signal that regulates Sir2 and links metabolism to gene regulation remains unknown. Initial studies describing the NAD^+-dependence of Sir2's deacetylase activity predicted that NAD^+ levels change in response to alterations in the cell's metabolic state [51]. Closer analysis in later studies suggests that Sir2 may not be regulated by NAD^+ and that NAD^+ levels do not change significantly during metabolic changes such as calorie restriction [56, 124]. Hence, the signal appears to not be NAD^+. The proposal that NADH regulates Sir2 [125] is consistent with the model that NADH levels change significantly in response to various stimuli such as calorie restriction and hypoxia. However, Sir2 binds NADH with very low affinity, suggesting that NADH does

not influence Sir2 activity. Other factors reported to affect Sir2's silencing activity include nicotinamide [126], and the NAD^+/NADH ratio [55]. The factor that links calorie restriction to life extension through Sir2 remains controversial [127]. This discussion will shed some light on the pyridine dinucleotide levels that should be considered when studying Sir2 or CtBP.

The high sequence homology of CtBP with 3-phosphoglycerate dehydrogenase suggested that CtBP contains an intrinsic dehydrogenase activity that may be important for its corepressor function. The observation that NADH stimulates the interaction between CtBP and repressor proteins suggests that nuclear NADH levels regulate the recruitment of CtBP to promoter elements. CtBP was proposed to serve as a metabolic sensor because of the important role of NADH in the cell's oxidative and energy state[5]. The dependence of CtBP on NADH categorizes CtBP with other transcriptional coregulators that require molecules linked to cellular respiration. These include the acetyl-CoA consuming HATs[70], the ATP-dependent chromatin remodeling proteins[70], and the NAD^+-dependent protein deacetylase Sir2 proteins[52, 72].

Molecular oxygen is a major component of cellular respiration and serves as the ultimate recipient of reducing equivalents that are generated during the catabolism of energy fuel sources. The reduction of molecular oxygen to water is coupled to the oxidation of NADH to NAD^+ in a process that drives the synthesis of ATP. This process is called oxidative phosphorylation. When ATP levels are sufficient to meet the energy needs of the cell, catabolic processes are inhibited and storage processes are activated. For example, elevated ATP levels stimulate glycogen synthesis and inhibit glycolysis. The inhibition of catabolic processes prevents the production of new NADH molecules.

A decrease in cellular oxygen tension, termed hypoxia, results in elevated levels of NADH. Thus, ATP and oxygen levels influence cellular NADH levels. Accordingly, NADH increases during hypoxia and may serve to stimulate recruitment of CtBP to certain promoters. The biological relevance of fluctuating NADH levels in response to changing oxygen tension may be seen in development and cancer.

Whether NADH plays a physiologically important role in CtBP function has been controversial. In this thesis, the affinity of CtBP for NADH was determined using a highly sensitive fluorescence technique. The high affinity binding observed ($K_{d,NADH}$ = 66 nM)[128] was in good agreement with the concentration of NADH required for half maximal stimulation of the interaction between CtBP and E1A (100 nM)[5]. Two other groups observed that NAD^+ and NADH were able to stimulate the CtBP/E1A interaction in GST-pulldown assays[60, 63]. However, the preferential NADH binding of CtBP reported by Zhang et al. was not observed. The approach of the three authors will be addressed to determine where the discrepancies may lie.

Kumar et al. synthesized ^{35}S-labeled CtBP by *in vitro* transcription/translation methods for use in the GST-pulldown E1A assays. This approach allowed Kumar et al. to measure the CtBP/E1A interaction by detecting CtBP using autoradiography and avoids difficulties associated with quantification using western blot techniques[60]. However, the lowest concentration of NADH used was 10-fold higher (1 μM) than the EC_{50} (100 nM) reported by Zhang et al. Balasubramanian et al. used purified CtBP expressed in bacteria and analyzed the levels of CtBP that interacted with GST-E1A by Western blot analysis using CtBP antibodies[63]. The lowest concentration of NADH used by Balasubramanian was 100-fold higher (10μM) than the EC_{50} reported by

Zhang[5]. Thus, neither group measured the CtBP/E1A interaction in the presence of NAD^+ or NADH levels that would clearly reveal a differential effect. A challenge associated with measuring high affinity binding events is to determine how much protein to use in the assay. The level of protein used must be high enough to obtain a good signal/noise ratio. However, higher levels of protein require more substrate to achieve saturation. If the $Kd_{substrate}$ is in the low nanomolar range and several microM of protein is used, the true Kd will not be observed as the amount of substrate required to saturate all the protein binding sites depends on the protein concentration. Neither, Kumar et al. nor Balasubramanian et al. addressed this issue. The high affinity of CtBP for NADH results in a significant portion of the purified CtBP to be bound to NADH. The intrinsically bound NADH raises the background signal by stimulating E1A binding in the absence of added NAD(H).

The experiments performed by Zhang et al. were optimized to ensure that signal detection was in the linear range[5]. Zhang et al. used CtBP expressed in bacteria and detected by Western blot analysis. Low levels of protein were used to test the ability of the antibody to detect nanoM to picoM levels of protein, quantitatively. Zhang et al. observed the nanoM affinity for NADH because sufficiently low concentrations of CtBP were used in the GST-pulldown assay. The sensitive methods used by Zhang et al. allowed the differential effects of NAD^+ and NADH on the CtBP/E1A interaction to be clearly observed. The conflicting reports that suggest there are no differences between NAD^+ and NADH led us to measure the affinity of each dinucleotide to CtBP directly.

The stimulated binding between CtBP and repressor proteins is the first example of pyridine dinucleotides regulating transcription by influencing protein/protein

interactions. Subsequently, it was determined that another protein/protein interaction influenced by pyridine dinucleotides was also important for regulating gene expression -- the interaction between GAPDH and Oct-1[129]. An example of a protein/DNA interaction influenced by pyridine dinucleotides is the interaction between DNA and the Clock/NPAS2 proteins[74]. The levels of pyridine dinucleotides required to regulate these binding events will be considered in a brief discussion of these two modes of gene regulation.

Glyceraldehyde-3-phosphate dehydrogenase was identified as a component of the OCA-S coactivator complex important for S phase-dependent histone H2B transcription[129]. NAD^+ and NADH levels were reported to regulate the formation of the OCA-S complex. Additional experiments revealed that GAPDH binds directly to Oct-1 in a manner that is stimulated by NAD^+ and inhibited by NADH. The levels of each nucleotide found to affect the GAPDH/Oct-1 POU domain interaction were between 10 nM and 10 μM with NAD^+ and NADH being similarly effective, with EC_{50} values of about 100 nM. The important mode of regulation is likely through the inhibition by NADH, given that the free NAD^+ concentration is saturating and unchanging.

Interestingly, two dehydrogenase proteins were isolated as components of the OCA-S transcriptional coactivator complex, GAPDH and lactate dehydrogenase[129]. Zheng et al. demonstrated that nucleotide binding, not dehydrogenase activity, was important for regulating the interaction between GAPDH and Oct-1. The importance of lactate dehydrogenase's enzymatic activity for gene activation through the OCA-S complex is to be determined. The studies performed in CtBP-deficient MEF cells demonstrate that the dehydrogenase activity of CtBP is not required for repression by the

CtBP corepressor complex[62]. Perhaps nuclear GAPDH, and possibly nuclear lactate dehydrogenase, do not require their respective catalytic activities for their roles in regulating gene expression. The relationship between OCA-S target genes and changes in nuclear dinucleotide levels may help to explain the "switch" sensed by GAPDH.

Rutter et al. observed that both NADH and NADPH stimulate the DNA binding of Clock and NPAS2 proteins[74]. These proteins regulate the expression of genes involved in circadian rhythms as a function of the light-dark cycle. Electrophoretic mobility shift assays demonstrated that NADH and NADPH were able to stimulate NPAS2:BMAL1 with EC_{50} values of 6.3 and 2.3 mM, respectively. Rutter et al. proposed that the low mM levels of reduced nucleotides is within the physiological range based on an early study designed to measure total cellular reduced nucleotide levels[130]. Interestingly, Rutter et al. observed that the oxidized form of each nucleotide inhibited DNA binding. The IC_{50} of NADP on NPAS2:BMAL1 DNA binding was reported to be 0.56 mM. Further DNA binding studies revealed that the ratio of reduced to oxidized nucleotide had a significant impact on the DNA binding of NPAS2:BMAL1. The authors concluded that NPAS2 and Clock proteins serve as redox sensors with the ability to sense changes in the NADPH/$NADP^+$ and NADH/NAD^+ levels. The ratios observed to elicit the greatest response were between 60:40 and 80:20 (reduced to oxidized) dinucleotides. The physiological relevance of the NADH:NAD^+ ratio influencing the DNA binding of these proteins seems very unlikely given that the free NAD^+ concentration is thought to be several orders of magnitude higher than the free NADH concentration. The NADH:NAD^+ ratio of 1:700 is significantly different than the ratios proposed to regulate NPAS2. Additionally, the effects observed at millimolar levels are unlikely to be

physiological when the concentration of free NAD^+ is about 70 μM and free NADH is about 100 nM[5].

Determining the differential binding of NAD^+ and NADH is critical to our understanding of CtBP's role as a metabolic sensor. In order for CtBP to be an effective sensor of the cell's energy and oxidative state, it must be sensitive to changes in the levels of free nucleotides in a cell. Thus, it is important to know the cellular levels of free NAD^+ and NADH, particularly in the nucleus. To address this, Zhang et al. determined the free nuclear levels of NADH using two-photon microscopy[5]. A value of 130 nM was determined for free NADH. The concentration of free NAD^+ was then determined using the pyruvate/lactate ratio that was determined to be 644, putting the free [NAD^+] at about 85 uM. The hypothesis of Zhang et al., and of this dissertation, is that recruitment of CtBP to promoter elements is regulated by fluctuations in NADH levels[5]. The relative concentrations of NAD^+ versus NADH suggest that inter-conversion of the two forms via oxidation/reduction reactions significantly alter only the NADH levels.

Two important questions regarding the physiological relevance of pyridine dinucleotide regulation of gene expression through CtBP and Sir2 follow: 1) What are the total and free concentrations of reduced and oxidized pyridine dinucleotides in mammalian cells? 2) Is the free or total concentration more important for regulating protein activities? To address the first question, a survey of published pyridine dinucleotide concentrations will be provided. The second question will be addressed by presenting the binding constants of several proteins that utilize NAD^+ or NADH. The proteins selected to address the second question include the nuclear NAD^+-glycohydrolases (SIRT1 and PARP) as well as the oxidoreductase proteins important for

glycolysis (GAPDH and pyruvate dehydrogenase), Krebs cycle (Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase), and electron transport (NADH: (acceptor) oxidoreductase).

Cellular Pyridine Dinucleotide Concentrations

Many studies have focused on determining the pyridine dinucleotide levels in human erythrocytes (values compiled in Table 2). The total concentration of NAD^+ in human erythrocytes ranges from 40-90 μM[131]. Canepa et al. determined the concentration of total and free dinucleotides in erythrocytes and found that about 50% of the NAD^+ (28.4 μM), and 10% of the NADH (200 nM), were free in the cytosol[131]. The total NAD^+/NADH ratio was approximately 20 and the free NAD^+/NADH ratio was greater than 100. Canepa et al. suggested that the pyruvate / lactate ratio should be taken as the basis for the calculated true NAD^+ / NADH ratio[131]. The ratio calculated for human red blood cells was 1000[132].

Simpson et al. determined the levels of free NAD^+ in human erythrocytes using a proton NMR approach. These studies suggested that free NAD^+ in intact cells was about 10 μM, which is 12.5% of the total extractable level (70-90 μM)[133]. Stocchi et al. determined pyridine dinucleotide levels in human red blood cells using a single-step extraction procedure that involved the rapid freezing of cells to prevent breakdown of the dinucleotides. The total NAD^+ and NADH concentrations were determined to be 48 μM and 1.4 μM, respectively[134]. If we assume that half the NAD^+ is free and 10% of the NADH is free, as suggested by Canepa et al.[131], then the free NAD^+ concentration is

24 μM and the free NADH concentration is 140 nM. These values are very close to the binding constants determined for CtBP in this work[128].

Litt et al. analyzed total pyridine dinucleotides in cultured rat hepatocytes by HPLC[135]. The stability of the different dinucleotides was considered during the extraction process and the recovery was determined to be greater than 90% for each of the pyridine dinucleotides. The total NAD^+/NADH ratio was 2.8. This value agrees well with that obtained by Kalhorn et al. (2.52) from fasting livers by HPLC[136].

Tischler et al. determined the total pyridine dinucleotides in rat hepatocytes using a turbulent flow technique[137]. Metabolite ratios were used to calculate the NAD^+/NADH ratios in the cytosol and mitochondria. The total cytosolic NAD^+ concentration in rat liver was 650 μM, while the total NADH concentration was 270 μM. The free cytosolic NAD^+ concentration was 420-510 μM and the free cytosolic NADH concentration was 0.6 – 1.5 μM. In the mitochodria, the free NADH concentration (443 μM) is higher, presumably due to displacement by the high levels of NAD^+ (5.9 mM).

A recent investigation published in *Science* by Anderson et al. suggested that the free NAD^+ concentration in yeast is about 10-fold higher than the total NAD^+ concentration typically reported for hepatocytes[124]. Using ^{13}C NMR, Anderson et al. determined the free NAD^+ concentration to be 4 mM. These experiments were performed in yeast provided with the NAD^+ precursor ^{13}C nicotinic acid (NA). It is unlikely, however, that the labeled NA taken up by the yeast is all in the form of NAD^+ during the NMR measurements. Therefore, a concern is that the addition of NA resulted in the production of several indistinguishable species by ^{13}C NMR: NMN, $NADP^+$, and NAD^+. It is possible that the sum of the three species is actually being measured. Another

concern is that the addition of NA may stimulate the production of NAD^+ resulting in physiologically irrelevant levels. Additionally, it should be noted that the studies by Anderson et al. were performed in yeast which differ from mammals in their NAD^+ metabolic pathways.

Reports that the Sir2 proteins are regulated *in vivo* by free nuclear NAD^+ have been challenged by studies suggesting that NAD^+ levels do not change significantly and therefore, do not influence gene silencing by Sir2. Understanding the regulation of Sir2 is important to our understanding of CtBP regulation. The regulation of Sir2 by NAD^+ levels is not in agreement with the current model of how CtBP is influenced by pyridine dinucleotides. Studies performed by Fulco et al. describe the role of Sir2 in regulating muscle gene expression and differentiation[55]. By looking at the expression of the MHC gene in myoblasts, Fulco et al. found that elevated pyruvate levels correlated with a loss in MHC expression and an increase in the cell's NAD^+/NADH levels. Fulco et al. concluded that an elevated NAD^+ concentration increased gene silencing by increasing the deacetylase activity of Sir2. In support of their conclusion, nicotinamide (10 mM) was found to reduce the pyruvate-stimulated repression of MHC presumably by inhibiting the deacetylase activity of Sir2. The use of 25 mM pyruvate and 10 mM nicotinamide to stimulate and inhibit the activity of Sir2, respectively, is not ideal, as such high levels of each metabolite may influence many cellular processes. Nevertheless, pyruvate treatment influences the NAD^+/NADH ratio by pushing the equilibrium between NAD^+ and NADH towards less NADH. Given that the free cytoplasmic NAD^+/NADH ratio was calculated to be 450 in untreated myoblasts and is usually 200-1,500, a shift in the equilibrium will significantly alter the NADH concentration and leave the NAD^+

concentration virtually unaffected. This suggests that if Sir2 is sensitive to nicotinamide adenine dinucleotides, the relevant form is NADH rather than NAD^+, which is not supported by the affinity of Sir2 for NADH (Ki~7 mM).

In support of this conclusion, Anderson et al. reported that NAD^+ levels change very little in yeast exposed to calorie restriction. This observation goes directly against the hypothesis that reduced carbon fuel sources increase NAD^+ or the NAD^+/NADH ratio. The ability of nicotinamide to inhibit the deacetylase activity of Sir2, and observations that overexpression of pyrazinamidase / nicotinamidase 1 (PNC1) in yeast is necessary and sufficient for lifespan extension by calorie restriction[126], suggests that nicotinamide plays an important role in regulating gene silencing. The gene product of PCN1 converts nicotinamide to the NAD^+ precursor, nicotinic acid. Thus, PCN1 could stimulate Sir2 by depleting cells of the inhibitor nicotinamide and by increasing cellular NAD^+ levels. Additionally, Pnc1 levels were found to be elevated under several conditions thought to mimic calorie restriction. However, biochemical experiments that correlate the $Ki_{nicotinamide}$ for Sir2 with cellular nicotinamide levels are required to support the hypothesis that nicotinamide links calorie restriction to life extension directly by inhibiting the NAD^+-dependent deacetylase activity of Sir2.

Pyridine Dinucleotide Binding Constants

The standard biochemical approach for determining the physiological relevance of a protein/ligand interaction observed *in vitro* is to compare the affinity of the interaction with the relevant biological ligand concentrations. If the affinity is an order of magnitude higher than the available ligand concentration, then essentially all binding

sites should be saturated with ligand. If the affinity is an order of magnitude lower than the available ligand concentration, then little binding is expected to occur. If the ligand concentration is two or more orders of magnitude higher or lower than the binding constant, then the protein will certainly be saturated or unbound, respectively. The range of change in ligand levels is also important when considering the potential for a ligand to regulate a protein's function. The importance of Ca^{+2} fluctuations in regulating cellular events is well established [138]. For example, studies suggest that free Ca^{+2} levels undergo a change spanning several orders of magnitude. A large increase in free Ca^{+2} levels is due to the release of Ca^{+2} by chelating proteins and flux through Ca^{+2} channels. The realization that free, and not total Ca^{+2} levels, are important for serving as a second messenger signal was vital to the understanding of many cellular processes including gene transcription, muscle contraction and cell proliferation.

An important question central to this thesis is whether the free or total NADH concentration may be important for regulating gene expression through CtBP. The binding constants of several NAD(H)-utilizing proteins were compiled in Table 3. A comparison of the binding constants of known protein/ligand interactions with the accepted physiological ligand concentrations, provides insight regarding the regulation of CtBP by small molecules. Furthermore, the binding constants alone should give a general sense of the relevant levels of pyridine dinucleotides seen by these proteins. The dehydrogenase proteins normally included in the conversion of glucose to ATP were listed, as well as two additional proteins that function in the cytoplasm and use NADH as a substrate. At the end of the table, several NAD^+ glycohydrolases are listed including several human SIRT proteins.

Human CtBP1 and bacterial 3-phosphoglycerate dehydrogenase have nearly identical binding constants for both NAD^+ and NADH (~10 μM and 50 nM, respectively) [83, 128]. The similar affinities are reflective of the high sequence similarity (nearly 40% identical) across the two proteins' pyridine dinucleotide-binding domains. Glyceraldehyde-3-phosphate dehydrogenase produces two molecules of NADH per glucose molecule in the cytoplasm during glycolysis. The binding constants of oxidized GAPDH for NAD^+ and NADH are 64 μM and 40 nM, respectively [139]. The pyridine dinucleotide binding properties of GAPDH are set for proper glycolytic flux of carbon fuel sources and for the proper redox potential between NAD^+ and NADH. The design of a nuclear metabolic sensor, sensitive to the energy and oxidative state of the cell's cytoplasmic environment, would most logically follow the "blueprints" of a glycolytic dehydrogenase. The similar pyridine dinucleotide binding properties that CtBP shares with GAPDH strengthens the hypothesis that CtBP provides a link between the cell's metabolic state and gene expression. The report that GAPDH, itself, appears to regulate gene expression in a manner sensitive to fluctuating pyridine dinucleotide levels, provides another example of this design at work in the nucleus.

Two other cytoplasmic proteins were included in Table 3 that utilize NADH as a substrate, lactate dehydrogenase and L-α-glycerophosphate dehydrogenase. Because proteins that consume NADH must be able to bind NADH at the available concentrations, these proteins are good indicators of the relevant NADH levels that CtBP is exposed to. Lactate dehydrogenase and L-α-glycerophosphate dehydrogenase have dissociation constants for NADH of 500 nM[140]and 1.8 μM[141], respectively. These values agree well with those reported in Table 2 for free NADH levels (120 nM – 1.5

μM). The low affinity of lactate dehydrogenase for NAD^+ (500 μM) suggests that the equilibrium favors NAD^+ as a product. The ratio of the $Kd_{NAD+}/Kd_{NADH} = 1000$, suggesting the relative free concentrations of the two nucleotides.

Pyruvate dehydrogenase is a multiprotein complex that bridges glycolysis to the Krebs cycle by catalyzing the oxidative decarboxylation of pyruvate in the mitochondria to produce acetyl-CoA. The binding constants for pyruvate dehydrogenase are $Kd_{NAD}{}^+ =$ 50μM and a $Ki_{NADH} = 36$ μM. The affinity of pyruvate dehydrogenase for NAD^+ is very similar to others listed in table 3 with binding constants between 21-74 μM. The concentration of NADH in the mitochondria is higher than in the cytoplasm. Pyruvate dehydrogenase, with a Ki_{NADH} of 36 μM, should be sensitive to changes in free mitochondrial NADH levels. Proper regulation of pyruvate dehydrogenase is very important because it generates acetyl-CoA, the intermediate that bridges many metabolic pathways. NADH is an important regulator of pyruvate dehydrogenase through feedback inhibition and by stimulating the phoshorylation of pyruvate dehydrogenase. NADH inhibits pyruvate dehydrogenase by competing with NAD^+ binding at the active site and also by activating pyruvate dehydrogenase kinase. Acetyl-CoA significantly enhances the activation of pyruvate dehydrogenase kinase by NADH. Pyruvate dehydrogenase kinase phosphorylates the pyruvate dehydrogenase complex which results in inhibition of the complex. The inhibition of pyruvate dehydrogenase by NADH through these two mechanisms supports the paradigm that fluctuating levels of NADH regulate the catabolic flux of carbon fuel sources through the metabolic pathways that generate ATP from glucose. In the mitochondria, NADH levels regulate acetyl-CoA synthesis and

NADH production by inhibiting the activity of pyruvate dehydrogenase, as well as several other dehydrogenase proteins in the Krebs cycle.

In addition to pyruvate dehydrogenase, the three dehydrogenases involved in the Krebs cycle were included: isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase. The affinity of these dehydrogenases for NAD^+ is similar to the affinity of dehydrogenases listed in Table 3. The affinity of these three mitochondrial dehydrogenases for NADH is also quite similar to the affinity determined for the cytoplasmic dehydrogenases. An exception worth noting is that no mitochondrial protein is able to bind with nanomolar affinity although several cytoplasmic proteins listed do bind with nanomolar affinity.

A build up of NADH in the mitochondria inhibits catabolic processes at several key regulatory points causing a reduced flux of carbon flow through mitochondrial pathways and elevated NADH levels in the cytoplasm. If the majority of cytosolic NADH is generated during glycolysis (a pathway that feeds into pyruvate dehydrogenase and Krebs cycle) and the nuclear NADH is the result of passive diffusion through nuclear pores, then the high affinity of CtBP for NADH (66 nM) allows for rapid detection in the nucleus of changes in the cytosolic NADH levels.

It is clear from this discussion of Table 3 that NADH serves as a signaling molecule to regulate metabolic pathways, particularly those pathways important for its own generation. Regulation of gene expression by the same signaling molecule suggests that NADH allows the metabolic state of the cell to tightly control the regulation of transcription.

The free NADH concentration is higher in the mitochondria than in the cytoplasm. Elevated total NAD^+ levels are thought to compete with NADH for binding sites resulting in higher levels of free NADH. The first step in the electron transport chain is the conversion of NADH to NAD^+ by NADH:acceptor oxidoreductase. NADH:acceptor oxidoreductase has a lower affinity for NADH (Km_{NADH} = 14μM) than the cytosolic proteins mentioned above that use NADH as a substrate. The Km_{NADH} was reported to be 14 μM, nearly 30-fold higher than cytoplasmic proteins that consume NADH. The mitochondrial proteins have binding constants that are several-fold higher than observed with CtBP and NADH. This is expected given the higher levels of free dinucleotides in the mitochondria.

NAD^+ kinase phosphorylates NAD^+ yielding $NADP^+$. NAD^+ Kinase has a relatively low affinity for NAD^+ (Km = 540 μM)[142] that may be important for maintaining the equilibrium between NAD^+ and $NADP^+$. The remaining proteins listed in Table 3 are NAD^+ glycohydrolases that cleave the nicotinamide ring from NAD^+. The first hydrolase listed, poly ADP-ribosylase (PARP) is a nuclear protein involved in DNA damage/repair and has a Km_{NAD^+} = 59 μM[143]. The reported Km_{NAD^+} of SIRT1(558 μM)[144] is nearly 10-fold higher than that reported for SIRT2 (83 μM)(unpublished results). Nonetheless, the similarity in binding constants reported for PARP, SIRT2 and several dehydrogenases is striking and very similar to the NAD^+ concentration determined by Zhang et al (Table 2). Binding and kinetic experiments have not been reported for the mitochondrial isoform, SIRT3.

NAD^+ metabolism

Regulation of CtBP by NADH requires that NADH levels change in response to cellular events and cues. As described in the introduction, NADH levels are sensitive to the energy (ATP and fuel sources) and oxidative state (oxygen tension and redox potential) of the cell. NADH is directly converted from, and to, NAD^+ via oxidation/reduction reactions, respectively. Because the free NAD^+/NADH ratio is large (>200), the conversion of one species to the other significantly alters only the free NADH concentration. Essentially all of the cellular NADH is a product of NAD^+ reduction. The primary enzymatic reaction that NADH is involved in is oxidation to NAD^+. NAD^+, on the other hand, is consumed by several glycohydrolyases with diverse catalytic functions that will be discussed in the next section. Additionally, phosphorylation of NAD^+ by NAD^+ Kinase yields $NADP^+$, a carrier of reducing equivalents in fatty acid metabolism. There are several NAD^+ synthetic pathways [145] utilized by cells to maintain the free cytosolic NAD^+ levels. The primary biosynthetic pathway for NAD^+ varies among species and is determined by the synthetic enzymes produced by each species. The major pathways found in yeast, mammalian liver cells, and *Drosophila* are discussed below. This information is important for critical evaluation, and proper design, of studies that involve manipulating pyridine dinucleotide levels.

The synthesis of NAD^+ from tryptophan is generally considered to be the *de novo* pathway, while the synthesis from niacin (nicotinamide and nicotinic acid) is considered the salvage pathway. Yeast contain the enzyme indolamine 2,3-dioxygenase which converts L-tryptophan and molecular oxygen to N-formyl-kynurenine, which is further converted in several enzymatic steps to quinolinic acid. Yeast contain a quinolinic acid

phosphoribosyltransferase that converts quinolinic acid to nicotinic acid mononucleotide. Nicotinic acid mononucleotide can also be synthesized in yeast from nicotinic acid and ATP by nicotinic acid phosphoribosyltransferase. Yeast also contain the enzyme nicotinamidase which converts nicotinamide into nicotinic acid. Thus, in yeast, all NAD^+ is synthesized from the intermediate nicotinic acid mononucleotide whether its precursors are derived from the *de novo* pathway or the salvage pathways.

In contrast, mammalian liver cells are unable to convert nicotinamide into nicotinic acid because they lack nicotinamidase. Instead, liver cells express nicotinamide phosphoribosyltransferase, which catalyzes the conversion of nicotinamide directly into nicotinamide mononucleotide. The enzyme, nicotinamide mononucleotide adenylyltransferase, synthesizes NAD^+ from nicotinamide mononucleotide and ATP. Paine and Hockin found that when rat hepatocytes are cultured in medium containing the NAD^+ precursors nicotinamide, quinolinic acid, and nicotinic acid, only nicotinamide increased the concentration of NAD^+[146]. Increased NAD^+ levels by nicotinamide treatment have also been reported in mouse and liver slices [147, 148]. These data are consistent with the work reported by Evans et al. demonstrating that nicotinamide was the major precursor incorporated into NAD^+ in a human liver cell line (SK-HEP)[149]. In this study Evans et al. found that cells treated with 50 μM of stable isotope labeled tryptophan, quinolinic acid, nicotinamide, and nicotinic acid, contained an increased level (3-4 fold) of NAD^+ almost entirely generated from nicotinamide. The remaining NAD^+ is thought to be synthesized from NAD^+ breakdown products, such a nicotinamide. These studies suggest that mammalian cells are not equipped to synthesize NAD^+ from nicotinic acid.

Similar to yeast, *C. elegans* and *Drosophila* contain a nicotinamidase and not nicotinamide mononucleotide adenylyl transferase. Unlike yeast, these species do not have a quinolinic acid phosphoribosyltransferase and are unable to synthesis NAD^+ via the de novo pathway. Recently, Rongvauz et al. carried out a comparative analysis based on metabolic reconstruction from genomic data suggesting the development of complex metabolic pathways[145]. The genomic study revealed that yeast, *C. elegans* and *Drosophila* lack a nicotinic acid mononucleotide adenylyltransferase and humans lack a nicotinamidase. Interestingly, Rongauz et al. suggest that organisms contain one main mechanism for the synthesis of NAD^+ and that the other pathways are used for other purposes such as cell signaling.

NAD^+ is synthesized in different species by different pathways described as the *de novo* pathway and the salvage pathways. NADH is synthesized by the reduction of NAD^+ by enzymes described as oxido-reductases. The differences between yeast and humans is of interest given the many studies focused on elucidating the hypothesized regulatory role of NAD^+ in Sir2 gene silencing. Currently, it has been proposed that NAD^+, nicotinamide, and NADH serve as the signaling molecule that links calorie restriction to life extension by regulating the deacetylase activity of Sir2. These conflicting reports are based on experiments performed in different species using genetic and drug strategies directed towards NAD^+ metabolic pathways. Understanding the fundamental differences of each approach may be required to properly interpret the conflicting reports.

Biological Events Associated with NADH Level Fluctuation

There are several events that invoke a significant change in cellular NADH levels. Excessive alcohol consumption initiates a dramatic increase in NADH in the liver as a result of the enzymatic conversion of ethanol to acetate in two steps producing two molecules of NADH per ethanol molecule. The elevated NADH levels disrupt the proper metabolism of lipids, carbohydrates, proteins and purines and result in malnutrition. Several studies suggest a role of elevated NADH levels in diabetes. For example, disruption of the electron transport chain in the murine pancreatic β cell line, β HC9, resulted in elevated NADH levels in the mitochondria and the cytosol leading to suppression of glucose-stimulated insulin release[150]. In cancer, poor vascularization of tumors results in insufficient oxygenation of tumor cells and leads to hypoxia, the state of low oxygen tension. Because oxygen is the final acceptor of the reducing equivalents carried by NADH, hypoxia results in elevated NADH levels. The hypoxic environment experienced by the mammalian embryo results in increased NADH levels during development. CtBP plays important roles in cellular transformation and differentiation. The importance of fluctuating NADH levels for the activity of CtBP during these events is to be determined.

Conclusion

The observation that NADH stimulates the interaction between CtBP and E1A was the first step towards understanding the significance of CtBP's high sequence similarity with dehydrogenase proteins. Studies demonstrating that the dehydrogenase activity of CtBP is not required for gene repression further support the hypothesis that CtBP's ability to bind NADH is important for regulating recruitment of CtBP to promoter elements and not for the reduction of a small molecule substrate. It is possible that the weak reductase activity of CtBP may terminate repression by causing the release of CtBP from repressor bound promoters upon converting NADH to NAD^+. The proposal that the similarity of CtBP with dehydrogenase proteins enables CtBP to sense changes in the cell's metabolic and oxidative state was based on the experiments demonstrating that NADH was effective at stimulating the interaction with E1A at concentrations more than 100-fold lower than observed with NAD^+. The inability of several groups to measure the differential effects between NAD^+ and NADH challenged the proposed model that CtBP links gene regulation to the cell's metabolic state. The enigmatic nature of the mechanism utilized by CtBP to repress gene expression, the large number of repressor proteins that CtBP interacts with, and the important role CtBP plays in development, transformation and differentiation, emphasize the importance of addressing the experimental inconsistencies. To accomplish this, the affinity of CtBP for NAD^+ and NADH was measured using highly sensitive fluorescence techniques that utilized the fluorescent properties of NADH and a tryptophan residue located near the NAD(H) binding site. The binding constants were in good agreement with the concentrations found to stimulate the CtBP / E1A interaction and suggest that CtBP is suited to serve as a metabolic sensor.

The regulation of transcription by the redox/energy state of the cell through pyridine dinucleotides has been suggested for clock proteins[74] and GAPDH[129]. The regulation reported for clock proteins seems unlikely given the requirement for millimolar levels of NAD(P)H. Authors of the NPAS2:Clock paper cite that the reduced pyridine levels are quite high in support of their binding data. However, the reference cited refers to the total levels of reduced nucleotides and not the free levels. The NADH concentrations required for the regulation of the GAPDH/Oct-1 interaction are consistent with free NADH levels. The effective concentrations of NAD^+ suggest that in the absence of elevated NADH, GAPDH will be primarily bound to NAD^+ and in the active form. Interestingly, the oxidized and reduced forms of NAD have opposing effects on the protein/protein interaction. Based on the NADH binding constant of GAPDH (30-50 nM), the concentration of NADH required to influence the GAPDH / Oct-1 interaction, and the cellular levels of free NADH, the inhibition resulting from NADH binding is more likely to be the important mode of regulation. Perhaps NADH synergistically inhibits gene activation through GAPDH and stimulates gene repression through CtBP. Uncovering coordinated regulatory events promoted by NADH may provide insight into how CtBP gene repression fits into the larger scheme of cellular events.

The proposal that CtBP serves as a metabolic sensor by responding to changes in NADH levels is supported by the differential binding observed in this thesis. The pyridine dinucleotide levels measured in various cell types using a variety of approaches (Table 2) are also consistent with the hypothesis that NADH may regulate recruitment of CtBP to promoter elements. The compilation of pyridine dinucleotide binding constants of enzymes involved in the conversion of glucose to CO_2 and H_2O (Table 3) strongly

suggests that the free concentration of pyridine dinucleotides, not the total concentration, is important for the proteins' function. Elevated NADH levels during hypoxia and tumorigenesis suggests that CtBP may be involved in stimulating cancer resulting from hypoxic conditions. While some ties are apparent between gene repression and NADH levels, much work may be required to fully appreciate the role of NADH in CtBP function.

The fluorescent studies presented here address the controversy that arose concerning the preference of CtBP for NADH. The role of NADH in metabolism, serving as a redox molecule directly involved in converting fuels to energy, was described. Several important components that are involved in the redox balance of a cell were described along with how they may influence levels of NADH. Efforts to measure cellular NADH and NAD^+ were summarized in table 2.

Final Word

I have demonstrated that CtBP preferentially binds NADH using fluorescence techniques. The significance of this observation depends on several parameters that were explored in this thesis. I provided evidence that NADH levels change, a requirement of any regulatory signaling molecule. In fact, NADH is thought to regulate catabolic pathways involved in ATP synthesis at several regulatory points. Thus, NADH levels change and are thought to regulate pathways important in cellular respiration. It is very logical for a metabolic sensor to be sensitive to levels of the same molecule that regulates metabolic processes. Many studies measuring the total and/or free pyridine dinucleotide concentrations in various tissues by a variety of approaches were presented and are

supportive of CtBP's role as a metabolic sensor. Finally, the binding constants of several proteins that bind pyridine dinucleotides were tabulated and discussed to provide a better sense of the concentrations that proteins are exposed to in cells.

Table 2. Pyridine Dinucleotide Concentrations

Cell Type / Tissue	Total NAD^+	Total NADH	Total NAD^+/NADH	Free NAD^+	Free NADH	Free NAD^+/NADH	Technique	Reference
Erythrocytes	49.5[Ψ]	1.8	27.5	28.3	0.2	141.5	Cycling assay	Canepa et al.
Erythrocytes	48	1.4	34.3	24*	0.14*	171.4*	HPLC	Stocchi et al.
Erythrocytes	70-90			10			Proton NMR	Simpson et al.
COS cells (kidney)				85	0.13	644	Two photon micr.	Zhang et al.
Cultured hepatocytes			2.8				HPLC	Litt et al.
Liver			2.52				HPLC	Kalhorn et al.
Hepatocytes	650	270	2.41	420-510	0.6-1.5	280-510	Turbulent flow	Tischler et al.
Liver	359	50	7.2			725	Metabolite ratio	Williamson et al.
Myoblasts						450	Metabolite ratio	Fulco et al.
Yeast				4,000			^{13}C NMR	Anderson et al.

[Ψ] All values listed are in μM.

*Based on % of total determined to be free by Canepa et al. See text.

Table 3. Pyridine Dinucleotide binding constants.

Localization	Protein	Function	NAD^+ Constants	NADH Constants
nucleus	hCtBP1	transcription corepressor	Kd = 8-11 μM	Kd = 66 nM
	3-phosphoglycerate dehydrogenase	serine biosynthesis	Kd = 8 μM	Kd = 50 nM
cytoplasm	GAPDH	glycolysis	Kd = 64 μM	Kd = 30-50 nM
cytoplasm	lactate dehydrogenase	pyruvate reduction	Kd = 500 μM	Kd = 500 nM
cytoplasm	L-α-glycerophosphate dehydrogenase	dihydroxyacetone phosphate reduction		Kd = 1.8 μM
mitochondria	pyruvate dehydrogenase	oxidative decarboxylation	Kd = 50 μM	Ki = 36 μM
mitochondria	isocitrate dehydrogenase	Krebs cycle	Km = 74 μM	Kd = 2.3 μM
mitochondria	α-ketoglutarate dehydrogenase	Krebs cycle	Km = 21 μM	Ki = 4.5 μM
mitochondria	malate dehydrogenase	Krebs cycle	Km = 60 μM	Kd = 3.8 μM
mitochondria	NADH: (acceptor) oxidoreductase	oxidative phosphorylation		Km = 14 μM
cytoplasm	NAD^+ kinase	$NADP^+$ synthesis	Km = 540 μM	
nucleus	PARP	poly ADP-ribosylation	Km = 59 μM	
nucleus	SIRT1	protein deacetylase	Km = 558 μM	
cytoplasm	SIRT2	protein deacetylase	Km = 65 μM	Ki ~ 7 mM
mitochondria	SIRT3	protein deacetylase	Km = ND	

References

1. Allen, R.G. and M. Tresini, *Oxidative stress and gene regulation.* Free Radic Biol Med, 2000. 28(3): p. 463-99.
2. Dalton, T.P., H.G. Shertzer, and A. Puga, *Regulation of gene expression by reactive oxygen.* Annu Rev Pharmacol Toxicol, 1999. 39: p. 67-101.
3. Marmorstein, R., *Dehydrogenases, NAD, and transcription--what's the connection?* Structure (Camb), 2002. 10(11): p. 1465-6.
4. Denu, J.M., *Linking chromatin function with metabolic networks: Sir2 family of NAD(+)-dependent deacetylases.* Trends Biochem Sci, 2003. 28(1): p. 41-8.
5. Zhang, Q., D.W. Piston, and R.H. Goodman, *Regulation of corepressor function by nuclear NADH.* Science, 2002. 295(5561): p. 1895-7.
6. Smale, S.T., *Core promoters: active contributors to combinatorial gene regulation.* Genes Dev, 2001. 15(19): p. 2503-8.
7. Naar, A.M., B.D. Lemon, and R. Tjian, *Transcriptional coactivator complexes.* Annu Rev Biochem, 2001. 70: p. 475-501.
8. Ranish, J.A., N. Yudkovsky, and S. Hahn, *Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB.* Genes Dev, 1999. 13(1): p. 49-63.
9. Butler, J.E. and J.T. Kadonaga, *The RNA polymerase II core promoter: a key component in the regulation of gene expression.* Genes Dev, 2002. 16(20): p. 2583-92.

10. Orphanides, G. and D. Reinberg, *RNA polymerase II elongation through chromatin.* Nature, 2000. 407(6803): p. 471-5.

11. Lemon, B. and R. Tjian, *Orchestrated response: a symphony of transcription factors for gene control.* Genes Dev, 2000. 14(20): p. 2551-69.

12. Ogbourne, S. and T.M. Antalis, *Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes.* Biochem J, 1998. 331 (Pt 1): p. 1-14.

13. Giroldi, L.A., et al., *Role of E boxes in the repression of E-cadherin expression.* Biochem Biophys Res Commun, 1997. 241(2): p. 453-8.

14. Shimano, H., *Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes.* Prog Lipid Res, 2001. 40(6): p. 439-52.

15. Narlikar, G.J., H.Y. Fan, and R.E. Kingston, *Cooperation between complexes that regulate chromatin structure and transcription.* Cell, 2002. 108(4): p. 475-87.

16. Horn, P.J. and C.L. Peterson, *Molecular biology. Chromatin higher order folding--wrapping up transcription.* Science, 2002. 297(5588): p. 1824-7.

17. Khorasanizadeh, S., *The nucleosome: from genomic organization to genomic regulation.* Cell, 2004. 116(2): p. 259-72.

18. Wu, J. and M. Grunstein, *25 years after the nucleosome model: chromatin modifications.* Trends Biochem Sci, 2000. 25(12): p. 619-23.

19. Kuo, M.H. and C.D. Allis, *Roles of histone acetyltransferases and deacetylases in gene regulation.* Bioessays, 1998. 20(8): p. 615-26.

20. Xu, L., C.K. Glass, and M.G. Rosenfeld, *Coactivator and corepressor complexes in nuclear receptor function.* Curr Opin Genet Dev, 1999. 9(2): p. 140-7.

21. Zeng, L. and M.M. Zhou, *Bromodomain: an acetyl-lysine binding domain.* FEBS Lett, 2002. 513(1): p. 124-8.

22. Marmorstein, R., *Structure of histone acetyltransferases.* J Mol Biol, 2001. 311(3): p. 433-44.

23. Brooks, C.L. and W. Gu, *Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation.* Curr Opin Cell Biol, 2003. 15(2): p. 164-71.

24. Vo, N., C. Fjeld, and R.H. Goodman, *Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP.* Mol Cell Biol, 2001. 21(18): p. 6181-8.

25. Agalioti, T., G. Chen, and D. Thanos, *Deciphering the transcriptional histone acetylation code for a human gene.* Cell, 2002. 111(3): p. 381-92.

26. Thiagalingam, S., et al., *Histone deacetylases: unique players in shaping the epigenetic histone code.* Ann N Y Acad Sci, 2003. 983: p. 84-100.

27. Guarente, L. and C. Kenyon, *Genetic pathways that regulate ageing in model organisms.* Nature, 2000. 408(6809): p. 255-62.

28. Bitterman, K.J., et al., *Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1.* J Biol Chem, 2002. 277(47): p. 45099-107.

29. Landry, J., J.T. Slama, and R. Sternglanz, *Role of NAD(+) in the deacetylase activity of the SIR2-like proteins.* Biochem Biophys Res Commun, 2000. 278(3): p. 685-90.

30. Becker, P.B. and W. Horz, *ATP-dependent nucleosome remodeling.* Annu Rev Biochem, 2002. 71: p. 247-73.

31. Hamiche, A., et al., *Histone tails modulate nucleosome mobility and regulate ATP-dependent nucleosome sliding by NURF.* Proc Natl Acad Sci U S A, 2001. 98(25): p. 14316-21.

32. Guschin, D., et al., *ATP-Dependent histone octamer mobilization and histone deacetylation mediated by the Mi-2 chromatin remodeling complex.* Biochemistry, 2000. 39(18): p. 5238-45.

33. Tsukiyama, T., et al., *Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae.* Genes Dev, 1999. 13(6): p. 686-97.

34. Claiborne, A., et al., *Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation.* Biochemistry, 1999. 38(47): p. 15407-16.

35. Forman, H.J., M. Torres, and J. Fukuto, *Redox signaling.* Mol Cell Biochem, 2002. 234-235(1-2): p. 49-62.

36. Chiarugi, P. and P. Cirri, *Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction.* Trends Biochem Sci, 2003. 28(9): p. 509-14.

37. Salmeen, A., et al., *Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate.* Nature, 2003. 423(6941): p. 769-73.

38. van Montfort, R.L., et al., *Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B.* Nature, 2003. 423(6941): p. 773-7.

39. Borutaite, V. and G.C. Brown, *Caspases are reversibly inactivated by hydrogen peroxide.* FEBS Lett, 2001. 500(3): p. 114-8.

40. Dickinson, D.A. and H.J. Forman, *Cellular glutathione and thiols metabolism.* Biochem Pharmacol, 2002. 64(5-6): p. 1019-26.

41. Schafer, F.Q. and G.R. Buettner, *Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple.* Free Radic Biol Med, 2001. 30(11): p. 1191-212.

42. Hoek, J.B. and J. Rydstrom, *Physiological roles of nicotinamide nucleotide transhydrogenase.* Biochem J, 1988. 254(1): p. 1-10.

43. Jackson, J.B., *Proton translocation by transhydrogenase.* FEBS Lett, 2003. 555(1): p. 176-7.

44. Cairo, G., et al., *The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage.* Free Radic Biol Med, 2002. 32(12): p. 1237-43.

45. Theil, E.C., *Ferritin: at the crossroads of iron and oxygen metabolism.* J Nutr, 2003. 133(5 Suppl 1): p. 1549S-53S.

46. Stadtman, E.R. and B.S. Berlett, *Fenton chemistry. Amino acid oxidation.* J Biol Chem, 1991. 266(26): p. 17201-11.

47. Meneghini, R., *Iron homeostasis, oxidative stress, and DNA damage.* Free Radic Biol Med, 1997. 23(5): p. 783-92.

48. Demple, B., *Study of redox-regulated transcription factors in prokaryotes.* Methods, 1997. 11(3): p. 267-78.

49. Hidalgo, E., H. Ding, and B. Demple, *Redox signal transduction via iron-sulfur clusters in the SoxR transcription activator.* Trends Biochem Sci, 1997. 22(6): p. 207-10.

50. Georgiou, G., *How to flip the (redox) switch.* Cell, 2002. 111(5): p. 607-10.

51. Lin, S.J., P.A. Defossez, and L. Guarente, *Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae.* Science, 2000. 289(5487): p. 2126-8.

52. Imai, S., et al., *Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase.* Nature, 2000. 403(6771): p. 795-800.

53. Jackson, M.D. and J.M. Denu, *Structural identification of 2'- and 3'-O-acetyl-ADP-ribose as novel metabolites derived from the Sir2 family of beta -NAD+-dependent histone/protein deacetylases.* J Biol Chem, 2002. 277(21): p. 18535-44.

54. Tanner, K.G., et al., *Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose.* Proc Natl Acad Sci U S A, 2000. 97(26): p. 14178-82.

55. Fulco, M., et al., *Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state.* Mol Cell, 2003. 12(1): p. 51-62.

56. Bedalov, A., et al., *NAD+-dependent deacetylase Hst1p controls biosynthesis and cellular NAD+ levels in Saccharomyces cerevisiae.* Mol Cell Biol, 2003. 23(19): p. 7044-54.

57. Bedalov, A. and J.A. Simon, *Sir2 flexes its muscle.* Dev Cell, 2003. 5(2): p. 188-9.

58. Boyd, J.M., et al., *A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis.* Embo J, 1993. 12(2): p. 469-78.

59. Schaeper, U., et al., *Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation.* Proc Natl Acad Sci U S A, 1995. 92(23): p. 10467-71.

60. Kumar, V., et al., *Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase.* Mol Cell, 2002. 10(4): p. 857-69.

61. Grooteclaes, M.L. and S.M. Frisch, *Evidence for a function of CtBP in epithelial gene regulation and anoikis.* Oncogene, 2000. 19(33): p. 3823-8.

62. Grooteclaes, M., et al., *C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs.* Proc Natl Acad Sci U S A, 2003. 100(8): p. 4568-73.

63. Balasubramanian, P., L.J. Zhao, and G. Chinnadurai, *Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and*

an intrinsic dehydrogenase activity of CtBP. FEBS Lett, 2003. 537(1-3): p. 157-60.

64. Nardini, M., et al., *CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission.* Embo J, 2003. 22(12): p. 3122-30.

65. Nardini, M., et al., *CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission.* Embo J, 2003. 22(12): p. 3122-3130.

66. Barnes, C.J., et al., *Functional inactivation of a transcriptional corepressor by a signaling kinase.* Nat Struct Biol, 2003. 10(8): p. 622-8.

67. Zhang, Q., et al., *Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP.* Cell, 2003. 115(2): p. 177-86.

68. Lin, X., et al., *Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding.* Mol Cell, 2003. 11(5): p. 1389-96.

69. Kagey, M.H., T.A. Melhuish, and D. Wotton, *The polycomb protein Pc2 is a SUMO E3.* Cell, 2003. 113(1): p. 127-37.

70. Sterner, D.E. and S.L. Berger, *Acetylation of histones and transcription-related factors.* Microbiol Mol Biol Rev, 2000. 64(2): p. 435-59.

71. Peterson, C.L., *Chromatin remodeling: nucleosomes bulging at the seams.* Curr Biol, 2002. 12(7): p. R245-7.

72. Landry, J., et al., *The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases.* Proc Natl Acad Sci U S A, 2000. 97(11): p. 5807-11.

73. Williamson, D.H., P. Lund, and H.A. Krebs, *The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver.* Biochem J, 1967. 103(2): p. 514-27.

74. Rutter, J., et al., *Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors.* Science, 2001. 293(5529): p. 510-4.

75. Nibu, Y., H. Zhang, and M. Levine, *Interaction of short-range repressors with Drosophila CtBP in the embryo.* Science, 1998. 280(5360): p. 101-4.

76. Subramaniam, N., E. Treuter, and S. Okret, *Receptor interacting protein RIP140 inhibits both positive and negative gene regulation by glucocorticoids.* J Biol Chem, 1999. 274(25): p. 18121-7.

77. Dressel, U., et al., *A dynamic role for HDAC7 in MEF2-mediated muscle differentiation.* J Biol Chem, 2001. 276(20): p. 17007-13.

78. Bertos, N.R., A.H. Wang, and X.J. Yang, *Class II histone deacetylases: structure, function, and regulation.* Biochem Cell Biol, 2001. 79(3): p. 243-52.

79. Shi, Y., et al., *Coordinated histone modifications mediated by a CtBP co-repressor complex.* Nature, 2003. 422(6933): p. 735-8.

80. Levine, S.S., et al., *The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans.* Mol Cell Biol, 2002. 22(17): p. 6070-8.

81. Steinberg, I.Z., *Long-range nonradiative transfer of electronic excitation energy in proteins and polypeptides.* Annu Rev Biochem, 1971. 40: p. 83-114.

82. Wolff, E.C., J. Wolff, and M.H. Park, *Deoxyhypusine synthase generates and uses bound NADH in a transient hydride transfer mechanism.* J Biol Chem, 2000. 275(13): p. 9170-7.

83. Sugimoto, E. and L.I. Pizer, *The mechanism of end product inhibition of serine biosynthesis. II. Optical studies of phosphoglycerate dehydrogenase.* J Biol Chem, 1968. 243(9): p. 2090-8.

84. Meijers, R., et al., *On the enzymatic activation of NADH.* J Biol Chem, 2001. 276(12): p. 9316-21.

85. Grant, G.A., Z. Hu, and X.L. Xu, *Cofactor binding to Escherichia coli D-3-phosphoglycerate dehydrogenase induces multiple conformations which alter effector binding.* J Biol Chem, 2002. 277(42): p. 39548-53.

86. Holbrook, J.J., et al., *The Enzymes*, in *The Enzymes*, P.D. Boyer, Editor. 1975, Academic Press: New York. p. 191-268.

87. Philippidis, H. and F.J. Ballard, *The development of gluconeogenesis in rat liver: experiments in vivo.* Biochem J, 1969. 113(4): p. 651-7.

88. Stubbs, M., R.L. Veech, and H.A. Krebs, *Control of the redox state of the nicotinamide-adenine dinucleotide couple in rat liver cytoplasm.* Biochem J, 1972. 126(1): p. 59-65.

89. Schaeper, U., et al., *Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is*

disrupted by E1A through a conserved PLDLS motif. J Biol Chem, 1998. 273(15): p. 8549-52.

90. Sollerbrant, K., G. Chinnadurai, and C. Svensson, *The CtBP binding domain in the adenovirus E1A protein controls CR1-dependent transactivation.* Nucleic Acids Res, 1996. 24(13): p. 2578-84.

91. Zhang, C.L., et al., *Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor.* J Biol Chem, 2001. 276(1): p. 35-9.

92. Sundqvist, A., K. Sollerbrant, and C. Svensson, *The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex.* FEBS Lett, 1998. 429(2): p. 183-8.

93. Sewalt, R.G., et al., *C-Terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins.* Mol Cell Biol, 1999. 19(1): p. 777-87.

94. Mannervik, M., et al., *Transcriptional coregulators in development.* Science, 1999. 284(5414): p. 606-9.

95. Postigo, A.A. and D.C. Dean, *Differential expression and function of members of the zfh-1 family of zinc finger/homeodomain repressors.* Proc Natl Acad Sci U S A, 2000. 97(12): p. 6391-6.

96. Nibu, Y., et al., *dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the Drosophila embryo.* Embo J, 1998. 17(23): p. 7009-20.

97. Turner, J. and M. Crossley, *Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators.* Embo J, 1998. 17(17): p. 5129-40.

98. Postigo, A.A. and D.C. Dean, *ZEB represses transcription through interaction with the corepressor CtBP.* Proc Natl Acad Sci U S A, 1999. 96(12): p. 6683-8.

99. Koipally, J. and K. Georgopoulos, *Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity.* J Biol Chem, 2000. 275(26): p. 19594-602.

100. Chen, H., et al., *Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase.* Cell, 1999. 98(5): p. 675-86.

101. Phippen, T.M., et al., *Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression.* J Biol Chem, 2000. 275(48): p. 37628-37.

102. Zhang, Q., et al., *Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP.* Proc Natl Acad Sci U S A, 2000. 97(26): p. 14323-8.

103. Cavailles, V., et al., *Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor.* Embo J, 1995. 14(15): p. 3741-51.

104. Postigo, A.A. and D.C. Dean, *ZEB, a vertebrate homolog of Drosophila Zfh-1, is a negative regulator of muscle differentiation.* Embo J, 1997. 16(13): p. 3935-43.

105. Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors.* Genes Dev, 2000. 14(2): p. 121-41.

106. Wagner, R.L., et al., *A structural role for hormone in the thyroid hormone receptor.* Nature, 1995. 378(6558): p. 690-7.

107. Shiau, A.K., et al., *The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen.* Cell, 1998. 95(7): p. 927-37.

108. Renaud, J.P., et al., *Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid.* Nature, 1995. 378(6558): p. 681-9.

109. Brzozowski, A.M., et al., *Molecular basis of agonism and antagonism in the oestrogen receptor.* Nature, 1997. 389(6652): p. 753-8.

110. Bourguet, W., et al., *Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha.* Nature, 1995. 375(6530): p. 377-82.

111. Vo, N. and R.H. Goodman, *CREB-binding protein and p300 in transcriptional regulation.* J Biol Chem, 2001. 276(17): p. 13505-8.

112. Wei, L.N., et al., *Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing.* J Biol Chem, 2000. 275(52): p. 40782-7.

113. Treuter, E., et al., *A regulatory role for RIP140 in nuclear receptor activation.* Mol Endocrinol, 1998. 12(6): p. 864-81.

114. Miyata, K.S., et al., *Receptor-interacting protein 140 interacts with and inhibits transactivation by, peroxisome proliferator-activated receptor alpha and liver-X-receptor alpha.* Mol Cell Endocrinol, 1998. 146(1-2): p. 69-76.

115. Lee, C.H. and L.N. Wei, *Characterization of receptor-interacting protein 140 in retinoid receptor activities.* J Biol Chem, 1999. 274(44): p. 31320-6.

116. Lee, C.H., C. Chinpaisal, and L.N. Wei, *Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2.* Mol Cell Biol, 1998. 18(11): p. 6745-55.

117. Wei, L.N., M. Farooqui, and X. Hu, *Ligand-dependent formation of retinoid receptors, receptor-interacting protein 140 (RIP140), and histone deacetylase complex is mediated by a novel receptor-interacting motif of RIP140.* J Biol Chem, 2001. 276(19): p. 16107-12.

118. Hollenberg, S.M., et al., *Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system.* Mol Cell Biol, 1995. 15(7): p. 3813-22.

119. Zhang, Q., N. Vo, and R.H. Goodman, *Histone binding protein RbAp48 interacts with a complex of CREB binding protein and phosphorylated CREB.* Mol Cell Biol, 2000. 20(14): p. 4970-8.

120. Kraus, W.L., K.E. Weis, and B.S. Katzenellenbogen, *Inhibitory cross-talk between steroid hormone receptors: differential targeting of estrogen receptor in the repression of its transcriptional activity by agonist- and antagonist-occupied progestin receptors.* Mol Cell Biol, 1995. 15(4): p. 1847-57.

121. Shilton, B.H., et al., *Crystallization of a soluble form of the Kex1p serine carboxypeptidase from Saccharomyces cerevisiae.* Protein Sci, 1996. 5(2): p. 395-7.

122. Struhl, K., *Histone acetylation and transcriptional regulatory mechanisms.* Genes Dev, 1998. 12(5): p. 599-606.

123. Vogelauer, M., et al., *Global histone acetylation and deacetylation in yeast.* Nature, 2000. 408(6811): p. 495-8.

124. Anderson, R.M., et al., *Yeast life-span extension by calorie restriction is independent of NAD fluctuation.* Science, 2003. 302(5653): p. 2124-6.

125. Lin, S.J., et al., *Calorie restriction extends yeast life span by lowering the level of NADH.* Genes Dev, 2004. 18(1): p. 12-6.

126. Anderson, R.M., et al., *Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae.* Nature, 2003. 423(6936): p. 181-5.

127. Koubova, J. and L. Guarente, *How does calorie restriction work?* Genes Dev, 2003. 17(3): p. 313-21.

128. Fjeld, C.C., W.T. Birdsong, and R.H. Goodman, *Differential binding of NAD+ and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor.* Proc Natl Acad Sci U S A, 2003. 100(16): p. 9202-7.

129. Zheng, L., R.G. Roeder, and Y. Luo, *S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component.* Cell, 2003. 114(2): p. 255-66.

130. Bergmeyer, H.U., *Methods of Enzymatic Analysis*. 2 ed. Vol. 4. 1974, New York: Academic Press.

131. Canepa, L., et al., *Bound and unbound pyridine dinucleotides in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes.* Biochim Biophys Acta, 1991. 1074(1): p. 101-4.

132. G. Jacobasch, S.M., and S. M. Rapoport, *In Cellular and Molecular Biology of Erythrocytes*, ed. H.a.R. Yokikawa, S. M. 1974, Baltimore: University Park Press. 52-92.

133. Simpson, R.J., K.M. Brindle, and I.D. Campbell, *Spin ECHO proton NMR studies of the metabolism of malate and fumarate in human erythrocytes. Dependence on free NAD levels.* Biochim Biophys Acta, 1982. 721(2): p. 191-200.

134. Stocchi, V., et al., *Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells.* Anal Biochem, 1985. 146(1): p. 118-24.

135. Litt, M.R., et al., *Analysis of pyridine dinucleotides in cultured rat hepatocytes by high-performance liquid chromatography.* Anal Biochem, 1989. 179(1): p. 34-6.

136. Kalhorn, T.F., et al., *Analysis of oxidized and reduced pyridine dinucleotides in rat liver by high-performance liquid chromatography.* Anal Biochem, 1985. 151(2): p. 343-7.

137. Tischler, M.E., et al., *Pyridine nucleotide distributions and enzyme mass action ratios in hepatocytes from fed and starved rats.* Arch Biochem Biophys, 1977. 184(1): p. 222-36.

138. Bootman, M.D., P. Lipp, and M.J. Berridge, *The organisation and functions of local Ca(2+) signals.* J Cell Sci, 2001. 114(Pt 12): p. 2213-22.

139. Arutyunova, E.I., et al., *Oxidation of glyceraldehyde-3-phosphate dehydrogenase enhances its binding to nucleic acids.* Biochem Biophys Res Commun, 2003. 307(3): p. 547-52.

140. Stinson, R.A. and J.J. Holbrook, *Equilibrium binding of nicotinamide nucleotides to lactate dehydrogenases.* Biochem J, 1973. 131(4): p. 719-28.

141. Kim, S.J. and B.M. Anderson, *Coenzyme binding to L-alpha-glycerophosphate dehydrogenase.* J Biol Chem, 1969. 244(6): p. 1547-51.

142. Lerner, F., et al., *Structural and functional characterization of human NAD kinase.* Biochem Biophys Res Commun, 2001. 288(1): p. 69-74.

143. Mendoza-Alvarez, H. and R. Alvarez-Gonzalez, *Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular.* J Biol Chem, 1993. 268(30): p. 22575-80.

144. Howitz, K.T., et al., *Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan.* Nature, 2003. 425(6954): p. 191-6.

145. Rongvaux, A., et al., *Reconstructing eukaryotic NAD metabolism.* Bioessays, 2003. 25(7): p. 683-90.

146. Paine, A.J. and L.J. Hockin, *Effect of hepatocyte culture conditions on their content of nicotinamide coenzymes [proceedings].* Biochem Soc Trans, 1980. 8(2): p. 183-4.

147. Kaplan, N.O., *Regulation of pyridine nucleotide coenzymes.* J Vitaminol (Kyoto), 1968. 14: p. Suppl:103-13.

148. Threlfall, C.J., *Synthesis of diphosphopyridine nucleotide from nicotinamide by slices of liver.* Nature, 1959. 184: p. 60-1.

149. Evans, J., et al., *LC/MS analysis of NAD biosynthesis using stable isotope pyridine precursors.* Anal Biochem, 2002. 306(2): p. 197-203.

150. Noda, M., et al., *Switch to anaerobic glucose metabolism with NADH accumulation in the beta-cell model of mitochondrial diabetes. Characteristics of betaHC9 cells deficient in mitochondrial DNA transcription.* J Biol Chem, 2002. 277(44): p. 41817-26.