THE CATALYTIC ACTIVITY OF HDAC3 IS NECESSARY FOR A NORMAL RATE OF CELLULAR PROLIFERATION

By

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53BP1	p53-binding protein 1
Ad-cre	adenovirus expressing the c*re recombinase*
ALT	alanine transaminase
ATP	adenosine-5'-triphosphate
CaMK	Ca^{2+}/CaM-dependent kinase
cDNA	complementary DNA
CK2	casein kinase 2
co-IP	co-immunoprecipitation
CoREST	co-repressor element-1 silencing transcription
CTCL	cutaneous T-cell lymphoma
DAD	deacetylase activating domain
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	double stranded break
E2F1	E2F transcription factor 1
GFP	green fluorescent protein
GPS2	g protein pathway suppressor 2
HAT	histone acetyltransferase
HCC	hepatocellular carcinoma

HDAC	histone deacetylase
HDI	histone deacetylase inhibitor
HR	homologous recombination
HSP	heat shock protein
IF	immunofluorescence
Ins(1,4,5,6)P_4	D-myo-inositol-1,4,5,6-tetrakisphosphate
IP	inositol polyphosphates
IR	ionizing radiation
IRES	internal ribosome entry site
KAP-1	krab associated protein 1
KD	knockdown
kDa	kilodalton
MAPK11	mitogen-activated protein kinase 11
MCM2	minichromosome maintenance 2
MEF	mouse embryonic fibroblast
MNase	micrococcal nuclease
MSCV	murine stem cell virus
MTG	myeloid translocation gene
N-CoR	nuclear receptor co-repressor
NAD^+	nicotinamide adenine dinucleotide
NES	nuclear export signal

NF-κB	nuclear factor-κB
NHEJ	Non-homologous end joining
NIH	National Institutes of Health
NKAP	NF-κB activating protein
NLS	nuclear localization signal
NuRD	nucleosome remodeling and deacetylating
PIAS2	protein inhibitor of activated STAT 2
PIP_2	inositol 4,5-bisphosphate
PML	promyelocytic leukemia
PP4	protein serine/threonine phosphatase 4
PPARγ	peroxisome proliferator-activated receptor γ
RAR	retinoic acid receptor
RB	retinoblastoma
RbAp48	retinoblastoma-associated protein 48
RBBP	retinoblastoma binding protein
RIPA	radio immunoprecipitation assay
RUNX2	runt-related transcription factor 2
RXR	retinoid-X receptor
SAHA	suberoylanilde hydroxamic acid
SAM	S-adenosyl methionine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sin3	switch independent 3

siRNA	small interfering ribonucleic acid
Sirt	sirtuin
SMRT	silencing mediator for retinoic acid and thyroid receptors
STAT3	signal transducer and activator of transcription 3
TBL1	transducin β-like 1
TBLR1	TBL1-related protein
TCP-1	t-complex protein 1
TR	thyroid-hormone receptor
TriC	TCP-1 ring complex
TSA	trichostatin A
YY1	ying yang 1

CHAPTER I

INTRODUCTION

Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes to the DNA sequence. Acetylation of histones is one of the main post-translational modifications responsible for altering the epigenetic landscape through regulation of chromatin structure. Histone deacetylases are the key enzymes responsible for removing this modification. In particular, histone deacetylase 3 (HDAC3) represents a pivotal member of the histone deacetylase family that modifies chromatin structure to regulate transcription, replication, recombination, and repair. Furthermore, given that histone deacetylases are deregulated in cancer, particularly hematological malignancies, further study of the structure and function of HDAC3 may directly affect human health through the design of more specific and targeted histone deacetylase inhibitors.

Chromatin Regulation

DNA (deoxyribonucleic acid) is the main building block of life, containing the genetic information needed for development and every day functioning of nearly all living organisms. The vast length of DNA, necessary to code for all aspects of life, needs to be compacted in such a way that it can fit into the nucleus of individual cells. At the same time, relatively small sections of DNA

often need to be loose and accessible for proteins which function to modulate a variety of cellular processes such as transcription, replication, recombination, and repair.

Structure

Chromatin refers to the combination of DNA and proteins that make up the contents of the nucleus of a cell. 147 base pairs of DNA wrap around a histone octamer made up of two copies of each of the globular proteins histone H2A, H2B, H3, and H4 forming the nucleosome (1-4) (Fig. 1). The addition of one histone H1 protein wraps another 20 base pairs, resulting in two full turns of DNA around the octamer. Structurally “loose” chromatin appears as beads-on-a-string with an average of 20 base pairs of linker DNA between nucleosomes (5, 6). This state of DNA is referred to as euchromatin. In euchromatic regions the position of nucleosomes along DNA, combined with how tightly the DNA wraps around the histone octamer, impacts DNA accessibility during transcription, replication, recombination, and repair (7, 8). These processes can only take place when DNA is in this “loose” state. When multiple nucleosomes begin to tightly associate with each other, DNA begins to compact forming heterochromatin. DNA contained in heterochromatic regions is not accessible for most cellular processes.

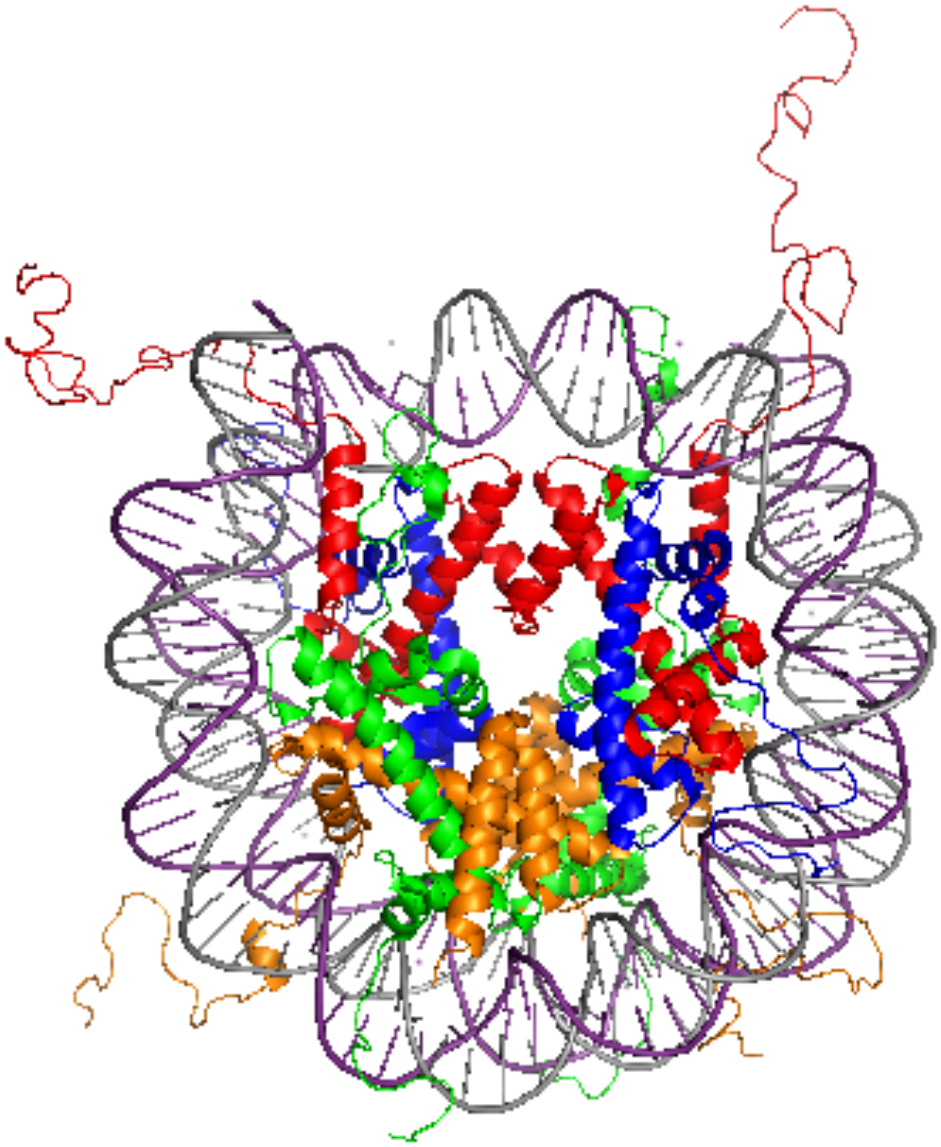


Figure 1. Nucleosome structure. 147 base pairs of DNA (purple and gray strands) are wrapped around a histone octamer formed by of two each of H2A (green), H2B (orange), H3 (red), and H4 (blue) represented by ribbon diagrams. Post-translationally modified N-terminal histone tails are unstructured and extend out from the nucleosome core particle. (PDB 1KX5)

Post-Translational Modifications

The N-terminal tails and, to a lesser extent, the globular domains of histones are subject to post-translational modifications that directly regulate chromatin structure (Fig. 1). These modifications include, but are not limited to, phosphorylation, acetylation, methylation, ubiquitylation and sumoylation. Phosphorylation occurs at a few serines and threonines, while a number of lysine residues are modified by methylation, ubiquitylation and sumoylation. These modifications are exclusive such that when a lysine is acetylated, it cannot be methylated. Methylation, which exists as mono-, di- or trimethylation, can also occur at arginine.

Ubiquitylation and methylation have variable effects on chromatin depending on the precise amino acids modified. For example, trimethylation of histone H3 lysine 4 (H3K4me3) generally occurs at active genes, whereas H3K9me3 generally occurs at inactive genes (9). Sumoylation seems to correspond primarily with repression, while acetylation corresponds with activation. Post-translational modifications do not exist in isolation and thus it is a combination of modifications that ultimately determines the “histone code” and determines the accessibility of a given DNA region (9). The effect of histone modifications can be direct, causing structural changes to chromatin, or indirect, acting through the recruitment of effector proteins, so called “readers”. Proteins that bind histones often contain chromodomains or tudordomains that bind to methylated lysines or bromodomains that bind to acetylated lysines (10, 11). Effector proteins include adenosine-5'-triphosphate (ATP)-dependent

nucleosome remodeling complexes that shift nucleosome positions along DNA or exchange/remove histones (12). ATP-dependent nucleosome remodeling complexes regulate gene expression, replication, and repair through regulating protein access to chromatin (13). Post-translational modifications along with nucleosome shifting and histone exchange/removal are the major mechanisms by which chromatin is regulated.

HATs and HDACs

Post-translational modifications are by nature reversible: one family of proteins adds modifications when necessary and another family removes them. Acetylation represents one of the major post-translational modifications that directly impacts chromatin structure. In the case of acetylation, histones are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs). When histones are acetylated by HATs the positive charge normally present at the end of the lysine residues is neutralized leading to the loosening of chromatin. In contrast, HDACs deacetylate histones leading to tighter DNA-histone and histone-histone associations resulting in chromatin compaction. The studies described here pay particular attention to HDACs, known negative regulators of transcription, replication, recombination, and repair.

HDAC Family of Proteins

Identification and Classification

The first mammalian histone deacetylase (HDAC1) was isolated from bovine thymus nuclear fractions and Jurkat nuclear extracts in 1996 (14). This 55kDa protein, originally named HD1 for histone deacetylase 1, was isolated along with its first known binding partner, retinoblastoma-associated protein 48 (RbAp48), and is 60% identical to the yeast histone deacetylase, Rpd3. Later that year HDAC2, originally named mRPD3 for mammalian RPD3, was identified based on an ability to bind the transcription factor, yin yang 1 (YY1) (15). Furthermore, HDAC2 activity was required for YY1-dependent transcriptional repression of a Gal4 construct. In 1997 complementary DNA (cDNA) (EST200871) encoding a partial open reading frame with significant sequence similarity to HDAC1 and 2 was identified from a human fetal liver library (16). When expressed, the HDAC3 protein deacetylated chicken histones and repressed transcription of a Gal4 construct. Even though HDACs have been studied for over 15 years, much remains to be discovered about the structure and function of individual HDACs.

Today, the mammalian histone deacetylase family has grown to include eighteen proteins, which are divided into four classes based on their homology to yeast histone deacetylases Rpd3, Hda1, and Sir2 (17, 18). Class 1 HDACs, consisting of HDAC1, 2, 3, and 8, are homologous to the yeast histone deacetylase Rpd3 while the class 2 HDACs (HDAC4, 5, 6, 7, 9, and 10) are

homologous to the yeast histone deacetylase Hda1. Class 3 HDACs, consisting of Sirtuin1-7, are homologous to the yeast histone deacetylase Sir2. HDAC11 is the only member of the class 4 HDACs and is partly homologous to both yeast histone deacetylases Rpd3 and Hda1. Though mammalian HDACs share a conserved deacetylase domain, they are a diverse group of proteins with unique localization, complex composition, level of activity, and even catalytic mechanism. (17, 18)

Class 1

In general, class 1 histone deacetylases are ubiquitously expressed and localized to the nucleus (17). However, HDAC3 can partially localize to the cytoplasm (discussed below). This expression pattern allows class 1 HDACs to regulate transcription of a significant number of targets (≤10% of genes) in a variety of different tissues (19). HDAC3 is catalytically active only when in complex with large multi-subunit co-repressor complexes while HDAC8 is a fully functional enzyme in isolation (20, 21). HDAC3 is found in the silencing mediator for retinoic acid and thyroid receptors (SMRT) and nuclear receptor co-repressor (N-CoR) complexes (discussed below) (Fig. 2). HDAC1 and 2, which share 82% identity and are nearly interchangeable, are partially active in isolation, but are highly active when in complex with their associated factors. HDAC1 and 2 are found in three major types of complexes, the switch independent 3 (Sin3) complex (22), the nucleosome remodeling and deacetylating (NuRD) complex (23), and the neuron-specific co-repressor element-1 silencing transcription

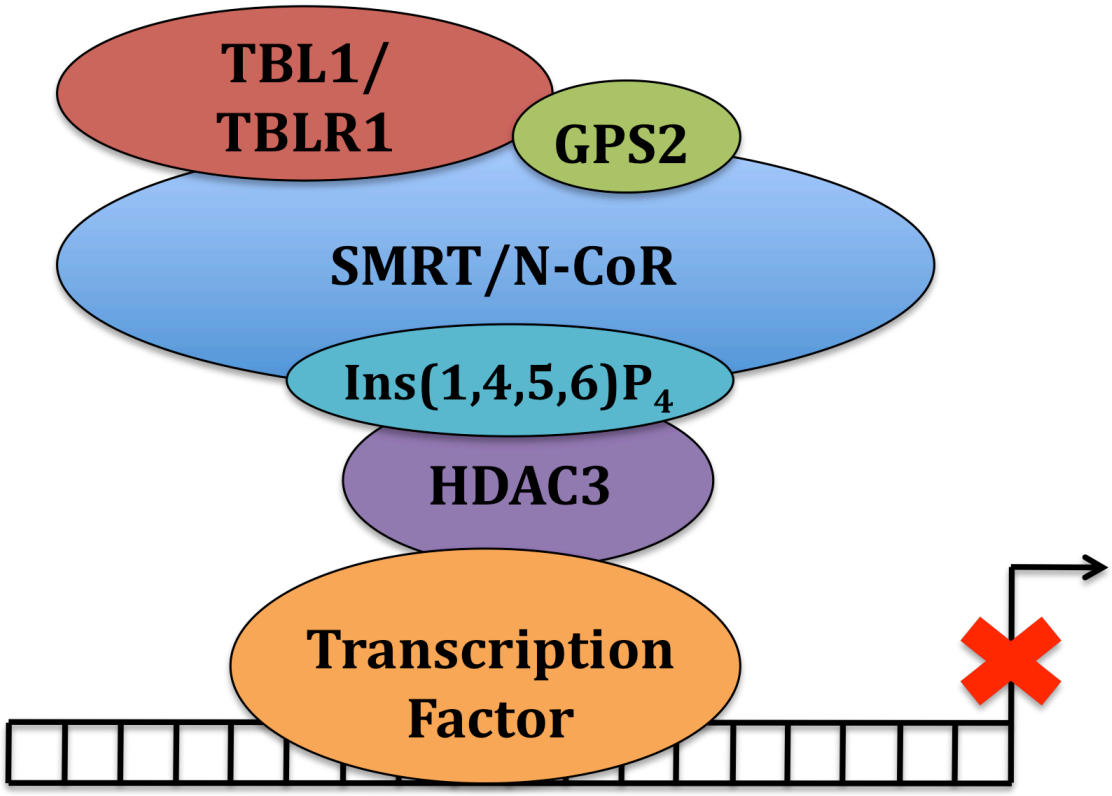


Figure 2. SMRT/N-CoR co-repressor complex. SMRT/N-CoR interacts directly with core complex members TBL1/TBLR1 and GPS2 while interacting with HDAC3 through Ins(1,4,5,6)P_4. TBL1/TBLR1 and GPS2 also directly interact. The co-repressor complex binds transcription factors to repress transcription.

(CoREST) complex (24). Each complex is unique, with the NuRD complex containing ATP-dependent chromatin remodeling activity in addition to HDAC activity (25) and the CoREST complex primarily functioning at neuronal genes (26). The Sin3 complex is targeted to a multitude of promoters through various DNA-binding proteins. In addition to functioning through these complexes, HDAC1 and 2 can also bind directly to DNA binding proteins YY1, RB binding protein-1 (RBBP1), and Sp1 (17). The multitude of HDAC1 and 2 associated proteins provides flexibility and specificity in modulating the epigenetic landscape.

Class 2

Class 2 HDACs can be divided into class 2a and 2b. Class 2a consists of HDAC4, 5, 7, and 9. These HDACs have little to no histone deacetylase enzymatic activity (27), but can associate with catalytically active HDACs including HDAC3 (28). Class 2b HDACs, HDAC6 and 10, are classified by the presence of two catalytic domains. As a group, class 2 HDACs display more tissue-specific expression than any other class of HDACs (29). Thus, inhibitors of class 2 HDACs may target diseases more specifically and with fewer side effects than multi-HDAC inhibitors. In addition, this class of enzymes shuttles in and out of the nucleus in response to cellular signals. Following phosphorylation of conserved N-terminal serine residues by Ca^{2+}/CaM-dependent kinase (CaMK) the 14-3-3 proteins can sequester class 2 HDACs to the cytoplasm (30). When localized to the cytoplasm class 2 HDACs cannot regulate transcription. The

need to bind catalytically active HDACs, along with the varied localization and tissue-specific expression, equates to multiple layers of regulation for class 2 HDACs.

Class 3

Class 3 HDACs, also known as sirtuins (Sirts), are unique among the four classes as they work in a nicotinamide adenine dinucleotide (NAD^+) dependent manner while Class 1, 2, and 4 HDACs work in a zinc-dependent manner (31). NAD^+ is a coenzyme found in all living cells that is primarily involved in redox reactions, carrying electrons from one reaction to another. Sirtuins transfer acetyl groups from their substrate proteins to NAD^+, making cellular levels of NAD^+ a key regulator of sirtuin activity. The dependence of sirtuins on NAD^+ implies a role for this class of deacetylases in the regulation of metabolic homeostasis (32). Individual sirtuins have divergent biological functions due to the distinct cell-type-specific sub-cellular localization of each family member. In particular, Sirt1 is located in both the nucleus and the cytoplasm, Sirt2 in the cytoplasm alone, Sirt3, 4, and 5 are mitochondrial, and Sirt6 and 7 are exclusively nuclear (32, 33). Inhibition of sirtuins may have particular significance for metabolic diseases.

Histone Deacetylase 3

HDAC3 is unique among the HDAC family of proteins. The C-terminal 35 amino acids of HDAC3 are not seen in any other histone deacetylase (Fig. 3).

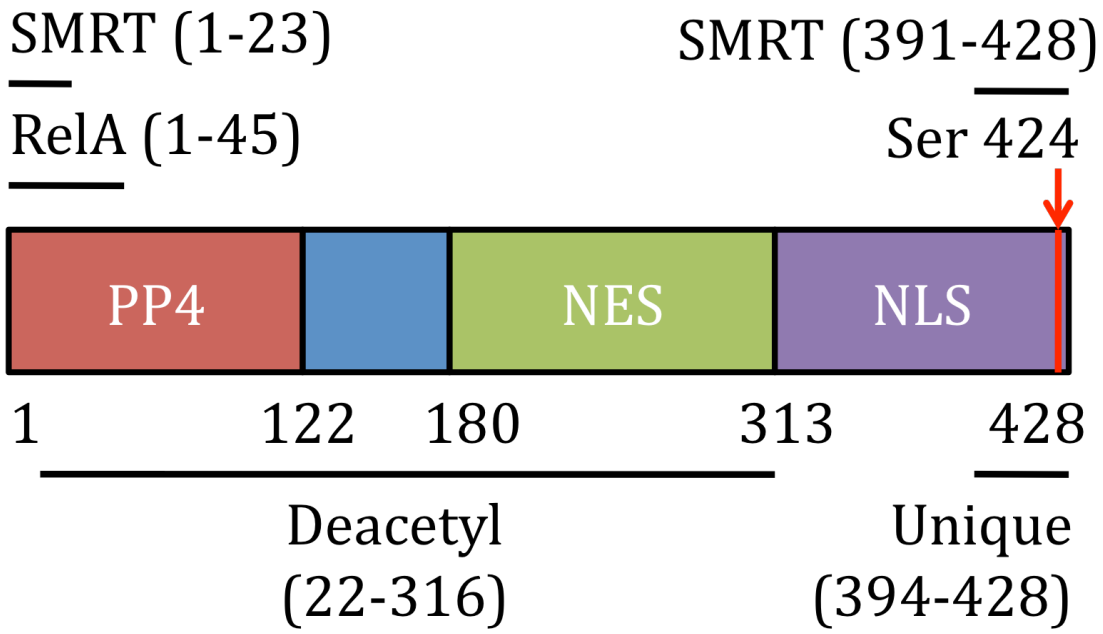


Figure 3. HDAC3 domains. Deacetyl (Deacetylase domain), SMRT (SMRT binding region), RelA (RelA binding region), PP4 (protein phosphatase 4 binding region), NES (nuclear export signal), NLS (nuclear localization signal), and Ser 424 (serine 424 phosphorylation site).

This unique region is required *in vivo* and *in vitro* for HDAC3 deacetylase activity and transcriptional repression (34). In addition, HDAC3 is the only catalytically active histone deacetylase found as part of the SMRT/N-CoR co-repressor complex (Fig. 2). This complex is recruited to a multitude of sites in the genome, including nuclear hormone receptors, to repress transcription (discussed below).

In vitro HDAC3 preferentially deacetylates lysine 5 and 12 of histone H4 and lysine 5 of histone H2A (35). Newly synthesized histones H3 and H4 undergo transient acetylation during synthesis phase (S phase) of the cell cycle and before deposition onto replicated DNA. In particular, acetylation of H4K5 and H4K12 is commonly associated with deposition of histones (36, 37). Soon after deposition histones are deacetylated, in part by HDAC3, to stabilize the nucleosome and/or allow higher-order compaction of chromatin. H4K5, H4K12, and H3K9K14 acetylation is often increased in the absence of *Hdac3* while an increase in H4K8 and H4K16 acetylation is more variable (38-40). The increase in acetylation of H4K5 and H4K12, when HDAC3 is absent, suggests a role for HDAC3 in removing these marks after DNA replication.

Histone Deacetylase Inhibitors

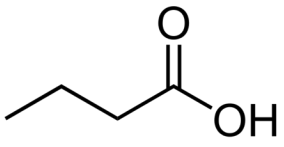
Given the role of HDACs in modulating chromatin structure, ultimately regulating transcription, replication, recombination, and repair, it is not surprising that deregulation of HDAC function has been associated with cancer. Many forms of leukemia are caused by chromosomal rearrangements that generate oncogenic fusion proteins. Multiple fusion proteins, including the t(15;17)

encoded fusion of promyelocytic leukemia (PML) and the retinoic acid receptor (RAR), interact with SMRT/N-CoR leading to aberrant recruitment of the HDAC3-containing co-repressor complex (41). Such associations provide rationale for using histone deacetylase inhibitors to treat these hematological malignancies. Therefore, inhibition of one or more HDACs offers an attractive therapeutic opportunity for treating a variety of cancers.

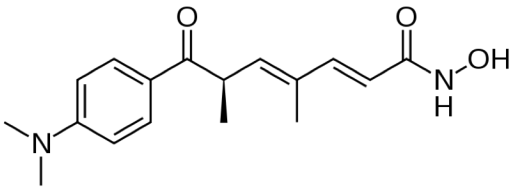
Identification and FDA Approval

The first histone deacetylase inhibitor (HDI), sodium butyrate (*n*-butyrate), was described in 1977, eighteen years before the first mammalian HDAC was identified (Fig. 4). Increased acetylation of histone H3 and H4 was observed following incubation of HeLa and Friend erythroleukemia cells with *n*-butyrate (42). This dose dependent, reversible increase in acetylation caused by *n*-butyrate was due to inhibition of histone deacetylation (43). *N*-butyrate has other biochemical activities and thus is not specific for HDAC inhibition. About 12 years later, the fungi-static antibiotic trichostatin A (TSA) was identified as the first potent and specific HDAC inhibitor (44) (Fig. 4).

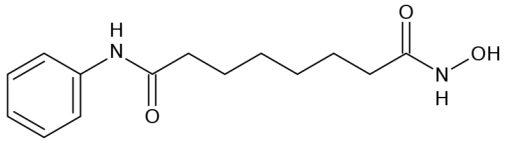
Today there are two histone deacetylase inhibitors approved as single agents by the FDA for treatment of relapsed cutaneous T-cell lymphoma (CTCL). Vorinostat, also known as suberoylanilde hydroxamic acid (SAHA), was the first histone deacetylase inhibitor approved in 2006 and Romidepsin, also known as depsipeptide, was approved in 2009 (Fig. 4). SAHA and depsipeptide are both multi-HDAC inhibitors that inhibit the activity of class 1 and 2 HDACs.



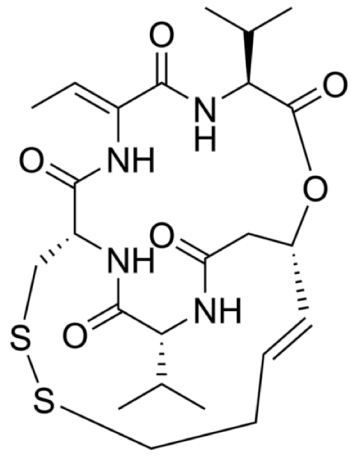
n-butyrate



TSA



SAHA



Depsipeptide

Figure 4. Histone deacetylase inhibitors. See text for details.

Specifically, SAHA inhibits HDAC1, 2, 3, 6, and 8 simultaneously (19). More selective inhibitors are being developed in the hopes of reducing side effects and having more direct biological effects. Selective and multi-HDAC inhibitors are in various phases of drug development/approval for the treatment of a variety of diseases including hematological malignancies and solid tumors (41, 45). Further study of the HDAC family of proteins is warranted as histone deacetylase inhibitors hold promise as effective treatments of human disease.

Combinatorial Use

Combining HDIs with inhibitors of other enzymes, classical chemotherapeutics, and/or radiation is being examined in the hopes of maximizing treatment efficacy (19, 46). HDIs act as potent radiosensitizers due to their effects on the DNA damage response and are thus being tested in combination with radiation therapy (47). In addition, the effects of HDIs on the structure of nucleosomes, leading to relaxation of chromatin, makes them attractive for combination treatment with DNA-targeted chemotherapeutics such as platinum compounds, topoisomerase inhibitors, and DNA intercalators (48, 49). DNA methylation by DNA methyltransferases (DNMTs) leads to gene silencing, in part due to recruitment of HDAC-containing transcriptional repression complexes (50). Combination inhibition of HDACs and DNMTs could promote transcription of genes inappropriately silenced in cancer. Therefore, the combinatorial use of HDIs and other agents may be particularly useful for the treatment of cancer.

Cellular Effects

Primary cells and cell lines are often the first systems used to study the effects of histone deacetylase inhibitors. HDIs have produced a variety of effects on transformed cells including induction of cellular differentiation, apoptosis, or growth arrest, while altering the transcription of a surprisingly small number of genes (2-10% of expressed genes) (51). Induction of cell death in particular can occur through a variety of mechanisms including activation of death receptor (52) or mitochondrial death pathways (53). Cell cycle changes in normal and cancerous cells following HDI treatment can include arrest in G0/G1 or G2/M depending on the dose (41). More directly related to cancer progression, HDIs affect angiogenesis, cell invasion and metastasis, and immune-modulatory activity (41). Responses seen following HDI treatment seem to, at least in part, depend on the nature of the HDI, concentration used, length of exposure, and cellular context. Though normal cells do respond to HDIs, there is a clearly documented higher sensitivity of transformed cells, thus providing the basis for HDI mediated tumor cell selective killing (54, 55). HDIs are well suited for use in therapy due to both their relatively low toxicity to normal cells and their specific biological effects on cellular processes. The effects of HDIs seem as diverse as the number of HDACs, thus careful attention must be paid to the HDAC(s) being targeted and the context in which particular HDIs are used. It is this need to pay attention to the HDAC(s) being targeted and their normal biological functions that forms the basis for studying individual HDACs, including HDAC3.

Hdac3 Deletion Models

Germline Deletion

One of the best ways to study the biological functions of a given protein is to study the phenotypes associated with the protein's inactivation in mice. Mice lacking *Hdac3* were created by first flanking exon 7 of *Hdac3* with loxP sites and inserting a G418 resistance gene (Neo) between exon 7 and exon 8 to engineer a *Cre-recombinase*-dependent allele. Mice harboring the targeted allele were crossed to *EIIA-Cre* transgenic mice to obtain progeny with either a "floxed" allele or a null allele. Germline *Hdac3* deletion triggered early embryonic lethality prior to embryonic day 9.5 (38). The requirement for *Hdac3* during early embryonic development precluded the study of complete deletion of *Hdac3* in adult mice.

Mouse Embryonic Fibroblasts

Given the severity of this phenotype, analysis of Hdac3 function was performed in mouse embryonic fibroblasts (MEFs) isolated from embryonic day 13.5-14.5 $Hdac3^{F/-}$ and $Hdac3^{F/+}$ embryos (38). The use of MEFs allowed for analysis of Hdac3 function on a cellular level. $Hdac3^{F/-}$ MEFs were infected with adenovirus expressing *Cre recombinase* (*Ad-cre*) to genetically delete *Hdac3*. These *Hdac3*-null cells began to show signs of apoptosis 3-4 days post infection. At 5 days post infection the majority of cells were still viable giving a significant window in which to examine the cell cycle, DNA damage, and DNA repair. To examine the cell cycle, $Hdac3^{-/-}$ and $Hdac3^{+/-}$ MEFs were analyzed using the

thymidine analogue BrdU in a pulse-chase experiment. Two fold fewer *Hdac3*$^{-/-}$ MEFs were labeled with BrdU, suggesting that *Hdac3* plays a role in the cycling of cells. As the BrdU was chased through the cell cycle, BrdU positive cells were delayed in proceeding from one phase to the next with a particularly significant delay in clearing S phase. This suggested a defect in cell cycle progression and DNA replication (38).

DNA damage was examined as a possible source of the S phase defect. *Hdac3*-null cells analyzed through immunofluorescence (IF) had higher levels of the DNA double strand break (DSB) marker, phosphorylated histone H2AX (γH2AX) than their *Hdac3*$^{+/-}$ counterparts (38). Additional markers of the DNA damage response, phosphorylated KRAB-associated protein 1 (KAP-1), phosphorylated minichromosome maintenance 2 (MCM2), and p53, were also elevated in the absence of *Hdac3*. To specifically examine the impact of cell-cycle progression on DNA damage, MEFs were first serum starved to synchronize cells in G0. Following serum starvation, cells were again analyzed through IF for the presence of γH2AX. Taking cells out of the cell cycle reduced the percentage of *Hdac3*$^{-/-}$ cells with DNA damage. *Hdac3*-null MEFs are characterized by cell-cycle-dependent DNA damage (38).

Akin to an accumulation of endogenous DNA damage, *Hdac3*$^{-/-}$ MEFs had defects in repair of exogenous DNA damage induced using ionizing radiation (IR), doxorubicin, and cisplatin (38, 39). Increased sensitivity to these forms of DNA damage suggests that in the absence of *Hdac3* repair by the homologous recombination (HR) pathway and non-homologous end joining (NHEJ)-mediated

repair are inefficient. There were clear defects in the repair of endogenous and exogenous DNA damage in the absence of *Hdac3*.

Given the requirement for Hdac3 in removing histone deposition marks, H4K5 and H4K12 acetylation, mitotic chromosomes were analyzed through metaphase spreads. *Hdac3*-null MEFs had a 5 to 8-fold increase in the number of breaks and gaps in metaphase chromosomes when compared to controls (39). Thus, *Hdac3* has a critical role in maintaining genomic stability.

To determine if histone deacetylase inhibitors also cause DNA damage, $Hdac3^{F/+}$ MEFs were treated with increasing amounts of the multi-HDAC inhibitors, SAHA or TSA (38). Treatment of cells with low levels of these multi-HDAC inhibitors caused DNA damage similar to the levels seen in the absence of *Hdac3*. Treatment with high levels of SAHA caused even greater DNA damage suggesting the DNA damage caused by HDIs may be a cumulative effect of inhibiting multiple HDACs (38).

Immortalized Cells

Immortalized cell lines offer another system in which to study loss of *Hdac3*. Knockdown (KD) of *Hdac3* in human colon cancer cell lines and HeLa cells using siRNA caused accumulation of cells in G_2/M phase of the cell cycle (56), loss of the metaphase marker histone H3S10 phosphorylation, and increased aberrancy of mitotic figures (57). A similar decrease in the number of cells in metaphase and H3S10 phosphorylation was observed in a line of $Hdac3^{-/-}$ National Institutes of Health (NIH) 3T3 cells. *Ad-cre* infection was again used to

delete *Hdac3*, this time from an immortalized cell line established from *Hdac3*$^{F/-}$ MEFs through the NIH 3T3 regimen (38, 58). A decrease in mitotic cells is often characteristic of an active DNA damage checkpoint (59). *Hdac3*$^{-/-}$ NIH 3T3 cells treated with the DNA damage checkpoint inhibitor, caffeine, showed normal numbers of metaphase cells and levels of histone H3S10 phosphorylation (38). This implies that the metaphase defects were either due to a defect in DNA damage response or replicative stress seen only in immortalized cells. Primary cells responded differently to loss of *Hdac3,* as none of these defects were seen in MEFs (38). The mitotic defects seen in *Hdac3*-null and knockdown cells require the immortalization process.

Mx1-Cre Conditional Deletion

In the case of embryonic lethality, following deletion of a protein in mice, conditional deletion is often used to study protein function. Promoter driven *Cre* expression allows analysis of biological function in a particular cell type or tissue. Given the embryonic lethality caused by *Hdac3* deletion in the germline, *Hdac3* heterozygous mice were crossed with transgenic *Mx1-Cre* and *Albumin-Cre* (*Alb-Cre*) mice (40). *Mx1-Cre* expression is stimulated by injection of synthetic double-stranded RNA (polyinosinic-poly-cytidylic acid, pIpC) to induce an interferon response. This system allows for *Hdac3* deletion in adult mice and bypasses embryonic lethality. Treatment of *Mx:Hdac3*$^{F/-}$ mice lead to abnormal liver size and morphology with hypertrophic hepatocytes. This liver phenotype prompted the *Alb-Cre* cross (40).

Alb-Cre Conditional Deletion

Alb-Cre is expressed in parenchymal liver cells and was used to analyze the role of Hdac3 in the liver while avoiding possible side effects of interferon signaling in the *Mx-Cre* model. By 28 days post birth the livers of *Alb:Hdac3*$^{-/-}$ mice were pale and hypertrophic, a trend that continued into adulthood (40). *Alb:Hdac3*$^{-/-}$ mice were also characterized by increased hepatocellular damage seen through elevated alanine transaminase (ALT) levels, altered metabolic homeostasis, and up-regulation of lipid and cholesterol biosynthesis regulatory genes (40). In particular, the up-regulation of genes belonging to the p53 network suggested the presence of DNA damage. *Alb:Hdac3*$^{-/-}$ mice were aged for approximately 16 weeks and later 8-10 months. By 16 weeks livers continued to appear pale due to an accumulation of lipids and contained "adenoma-like white nodules" (39). By 8-10 months *Alb:Hdac3*$^{-/-}$ mice developed low-grade hepatocellular carcinoma (HCC) and the experiment was humanly terminated (39). Thus the conditional deletion of *Hdac3* in the liver led to altered metabolism and eventually cancer.

Hepatocyes from *Alb:Hdac3*$^{-/-}$ mice were used to examine the effects of *in vivo Hdac3* deletion (39). The increase in H3K9K14 acetylation in *Alb:Hdac3*$^{-/-}$ mice corresponded to a decrease in H3K9 methylation, a mark of heterochomatin. Hepatocytes from *Alb:Hdac3*$^{-/-}$ mice had decreased levels of heterochromatin as visualized by transmission electron microscopy and Hoechst staining. Given the reduction in heterochromatin, nucleosomal compaction was examined through micrococcal nuclease (MNase) digestion and salt extraction.

Bulk chromatin isolated from nuclei of *Alb:Hdac3*$^{-/-}$ hepatocytes was more sensitive to MNase digestion and histone H3 was less resistant to high NaCl concentrations. In the absence of *Hdac3* global chromatin structure was more "open", indicating that *Hdac3* is required for maintaining proper chromatin structure *in vivo* (39).

Given the increase in DNA damage and decrease in DNA repair seen in *Hdac3*$^{-/-}$ MEFs, endogenous and exogenous DNA damage was examined in *Alb:Hdac3*$^{-/-}$ hepatocytes (39). DNA damage was again visualized using IF to detect the DNA double-stranded break markers γH2AX and p53-binding protein 1 (53BP1). *Alb:Hdac3*$^{-/-}$ hepatocytes displayed increased amounts of endogenous DNA and decreased rates of repair of exogenous damage induced by non-lethal doses of IR as compared to *Alb:Hdac3*$^{+/-}$ hepatocytes. Though Hdac3 is not recruited to sites of double-strand breaks, it could impact repair through changes in histone acetylation especially of H3K9. *In vivo Hdac3* has a critical role in maintaining genomic stability (39).

HDAC3 Containing Complexes

SMRT/N-CoR

HDAC3 biological functions are directly dependent on binding to SMRT/N-CoR. The SMRT/N-CoR complex was originally identified through recruitment by nuclear hormone receptors, thyroid-hormone receptor (TR), retinoic-acid receptor (RAR), and retinoid-X receptor (RXR), to repress transcription (60, 61). Ligand

binding to nuclear hormone receptors leads to dissociation of SMRT/N-CoR and triggers gene activation. It was later discovered that the SMRT/N-CoR complex contained HDAC3 and was dependent on the presence of HDAC3 for transcriptional repression (62, 63).

HDAC3 is not a functional enzyme in isolation and requires binding of SMRT/N-CoR to be active. Therefore, HDAC3 represents the catalytic component of the large multi-protein SMRT and N-CoR co-repressor complexes (64) (Fig. 2). SMRT and N-CoR are two distinct, yet highly related, proteins with similar amino acid sequences (60, 61). They each have a conserved deacetylase-activating domain (DAD) that binds HDAC3 and is required for HDAC3 activity (64). The 95 amino acid DAD consists primarily of a SANT (Swi3/Ada2/N-CoR/TFIIIB) domain that is necessary and sufficient for HDAC3 activation. The DAD, in conjunction with additional regions of SMRT, bind both the extreme N and C-terminal ends of HDAC3 (amino acids 1-23 and 391-428) (64) (Fig. 3).

Priming

HDAC3 interacts with SMRT only after priming by the chaperone proteins, heat shock protein 70 (HSP70) and T-complex protein 1 (TCP-1) ring complex (TriC), in an ATP-dependent process (35, 65). Following proper HDAC3 folding, TriC is displaced by SMRT. Recombinant HDAC3 made in bacteria is devoid of enzymatic activity, as these steps do not occur in bacteria. Priming by

chaperone proteins and binding of the SMRT/N-CoR DAD are necessary for enzymatic competency and full assembly of the co-repressor complex.

Binding Partners

Biochemical purifications from human cells have revealed additional members of the SMRT/N-CoR complexes. Transducin β-like 1 (TBL1) (63)/TBL1-related protein (TBLR1) (66) and G protein pathway suppressor 2 (GPS2) (66) are core members of the SMRT/N-CoR co-repressor complexes (Fig. 2). TBL1 and TBLR1 are functionally redundant proteins that can mediate SMRT/N-CoR co-repressor complex dependent transcriptional repression through binding to histone H2B and H4 (67). The other member of the core complex, GPS2, mediates interactions with DNA binding transcription factors to facilitate recruitment of the complex to target promoters to repress transcription (66). Though the presence of TBL1/TBLR1 and GPS2 does not impact the enzymatic activity of HDAC3 they do facilitate interactions of the co-repressor complex with other proteins.

Many proteins are bound by the HDAC3 containing SMRT/N-CoR core complex. Transcription factors that recruit the complex include peroxisome proliferator-activated receptor γ (PPARγ) (68), promyelocytic leukemia (PML) (69), runt-related transcription factor 2 (RUNX2) (70, 71), and transcription factor TFII-I (72) (Fig. 2). The SMRT/N-CoR complex also binds retinoblastoma tumor suppressor protein (RB) (68), transcriptional repressor NF-κB activating protein (NKAP) (73), and co-repressors myeloid translocation gene 8 (MTG8), MTG16,

MTGR1 (74, 75), and krab associated protein 1 (KAP-1) (76). HDAC3 primarily exists in complex with a multitude of core components and external binding partners. Identifying new or further characterizing existing interactions of the SMRT/N-CoR complex could lead to new therapeutic opportunities in disease treatment through the use of histone deacetylase inhibitors.

HDAC Crystal Structures

HDAC8 and HDAC2

Crystal structures offer some of the best information on catalytic mechanisms, amino acid surface accessibility, and interactions with other proteins. A multitude of HDAC8 crystal structures bound to different ligands have been published (77-81). HDAC8 is a structurally unique class 1 HDAC that does not exist as part of a multi-protein complex and is catalytically active when purified as a recombinant protein. More recently the crystal structure of HDAC2 bound to a ligand was published (82). This structure did not offer any information on potential binding of HDAC2 to other members of multi-protein complexes.

HDAC3

HDAC3 represents the third class 1 histone deacetylase to be crystallized. Unlike the other HDACs, HDAC3 was crystalized in complex with the deacetylase-activating domain (DAD) of SMRT/N-CoR (83). These studies revealed two unexpected aspects of the binding between these two key

components of the large multi-protein complexes. The DAD, when in complex with HDAC3, undergoes extensive rearrangements as compared to the previously published structure of the DAD alone (84). The N-terminus of the DAD lies along the surface of HDAC3 making numerous associations with the N-terminus of HDAC3 (amino acids 9-49) (83). This region of HDAC3 differs extensively from that of HDAC8, which does not bind a co-repressor. When in complex with HDAC3 the DAD of SMRT/N-CoR is in a different conformation than was originally shown by the crystal structure of the DAD alone.

Secondly and most unexpectedly, an inositol tetraphosphate (D-myo-inositol-1,4,5,6-tetrakisphosphate, Ins(1,4,5,6)P_4) was identified at the interface of HDAC3 and the DAD (83) (Fig. 2). This specific version of phosphorylated inositol is required for the interaction between HDAC3 and SMRT/N-CoR, making extensive contact with both proteins (His17, Gly21, Lys25, Arg265, Arg301 of HDAC3 and Lys449, Tyr470, Try471, Lys474, Lys475 of the DAD). HDAC3 enzymatic activity depends on Ins(1,4,5,6)P_4 bridging the gap between HDAC3 and the DAD. SMRT/N-CoR-Ins(1,4,5,6)P_4-Hdac3 complex formation appears necessary for a catalytically active co-repressor complex. Though early crystal structures of HDAC8 and HDAC2 offered insight into the general structure of HDACs, only the recent structure of HDAC3, in complex with the deacetylase activating domain of SMRT/N-CoR, offers insight into HDACs in complex with other proteins (83).

HDAC3 Functional Domains

The Ins(1,4,5,6)P_4 interacting region is just one of a number of key HDAC3 functional and regulatory regions. HDAC3 is a 428 amino acid protein that shares 53% identity with HDAC1, 52% with HDAC2, and 34% with HDAC8. Over half of HDAC3 consists of the highly conserved histone deacetylase domain (amino acids 22-316) that is the key feature of all HDAC proteins (85) (Fig. 3).

Phosphorylation

The unique HDAC3 C-terminus previously mentioned contains a single serine phosphorylation site at amino acid 424 (86) (Fig. 3). Phosphorylation at this site by casein kinase 2 (CK2) is necessary for *in vitro* deacetylation of hyperacetylated core histones from HeLa cells. The N-terminus of HDAC3 (amino acids 1-122) interacts with protein serine/threonine phosphatase 4 (PP4) (Fig. 3). Dephosphorylation of serine 424 by PP4 down-regulates HDAC3 activity. Unexpectedly, while phosphorylation status at this key residue impacts histone deacetylase activity, no difference is seen in the ability of phosphorylated versus nonphosphorylated HDAC3 to associate with N-CoR. No proteins have been identified that selectively interact with phosphorylated HDAC3. Though this is the only identified post-translational modification of HDAC3, the full impact of phosphorylation at serine 424 is yet to be elucidated (86).

Cytoplasmic Localization

In addition to a distinct C-terminal region, HDAC3 is unique among class 1 HDACs in its ability to localize to the cytoplasm (87). A nuclear export signal (NES) (amino acids 180-313) in combination with the CRM1/exportin 1- related export pathway shuttles HDAC3 out of the nucleus (34) (Fig. 3). The effects of cytoplasmic HDAC3 are not well understood. Though HDAC3 has this unique ability, like all other class 1 HDACs, it is primarily localized to the nucleus. A nuclear localization signal (NLS) (amino acids 313-428) assures that HDAC3 is localized to the nucleus and can deacetylate histones and non-histone proteins (34) (Fig. 3).

RelA Binding

One of the direct targets of HDAC3 in the nucleus is RelA. This protein represents one of the only known non-histone targets of HDAC3. The N-terminus of HDAC3 (amino acids 1-45) physically binds the RelA subunit of the nuclear factor-κB (NF-κB) transcription factor (88) (Fig. 3). Deacetylation of RelA by HDAC3 promotes effective binding of IκBα inhibitory proteins to the NF-κB complex and subsequent sequestration of NF-κB to the cytoplasm. This nuclear export of NF-κB terminates target gene transcription. Not only does HDAC3 promote transcriptional repression through deacetylation of histones, it also promotes repression through deacetylation of RelA (88). Though HDAC3 is a member of class 1 HDACs, it has a number of features not shared with any other class 1 enzyme.

Summary

Histone deacetylases regulate transcription, replication, recombination, and repair through modulation of chromatin structure. HDAC3 represents a unique member of the HDAC family of proteins that functions in a multitude of cellular processes. Loss of *Hdac3* leads to changes in cell cycle progression, decreased chromatin compaction, increased genomic instability, decreased rates of DNA repair, and ultimately cancer. HDAC3 is subject to regulation through phosphorylation and SMRT/N-CoR binding. In addition, many proteins interact with HDAC3 but the localization and full impact of most of these interactions is not understood. The elucidation of an HDAC3 crystal structure provides a better visualization of the cell surface. Therefore, this structure should be used as a tool to evaluate how the structure of HDAC3 impacts function.

CHAPTER II

MATERIALS AND METHODS

Plasmids

To allow viral expression in complementation experiments Flag tagged HDAC3 was sub-cloned into a murine stem cell virus (MSCV) vector that expresses green fluorescent protein (GFP) driven by an internal ribosome entry site (IRES). PCR was used to integrate Xho1 sites on either end of wild type Flag-HDAC3. The PCR amplified wild type Flag-HDAC3 and MSCV vector were cut with Xho1 and ligated together. The correct orientation was verified by diagnostic digest using EcoR1 and BglII. Quick change II XL (Aligent Technologies) was used to introduce point mutants into HDAC3 while in the MSCV-Flag-HDAC3 vector (Table 1). Two sequential rounds of site directed mutagenesis were necessary to create some of the final mutants. In the case of the H17C,G21A,K25I,R264P,L265M,R301A mutant, the MSCV-Flag-Hdac3 H17C,G21A,K25I and MSCV-Flag-Hdac3 R264P,L265M,R301A constructs were cut with EcoR1 and BglII and the two opposite fragments ligated together. The VSV-G vector, that expresses pantropic (VSV-G) envelope proteins from the CMV promoter, was used to allow packaging of virus in complementation experiments (89). All constructs were verified through Sanger sequencing performed at the Vanderbilt DNA sequencing facility (Table 2 and 3).

Table 1. Mutagenesis Primers
HDAC3 bases (black), mutations (red), and extra bases (green)

Primer Name		Sequences (5' ➔ 3')
Y298H	FOR	CTGGGTGGTGGTGGTCATACTGTCCGAAATGTT
Y298H	REV	AACATTTCGGACAGTATGACCACCACCACCCAG
S424A	FOR	TGACAATGACAAGGAAGCCGATGTGGAGATTTAAG
S424A	REV	CTTAAATCTCCACATCGGCTTCCTTGTCATTGTCA
S424D	FOR	CAATGACAAGGAAGACGATGTGGAGATTTAAGAGTG
S424D	REV	CACTCTTAAATCTCCACATCGTCTTCCTTGTCATTG
P11G,F16Y,H17Y	FOR	TTCTACGACGGCGACGTGGGCAACTACTACTACGGAGCTGG
P11G,F16Y,H17Y	REV	CCAGCTCCGTAGTAGTAGTTGCCCACGTCGCCGTCGTAGAA
H38N, K43R	FOR	AGCCTGGTCCTGAATTACGGTCTCTATAGGAAGATGATCGTC
H38N, K43R	REV	GACGATCATCTTCCTATAGAGACCGTAATTCAGGACCAGGCT
F199 deletion	FOR	CAAATACGGAAATTAC()TTCCCTGGCACAGGTG
F199 deletion	REV	CACCTGTGCCAGGGAA()GTAATTTCCGTATTTG
I46S, F48S	FOR	CTCTATAAGAAGATGAGCGTCTCCAAGCCATACCAGGCC
I46S, F48S	REV	GGCCTGGTATGGCTTGGAGACGCTCATCTTCTTATAGAG
F88S	FOR	AAGAGTCTTAATGCCTCCAACGTAGGCGATGAC
F88S	REV	GTCATCGCCTACGTTGGAGGCATTAAGACTCTT
F139S	FOR	CACCATGCCAAGAAGTCTGAGGCCTCTGGCTTC
F139S	REV	GAAGCCAGAGGCCTCAGACTTCTTGGCATGGTG
F246S	FOR	AACCAGGTAGTGGACTCCTACCAACCCACGTGC
F246S	REV	GCACGTGGGTTGGTAGGAGTCCACTACCTGGTT
F363S	FOR	GATCCGCCAGACAATCTCTGAAAACCTGAAGATGC
F363S	REV	GCATCTTCAGGTTTTCAGAGATTGTCTGGCGGATC
F329S, F336S	FOR	AGTGAATACTCCGAGTACTTTGCCCCAGACTCCACACTTCAT
F329S, F336S	REV	ATGAAGTGTGGAGTCTGGGGCAAAGTACTCGGAGTATTCACT
L265M	FOR	GGGCTGTGATCGAATGGGCTGCTTTAACC
L265M	REV	GGTTAAAGCAGCCCATTCGATCACAGCCC
R264P, L265M	FOR	CTGGGCTGTGATCCAATGGGCTGCTTTAAC
R264P, L265M	REV	GTTAAAGCAGCCCATTGGATCACAGCCCAG
H17C,G21A,K25I	FOR	GGGCAACTTCTGCTACGGAGCTGCACACCCTATGATCCCCCATCGCC
H17C,G21A,K25I	REV	GGCGATGGGGGATCATAGGGTGTGCAGCTCCGTAGCAGAAGTTGCCC
R301A	FOR	GTGGTTATACTGTCGCAAATGTTGCCCGCTG
R301A	REV	CAGCGGGCAACATTTGCGACAGTATAACCAC
HDAC3 to MSCV	FOR	AAAAACTCGAGGGTACCATGGACTACAAGGACGAC
HDAC3 to MSCV	REV	AAAAACTCGAGAGAGGTAAAAGAAATTCCTTGGGAC

Table 2. Sequencing primers

Primer Name		Sequences (5' ➔ 3')
MSCV	FOR	CCTTGAACCTCCTCGTTCGACC
MSCV	REV	CCAAGCGGCTTCGGCCAGTAACG
HDAC3 INTERNAL	FOR	GAGAGTGGCCGCTACTACTGT
HDAC3 INTERNAL	FOR	GAGTACTTTGCCCCAGACTTCA
HDAC3 INTERNAL	REV	GTAACTCTGGTCATCAAATGCCA
HDAC3 INTERNAL	REV	GATTGTCTGGCGGATCTGGTC

Table 3. Plasmids

Plasmid	Mutation
MSCV	-
MSCV-Flag-Hdac3	-
MSCV-Flag-Hdac3	Y298H
MSCV-Flag-Hdac3	424A
MSCV-Flag-Hdac3	424D
MSCV-Flag-Hdac3	P11G, F16Y, H17Y
MSCV-Flag-Hdac3	H38N, K43R
MSCV-Flag-Hdac3	F199 deletion
MSCV-Flag-Hdac3	I46S, F48S
MSCV-Flag-Hdac3	F88S, F139S
MSCV-Flag-Hdac3	F246S, F363S
MSCV-Flag-Hdac3	F329S, F336S
MSCV-Flag-Hdac3	L265M
MSCV-Flag-Hdac3	R264P, L265M
MSCV-Flag-Hdac3	H17C, G21A, K25I
MSCV-Flag-Hdac3	H17C, G21A, K25I, R264P, L265M, R301A
VSV-G	-

Cell culture

NIH 3T3 cells were cultured in dulbecco's modified eagle's medium (DMEM) (Cellgro) supplemented with 10% bovine calf serum (Atlanta Biologicals), 50 µg/ml penicillin-streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro).

Generating *Hdac3*$^{-/-}$ NIH 3T3 Cells

An immortalized NIH 3T3 *Hdac3*$^{F/-}$ cell line was previously established from *Hdac3*$^{F/-}$ MEFs following the National Institutes of Health (NIH) 3T3 regimen (38, 58). *Hdac3*$^{-/-}$ NIH 3T3 cells were obtained following infection of *Hdac3*$^{F/-}$ NIH 3T3 cells with recombinant adenovirus expressing the *Cre recombinase* (*Ad-Cre*). The day before *Ad-cre* infection *Hdac3*$^{F/-}$ NIH 3T3 cells were seeded at 5 x 10^5 cells/10 cm dish. Cells were incubated with *Ad-Cre* (Ad5-CMV-Cre obtained from the Vector Development Lab, Baylor University, 2 x 10^5 particles/cell in 3 mls media) for 3 hrs. Deletion was confirmed through western blot analysis three days post infection.

Complementation

The day before transfection with MSCV constructs, GP2-293 cells were seeded at 1.2 x 10^6 cells/6cm dish. Cells were co-transfected using Polyfect (Qiagen) with 4 µg VSV-G and MSCV constructs at a 1:3 ratio. A second plating and MSCV construct transfection was performed the following day. Cells were permitted to produce MSCV virus for three days. The day before MSCV infection

Hdac3$^{-/-}$ NIH 3T3 cells were seeded at 1 x 10^5 cells/6 cm dish. For MSCV infection, media was collected from packaging cells, filtered through a 0.45-μm cellulose acetate filter, and added to *Hdac3*$^{-/-}$ NIH 3T3 cells. To aid MSCV infection, polybrene was added to the viral media at a final concentration of 4 μg/ml. A second MSCV infection was performed the following day with fresh media being added 1 day after the second MSCV infection. Three days after the second MSCV infection the percent of *Hdac3*$^{-/-}$ NIH 3T3 cells expressing GFP was assessed on a FACS Calibur flow cytometer using Cell Quest Pro software (BD). Only conditions in which the percent of cells expressing GFP was ≥80% were permitted to continue. Greater than 90% GFP expression was often achieved. *Hdac3*$^{-/-}$ NIH 3T3 cells expressing GFP were seeded at 1 x 10^5 cells/10 cm dish. Two days later cells were trypsinized, counted using the Countess automated cell counter (Invitrogen), and replated at 1 x 10^5 cells/10 cm dish. This was repeated two additional times. Expression of Flag tagged HDAC3 constructs was confirmed through western blot (data not shown).

To visualize exponential cellular growth the following equation was used

((the current days cell count) X (the pervious days cellular dilution)) +

(the previous days total cell number) = (the current days total cell number)

Immunoblotting

Whole cell lysates, for detection of Hdac3 levels, were prepared using RIPA (radio immunoprecipitation assay) buffer and resolved by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). For immunoblotting,

samples were transferred onto nitrocellulose membranes and probed with the appropriate primary antibodies. Proteins were visualized using LI-COR Biosciences IRDye secondary antibodies combined with the Odyssey detection system (LI-COR). The antibody for Actin (A2066) was purchased from Sigma. The antibody for HDAC3 (ab16047) was purchased from Abcam.

CHAPTER III

THE CATALYTIC ACTIVITY OF HDAC3 IS NECESSARY FOR A NORMAL RATE OF CELLULAR PROLIFERATION

Introduction

Histone deacetylase inhibitors are increasingly being tested as therapy for a variety of diseases, most notably solid and hematological cancers. The main advantage of HDIs is their relatively low toxicity compared to other therapies, targeting cancerous cells more vigorously than normal cells. One of the hallmarks of cancerous cells is their ability to divide and grow uncontrollably, bypassing the mechanisms cells have in place to limit growth and proliferation. I looked to understand the mechanism by which HDAC3 regulates cellular proliferation through a structure/function approach. The *Hdac3*$^{-/-}$ NIH 3T3 cell system we have established gave me a unique opportunity to alter the amino acid composition of HDAC3 in cells lacking endogenous Hdac3. Point mutants were introduced into HDAC3 to elucidate the impact of histone deacetylase activity, HDAC3 phosphorylation, interactions with hydrophobic regions, RelA binding, and SMRT/N-CoR binding on cellular proliferation.

Results

Deletion of *Hdac3* in NIH 3T3 cells

In the absence of Hdac3 our line of immortalized NIH 3T3 cells behaved similarly to cell lines in which HDAC3 is knocked down (38). Following treatment with adenovirus expressing the *Cre recombinase* (*Ad-Cre*) *Hdac3* is deleted from *Hdac3*$^{F/-}$ NIH 3T3 cells, generating cells lacking endogenous Hdac3. *Hdac3*$^{-/-}$ NIH 3T3 cells remain predominantly *Hdac3*-null, as visualized through western blot, for at least 14 days (Fig. 5). Following this period, Hdac3 expression begins to increase, eventually reaching levels detected in uninfected *Hdac3*$^{F/-}$ NIH 3T3 cells. It is clear that *Ad-Cre* infection of *Hdac3*$^{F/-}$ NIH 3T3 cells does not hit every cell and thus Hdac3 expression is maintained in a small population of cells, which out complete *Hdac3*-null cells over time. That said, the level of deletion achieved in our system does provide a sufficient window in which HDAC3 can be exogenously expressed in a predominately *Hdac3*$^{-/-}$ NIH 3T3 cell system.

Loss of *Hdac3* decreases growth rate

The predominant phenotype observed upon *Hdac3* deletion in NIH 3T3 cells was a decrease in growth rate as compared to controls (Fig. 6), suggesting that *Hdac3* is necessary for a normal rate of cellular proliferation. I looked to take advantage of this defect to identify structural elements and interactions of HDAC3 that impact growth rate.

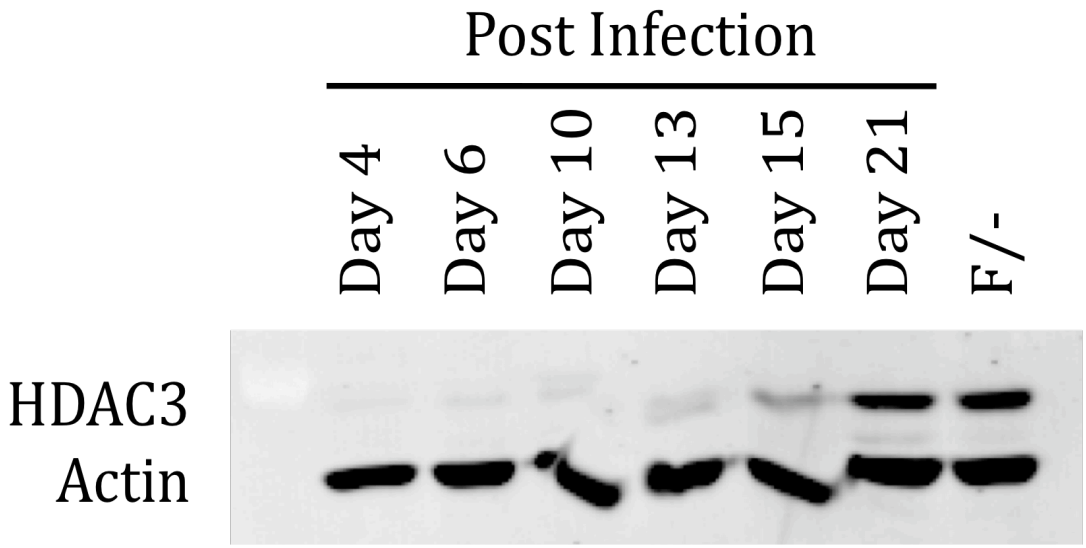


Figure 5. *Hdac3* deletion in NIH 3T3 cells. 5 x 10^5 *Hdac3*$^{F/-}$ NIH 3T3 cells were infected with 2 x 10^5 particles/cell adenovirus expressing *Cre* recombinase. Whole cell lysates were prepared from *Hdac3*$^{-/-}$ and control *Hdac3*$^{F/-}$ NIH 3T3 cells on the indicated days post infection. Samples were analyzed by SDS-PAGE and immunoblotted with antibodies to HDAC3 and actin. *Hdac3*$^{F/-}$ NIH 3T3 cells (F/-)

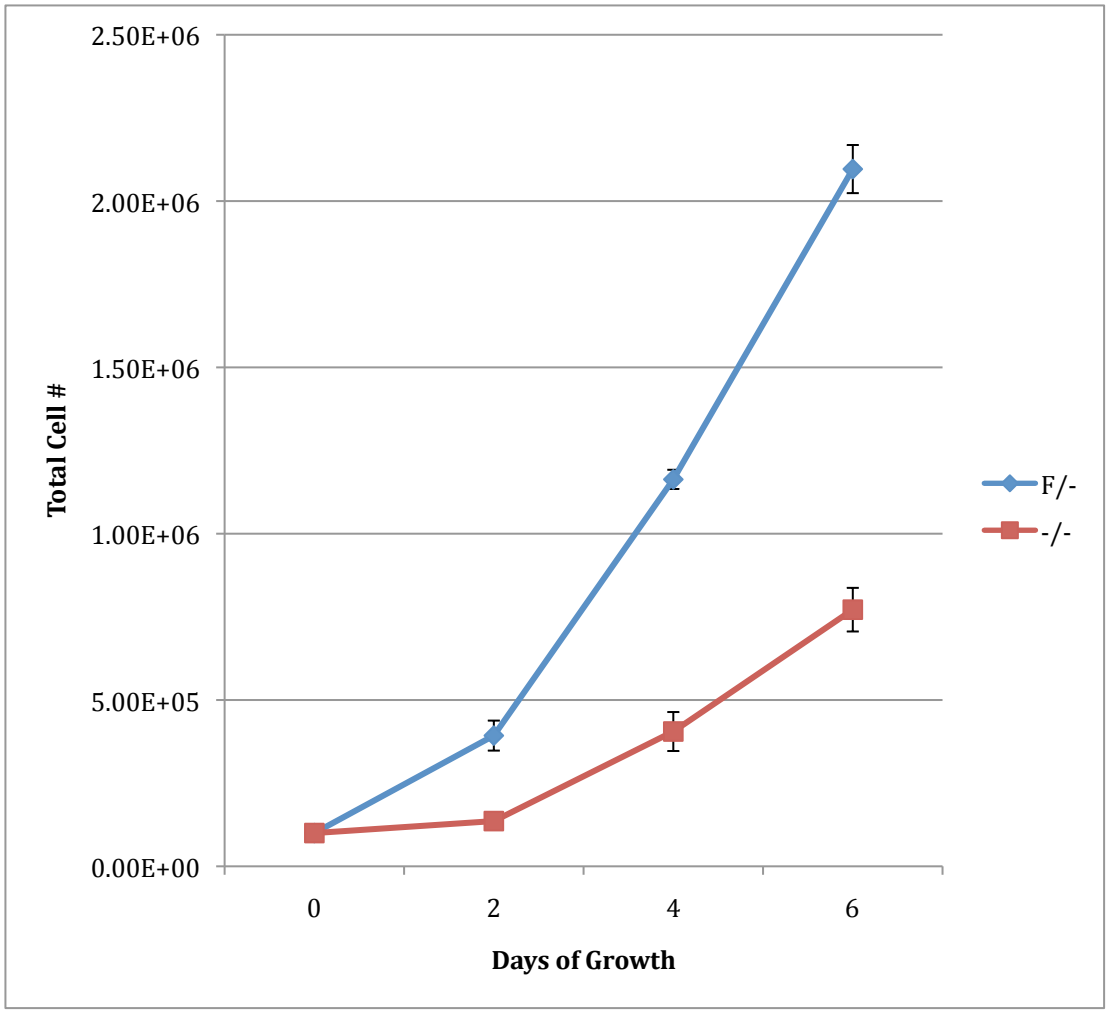


Figure 6. Proliferation defect upon *Hdac3* deletion. *Hdac3*$^{F/-}$ NIH 3T3 cells (F/-) and *Hdac3*$^{-/-}$ NIH 3T3 cells (-/-). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

I first confirmed that re-expression of HDAC3 in *Hdac3*$^{-/-}$ NIH 3T3 cells would complement the growth defect. Using the murine stem cell virus (MSCV) retrovirus expression vector, that also expresses green fluorescent protein (GFP), I infected *Hdac3*$^{-/-}$ NIH 3T3 cells with Flag tagged HDAC3 expressing or empty vector control virus. The percent of *Hdac3*$^{-/-}$ NIH 3T3 cells expressing GFP, and thus HDAC3, was assessed through flow cytometry (Fig 7). To allow comparison between independent experiments and ensure significant HDAC3 expression, only conditions in which the percent of cells expressing GFP was ≥80% were analyzed. Moreover, *Hdac3*$^{-/-}$ NIH 3T3 cells expressing HDAC3 were trypsinized, counted, and re-plated at a 1 x 10^5 cells/10 cm dish every two days to avoid contact inhibition of growth. Expression of HDAC3 led to an increase in the rate of proliferation, as compared to the control MSCV alone (Fig. 8). Thus, the defect in cell growth is due to the loss of *Hdac3* expression and can be complemented by expression of HDAC3.

Catalytic activity of HDAC3 is necessary for a normal growth rate

HDAC3 histone deacetylase activity is central to the role of HDAC3 in regulating transcription, replication, recombination, and repair. Therefore, I set out to determine if HDAC3 catalytic activity is necessary for the mechanism by which HDAC3 regulates cellular proliferation. Through sequence alignment of various histone deacetylases, it was determined that in the catalytic pocket of the inactive class 2a HDACs there is a distinctive histidine (H) while in class 1 and 4 HDACs this amino acid is a tyrosine (Y). A tyrosine to histidine mutation of

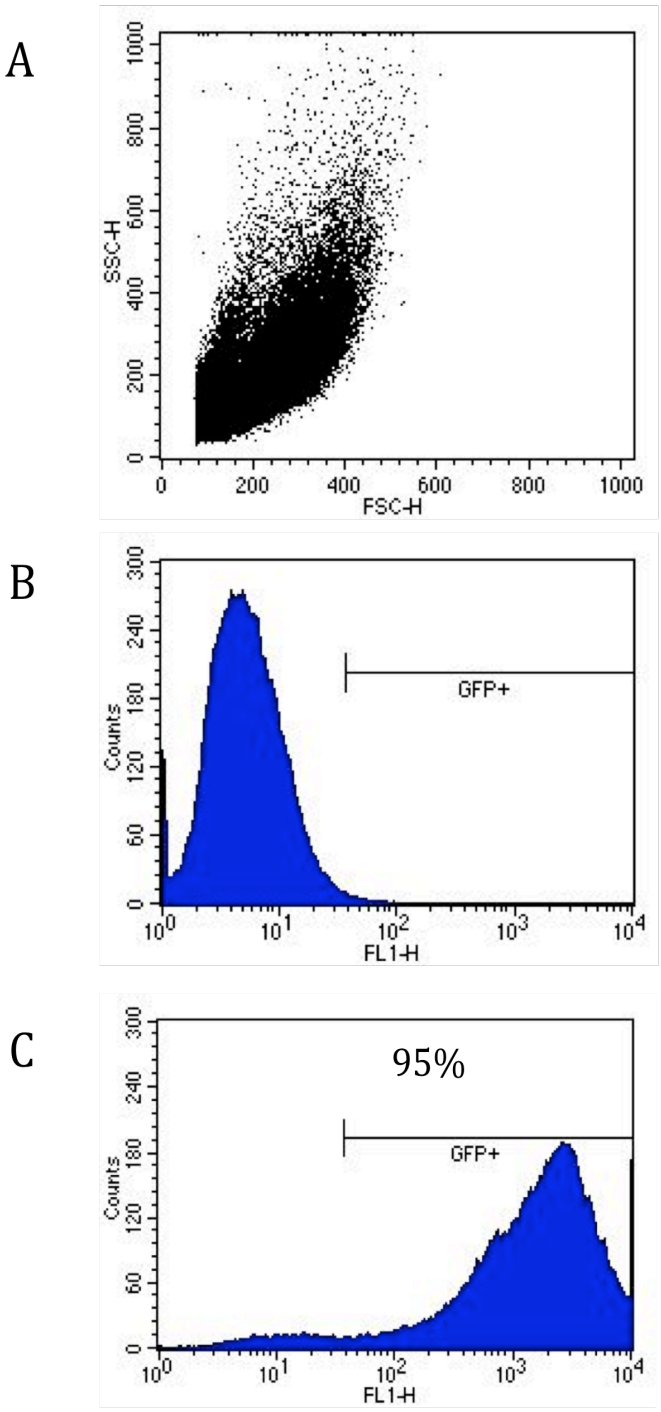


Figure 7. GFP expression correlates with HDAC3 expression in infected *Hdac3*$^{-/-}$ NIH 3T3 cells. A. Dot plot of *Hdac3*$^{-/-}$ NIH 3T3 cells analyzed through flow cytometry. **B.** Uninfected control *Hdac3*$^{-/-}$ NIH 3T3 cells do not express GFP. **C.** ≥80% of infected *Hdac3*$^{-/-}$ NIH 3T3 cells express GFP.

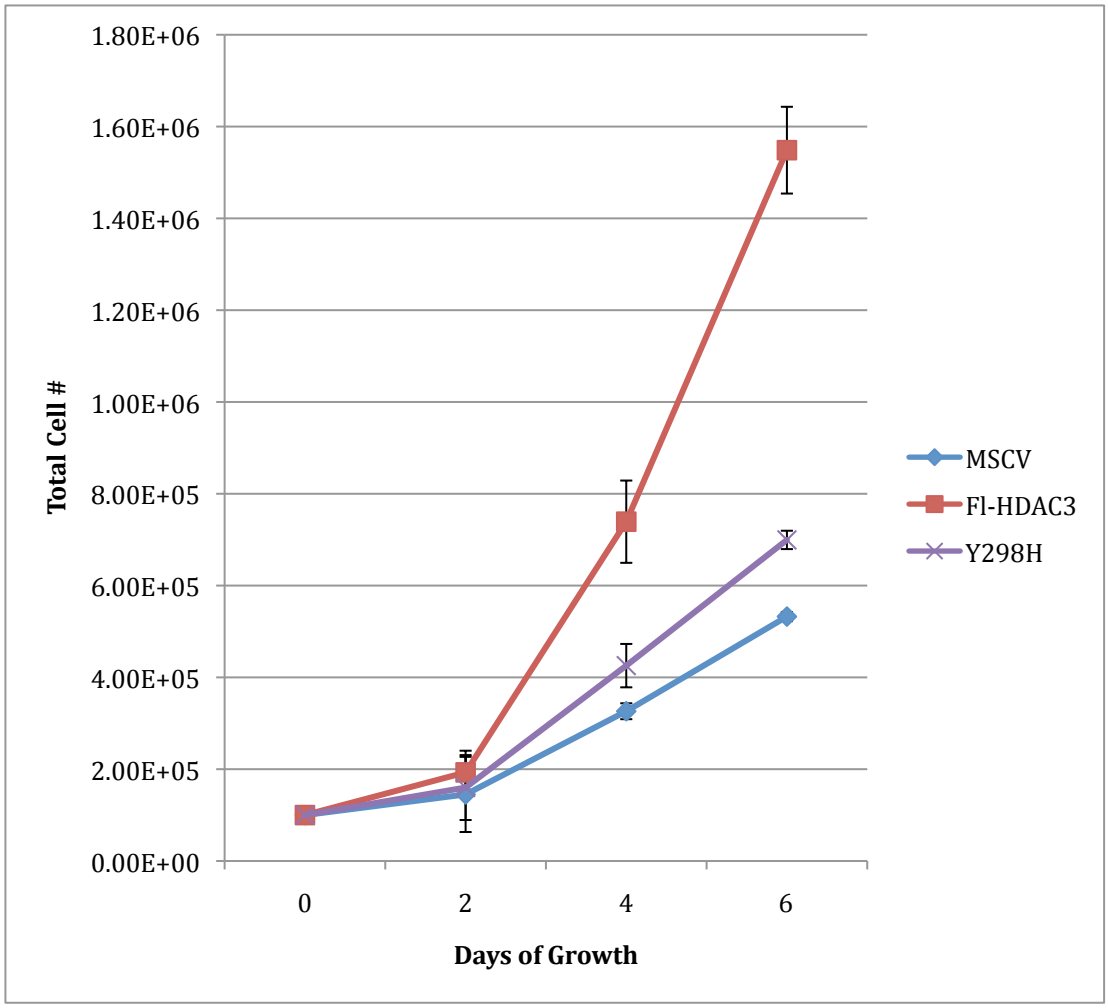


Figure 8. Catalytic activity of HDAC3 is necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), and catalytically inactive mutant HDAC3 (Y298H). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

HDAC3 amino acid 298 abolished the *in vitro* deacetylase activity of HDAC3 towards an acetylated histone H4 peptide and core histones (27). The Y298H substitution did not affect the association of HDAC3 with the DAD of N-CoR, suggesting the amino acid substitution did not disturb the integrity of the co-repressor complex (27). Complementation of *Hdac3*$^{-/-}$ NIH 3T3 cells with Flag tagged HDAC3 containing the Y298H substitution did not complement the growth defect (Fig. 8). Therefore, HDAC3 deacetylase activity is required for a normal rate of cellular proliferation.

Phosphorylation of HDAC3 is not necessary for a normal growth rate

Amino acid serine 424 is a key HDAC3 phosphorylation site (86). *In vitro* studies identified phosphorylation of serine 424 as necessary for HDAC3 histone deacetylase activity (86). To explore if phosphorylation of serine 424 was necessary for the mechanism by which HDAC3 regulates cellular proliferation I created a pair of point mutants. First, serine 424 was mutated to aspartic acid (D), a phosphomimetic amino acid. The negative charge of the aspartic acid side chain resembles the negative charge of a phosphorylated amino acid. Second, serine 424 was mutated to alanine (A), a small amino acid with no reactive groups. Alanine cannot be phosphorylated and does not resemble a phosphorylated amino acid. I hypothesized that an aspartic acid mutant at amino acid 424 would result in constitutive HDAC3 activity, and would therefore complement the growth defect. At the same time, an alanine mutant at amino acid 424 would not complement the growth defect due to a lack of catalytic

activity, as seen in the Y298H mutant. As expected, the S424D mutant did complement the growth defect, proliferating at a rate equivalent to *Hdac3*$^{-/-}$ NIH 3T3 cells expressing wild-type Flag tagged HDAC3 (Fig. 9). Unexpectedly, the S424A mutant also complemented the growth defect (Fig. 9). The fact that S424D and S424A mutants both proliferated at a rate equivalent to wild-type HDAC3 shows that the charge status at amino acid 424 does not affect growth rate. These data imply that *in vivo* the phosphorylation status of HDAC3 at serine 424 may not affect HDAC3 catalytic activity to the extent shown *in vitro*.

HDAC3 homology model

With a desire to identify key amino acids important for HDAC3-mediated changes in cellular proliferation, I set out to better understand the structure of HDAC3. First, the amino acid sequence of HDAC3 was run through metaPrDOS (meta protein disorder prediction system) to predict the level of disorder present in fully folded HDAC3 (Fig. 10). According to the prediction, the C-terminal 59 amino acids, which include the amino acids unique to HDAC3, would be unfolded giving no structural information. At the time these studies were started no crystal structure of HDAC3 was available. Multiple HDAC8 crystal structures had been published along with one HDAC2 structure (77-82). Though HDAC8 is a member of the class 1 HDACs it is not a member of a large multi-protein complex and thus is presumably not regulated in the same manner as HDAC3. The ClustalW sequence alignment software was used to align HDAC2 and 3, allowing visualization of identical and similar amino acids (Fig. 11). The 52% sequence

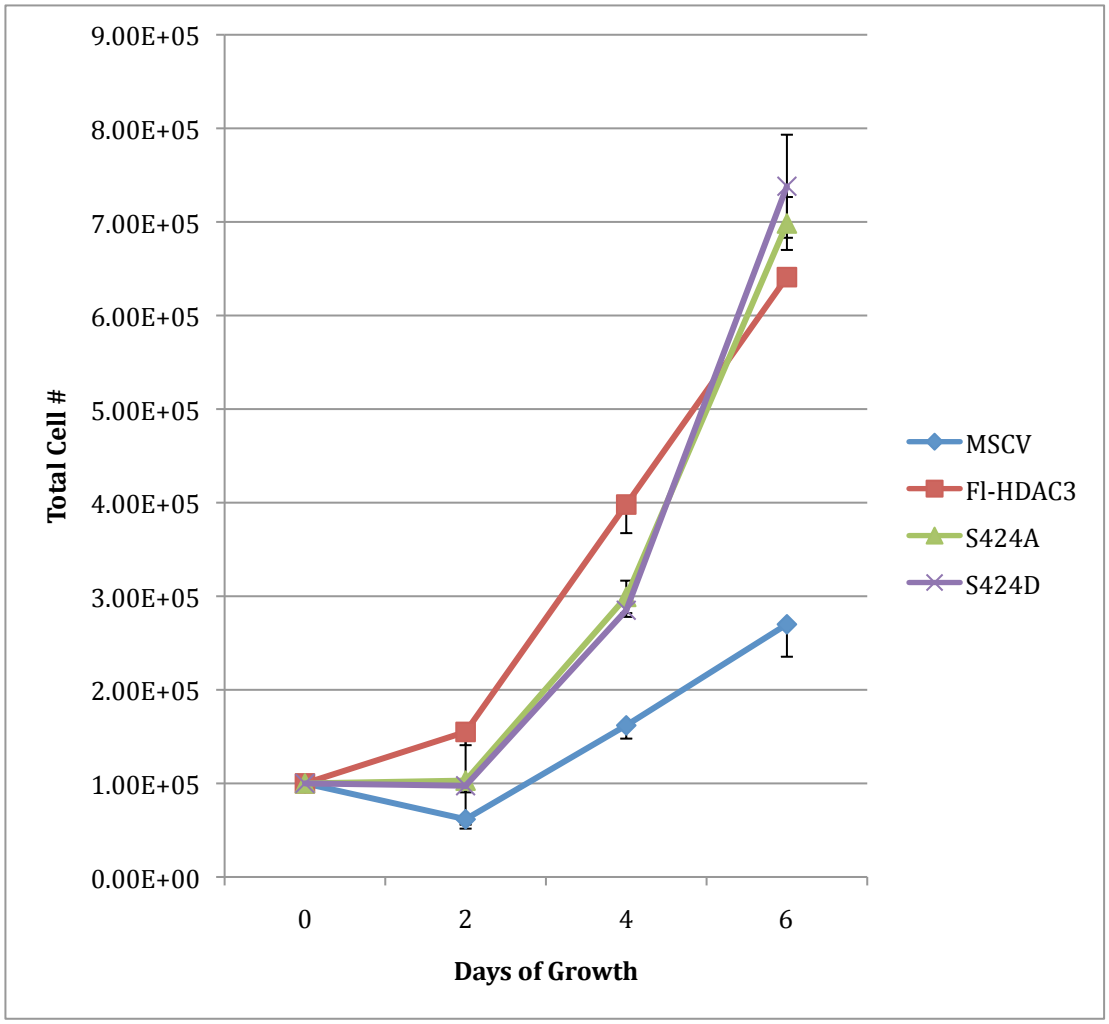
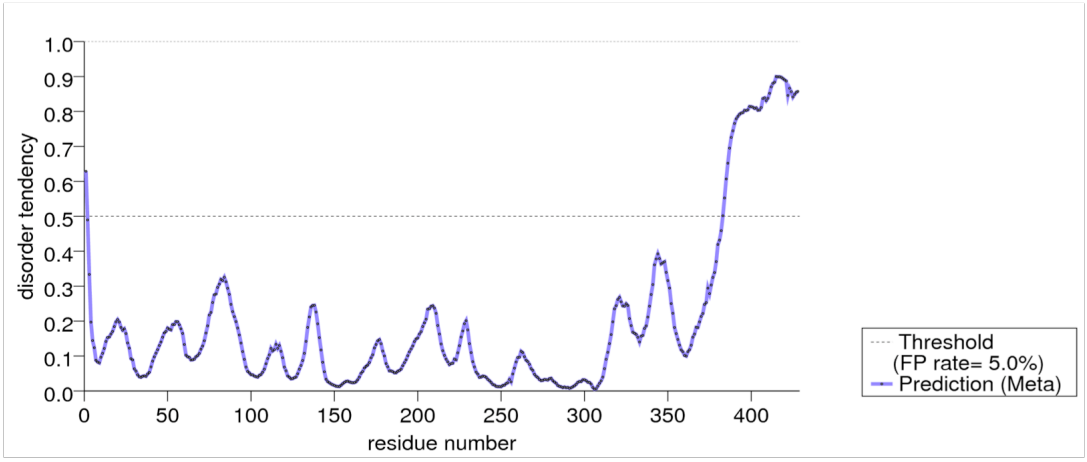


Figure 9. Phosphorylation of HDAC3 is not necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), an uncharged/non-reactive mutant at serine 424 (S424A), and a phosphomimetic mutant at serine 424 (S424D). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

A



B

1	MAKTVAYFYD	PDVGNFHYGA	GHPMKPHRLA	LTHSLVLHYG	LYKKMIVFKP	50
51	YQASQHDMCR	FHSEDYIDFL	QRVSPTNMQG	FTKSLNAFNV	GDDCPVFPGL	100
101	FEFCSRYTGA	SLQGATQLNN	KICDIAINWA	GGLHHAKKFE	ASGFCYVNDI	150
151	VIGILELLKY	HPRVLYIDID	IHHGDGVQEA	FYLTDRVMTV	SFHKYGNYFF	200
201	PGTGDMYEVG	AESGRYYCLN	VPLRDGIDDQ	SYKHLFQPVI	NQVVDFYQPT	250
251	CIVLQCGADS	LGCDRLGCFN	LSIRGHGECV	EYVKSFNIPL	LVLGGGGYTV	300
301	RNVARCWTYE	TSLLVEEAIS	EELPYSEYFE	YFAPDFTLHP	DVSTRIENQN	350
351	SRQYLDQIRQ	TIFENLKMLN	HAPSVQIHDV	PADLLTYDRT	DEADAEERGP	400
401	EENYSRPEAP	NEFYDGDHDN	DKESDVEI			450

Figure 10. Last 46 amino acids of HDAC3 are predicted to be disordered. A. MetaPrDOS protein disorder prediction for HDAC3. **B.** Amino acid sequence of HDAC3 with disordered amino acids shown in red.

Figure 11. HDAC3 is 52% identical and 58% similar to HDAC2.
ClustalW sequence alignment of HDAC3 and HDAC2.
A (*) (asterisk) indicates positions which have a single, fully conserved residue.
A (:) (colon) indicates conservation between groups of strongly similar properties.
A (.) (period) indicates conservation between groups of weakly similar properties.

identity (58% similarity) of HDAC2 made it the obvious choice to use to create an HDAC3 homology model. Using PyMOL a homology model was constructed using HDAC2 (PDB structure 3MAX (82)) as a basis for modeling. As predicted, one of the major limitations of this structure was an inability to visualize the last 59 amino acids of HDAC3. That said, with the homology model I was able to visualize the surface of HDAC3 and localize the active site.

With a desire to further understand dynamics at work on the protein surface I ran a script (color_h.py) in PyMOL designed to color amino acid side chains according to their level of hydrophobicity (Fig. 12). This script utilizes the normalized consensus hydrophobicity scale to determine what color to color an amino acid with white being hydrophilic and red being hydrophobic (90). This HDAC3 homology model represented the best structural information available.

Binding of SMRT/N-CoR and RelA to HDAC3 is not necessary for a normal growth rate

Using the final homology model, I designed a series of point mutants to determine HDAC3 regions important for the mechanism by which HDAC3 regulates cellular proliferation. First, mutations were made in the N-terminal regions that bind SMRT (amino acids 1-23) and RelA (amino acids 1-45) (Fig. 3). Failure to complement the growth defect with HDAC3 containing mutations in these regions, would allow easy identification of the binding partner(s) necessary for a normal growth rate. Amino acids prominently on the cell surface in the SMRT binding region (proline 11, phenylalanine 16, and histidine 17) and RelA

```
def color_h(selection="(all)"):
      s = str(selection)
         print s
      cmd.set_color('color_ile',[0.996,0.062,0.062])
      cmd.set_color('color_phe',[0.996,0.109,0.109])
      cmd.set_color('color_val',[0.992,0.156,0.156])
      cmd.set_color('color_leu',[0.992,0.207,0.207])
      cmd.set_color('color_trp',[0.992,0.254,0.254])
      cmd.set_color('color_met',[0.988,0.301,0.301])
      cmd.set_color('color_ala',[0.988,0.348,0.348])
      cmd.set_color('color_gly',[0.984,0.394,0.394])
      cmd.set_color('color_cys',[0.984,0.445,0.445])
      cmd.set_color('color_tyr',[0.984,0.492,0.492])
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      cmd.set_color('color_thr',[0.980,0.586,0.586])
      cmd.set_color('color_ser',[0.980,0.637,0.637])
      cmd.set_color('color_his',[0.977,0.684,0.684])
      cmd.set_color('color_glu',[0.977,0.730,0.730])
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      cmd.set_color('color_gln',[0.973,0.824,0.824])
      cmd.set_color('color_asp',[0.973,0.875,0.875])
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      cmd.color("color_phe","("+s+" and resn phe)")
      cmd.color("color_val","("+s+" and resn val)")
      cmd.color("color_leu","("+s+" and resn leu)")
      cmd.color("color_trp","("+s+" and resn trp)")
      cmd.color("color_met","("+s+" and resn met)")
      cmd.color("color_ala","("+s+" and resn ala)")
      cmd.color("color_gly","("+s+" and resn gly)")
      cmd.color("color_cys","("+s+" and resn cys)")
      cmd.color("color_tyr","("+s+" and resn tyr)")
      cmd.color("color_pro","("+s+" and resn pro)")
      cmd.color("color_thr","("+s+" and resn thr)")
      cmd.color("color_ser","("+s+" and resn ser)")
      cmd.color("color_his","("+s+" and resn his)")
      cmd.color("color_glu","("+s+" and resn glu)")
      cmd.color("color_asn","("+s+" and resn asn)")
      cmd.color("color_gln","("+s+" and resn gln)")
      cmd.color("color_asp","("+s+" and resn asp)")
      cmd.color("color_lys","("+s+" and resn lys)")
      cmd.color("color_arg","("+s+" and resn arg)")
cmd.extend('color_h',color_h)
```

Figure 12. Pymol script used to model hydrophobicity of amino acids

binding region (histidine 38 and lysine 43) were mutated simultaneously to their corresponding amino acids in HDAC2 (glycine 11, tyrosine 16, tyrosine 17 and asparagine 38, arginine 43) (Fig. 13). These mutations were chosen to preserve HDAC3 structural integrity while still preventing binding to SMRT and RelA, as HDAC2 does not bind either of these proteins.

The two mutants intended to eliminate binding to SMRT and RelA, confirmed binding partners of HDAC3, did complement the growth defect, proliferating at a rate equivalent to *Hdac3*$^{-/-}$ NIH 3T3 cells expressing wild-type Flag tagged HDAC3 (Fig. 14). This suggested that SMRT and RelA binding are not required for modulating the effects of HDAC3 on cellular proliferation. Given that SMRT/N-CoR binding to HDAC3 is required for activity and that, as shown in the Y298H mutant, catalytic activity is required for a normal rate of proliferation it was surprising that HDAC3 containing mutations in the SMRT binding region complemented the growth defect.

Hydrophobic regions of HDAC3 are not necessary for a normal growth rate

Next, mutations were made in HDAC3 hydrophobic regions visualized on the protein surface. Hydrophobic regions are primed for protein-protein interactions, as the amino acid side chains do not interact favorably with the hydrophilic environment. The amino acids in region 1 (isoleucine 46 and phenylalanine 48), region 2 (phenylalanine 88 and 139), region 3 (phenylalanine 246 and 363), and region 4 (phenylalanine 329 and 336) were simultaneously mutated to serine (S) (Fig. 15). The mid-sized polar amino acid serine was used

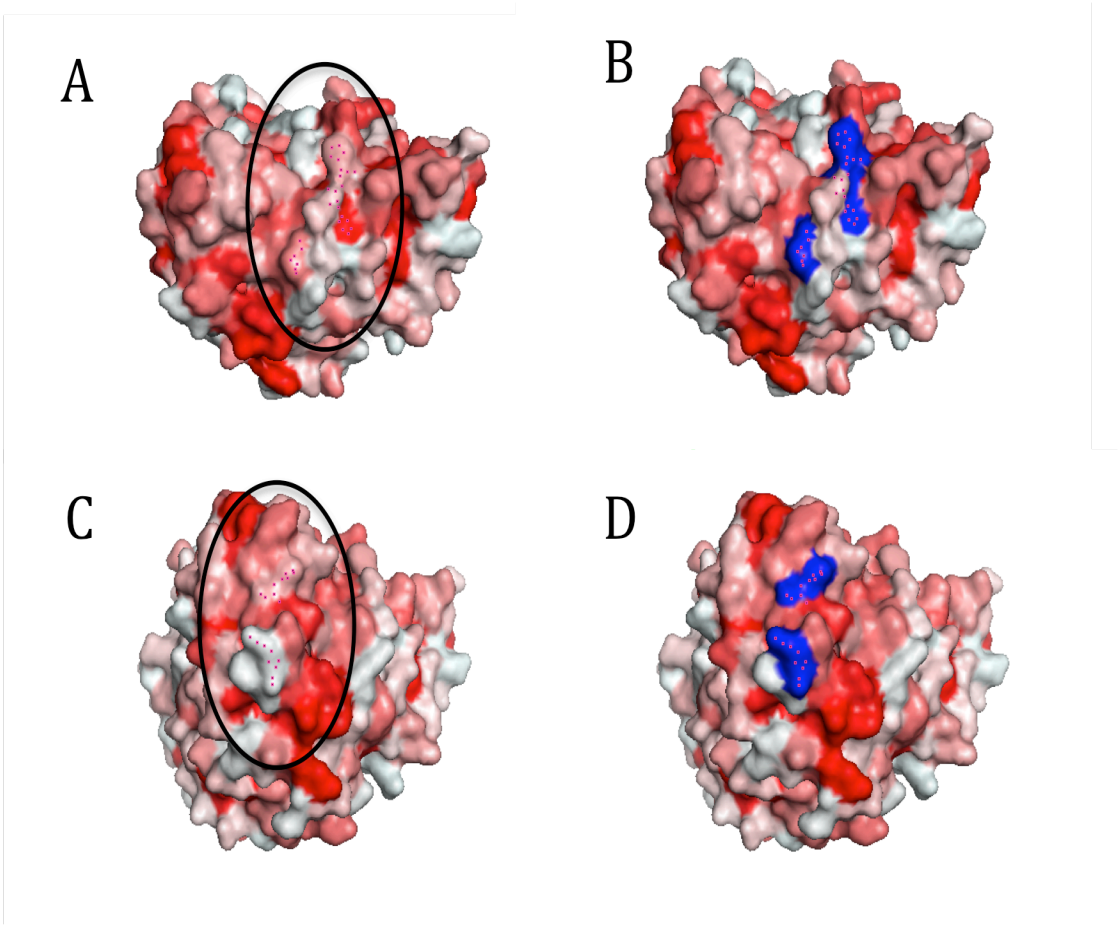


Figure 13. Protein surface hydrophobic amino acids predicted to bind SMRT/N-CoR and RelA. HDAC3 amino acids colored based on a spectrum of white being hydrophilic to red being hydrophobic. **A.** SMRT/N-CoR binding hydrophobic region. **B.** Amino acids P11, F16, H17 mutated to eliminate binding to SMRT/N-CoR shown in blue. **C.** RelA binding hydrophobic region. **D.** Amino acids H38, K43 mutated to eliminate binding to RelA shown in blue.

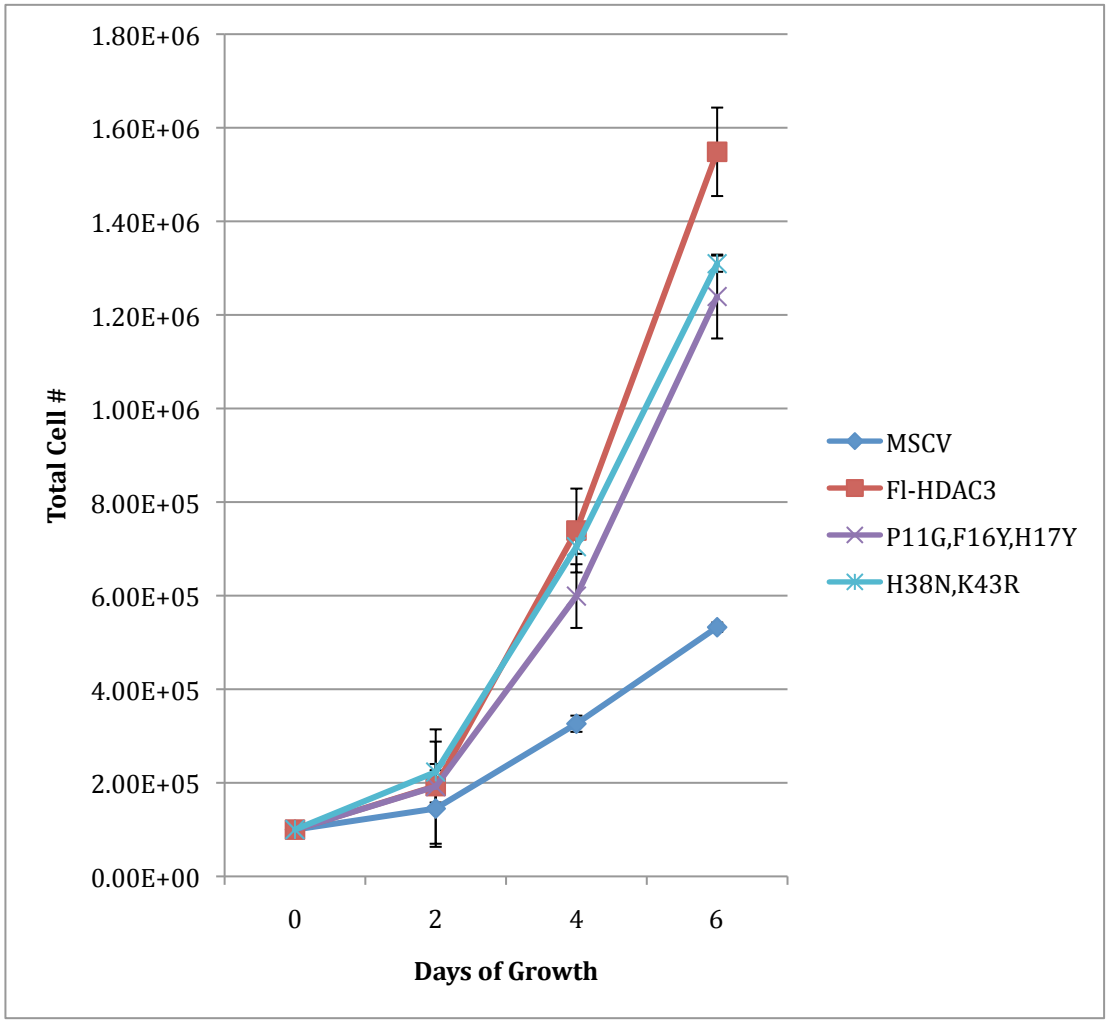


Figure 14. Binding of SMRT/N-CoR and RelA to HDAC3 is not necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), a mutant intended to eliminate binding to SMRT/N-CoR (P11G,F16Y,H17Y), and a mutant intended to eliminate binding to RelA (H38N, K43R). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

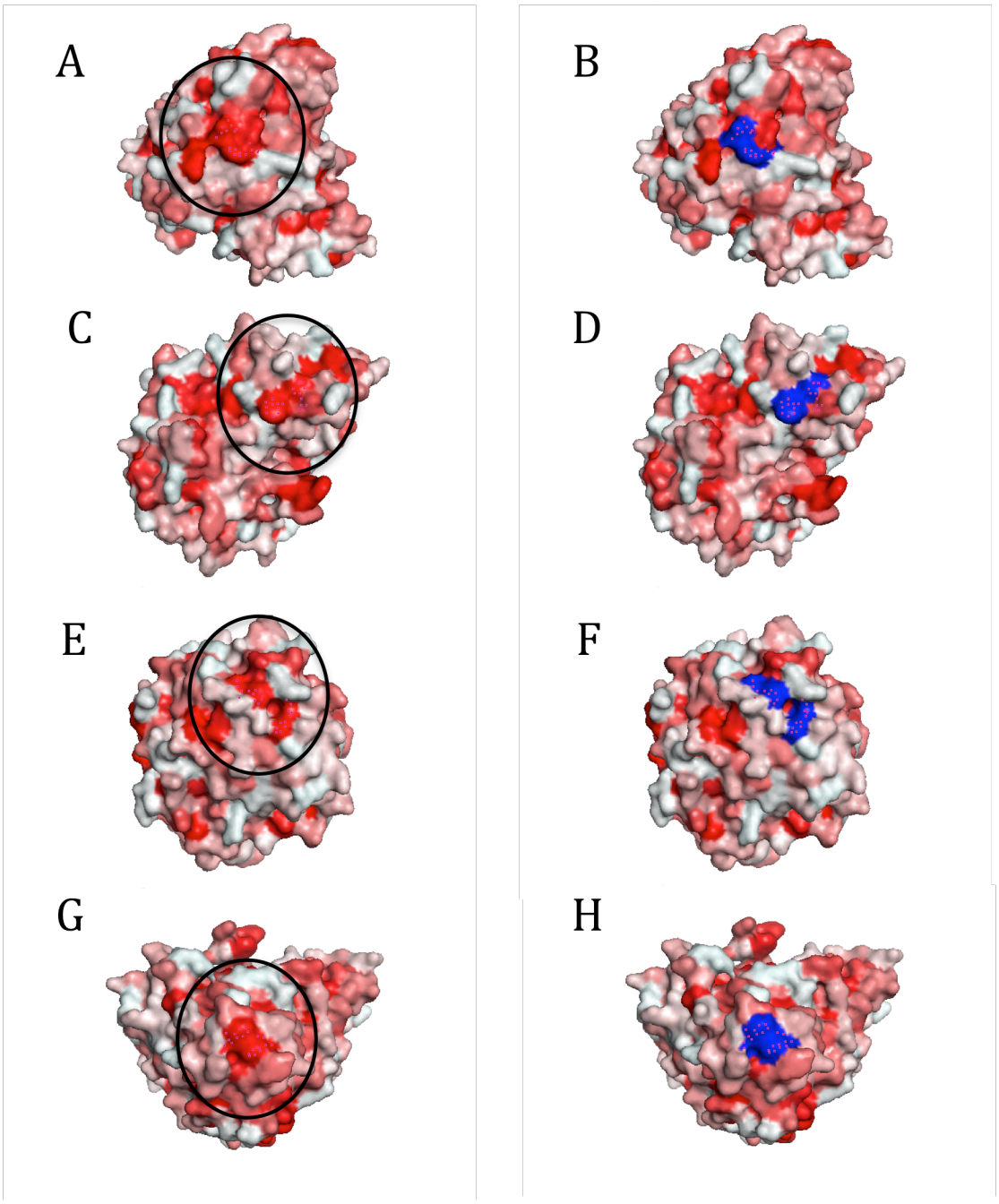


Figure 15. Protein surface hydrophobic amino acids predicted to be binding regions. HDAC3 amino acids colored based on a spectrum of white being hydrophilic to red being hydrophobic. **A.** Hydrophobic binding region 1. **B.** Amino acids I46, F48 mutated to eliminate binding shown in blue. **C.** Hydrophobic binding region 2. **D.** Amino acids F88, F139 mutated to eliminate binding shown in blue. **E.** Hydrophobic binding region 3. **F.** Amino acids F246, F363 mutated to eliminate binding shown in blue. **G.** Hydrophobic binding region 4. **H.** Amino acids F329, F336 mutated to eliminate binding shown in blue.

to minimize the structural impact of the point mutants while still affecting hydrophobicity.

All the HDAC3 mutants, containing polar amino acid substitutions in hydrophobic regions, complemented the growth defect, proliferating at a rate equivalent to or better than *Hdac3*$^{-/-}$ NIH 3T3 cells expressing wild-type Flag tagged HDAC3 (Fig. 16). Hydrophobicity in these four regions is not required for Hdac3 regulated cellular proliferation.

An intact "lid" of HDAC3 is not necessary for a normal growth rate

Finally, in analyzing the sequence alignment and structures of HDAC3 and HDAC2 it was determined that HDAC3 contains a phenylalanine (F) at amino acid 199 that is not present in HDAC2. This additional amino acid makes the putative "lid" that extends over the active site of HDAC3 larger than that seen in HDAC2. To make the HDAC3 "lid" look more like that of HDAC2, phenylalanine 199 was deleted (Fig. 17). Removal of F199 could alter the catalytic targets of HDAC3 leading to a failure to complement or could make HDAC3 constitutively active leading to a growth rate higher than wild-type.

The HDAC3 mutant containing a single amino acid deletion in the "lid" that extends over the active site complemented the growth defect, proliferating at a rate equivalent to *Hdac3*$^{-/-}$ NIH 3T3 cells expressing wild-type Flag tagged HDAC3 (Fig. 18). This suggests that either the deletion did not significantly alter the catalytic targets of HDAC3 or the "lid" does not impact the mechanism by which HDAC3 regulates cellular proliferation.

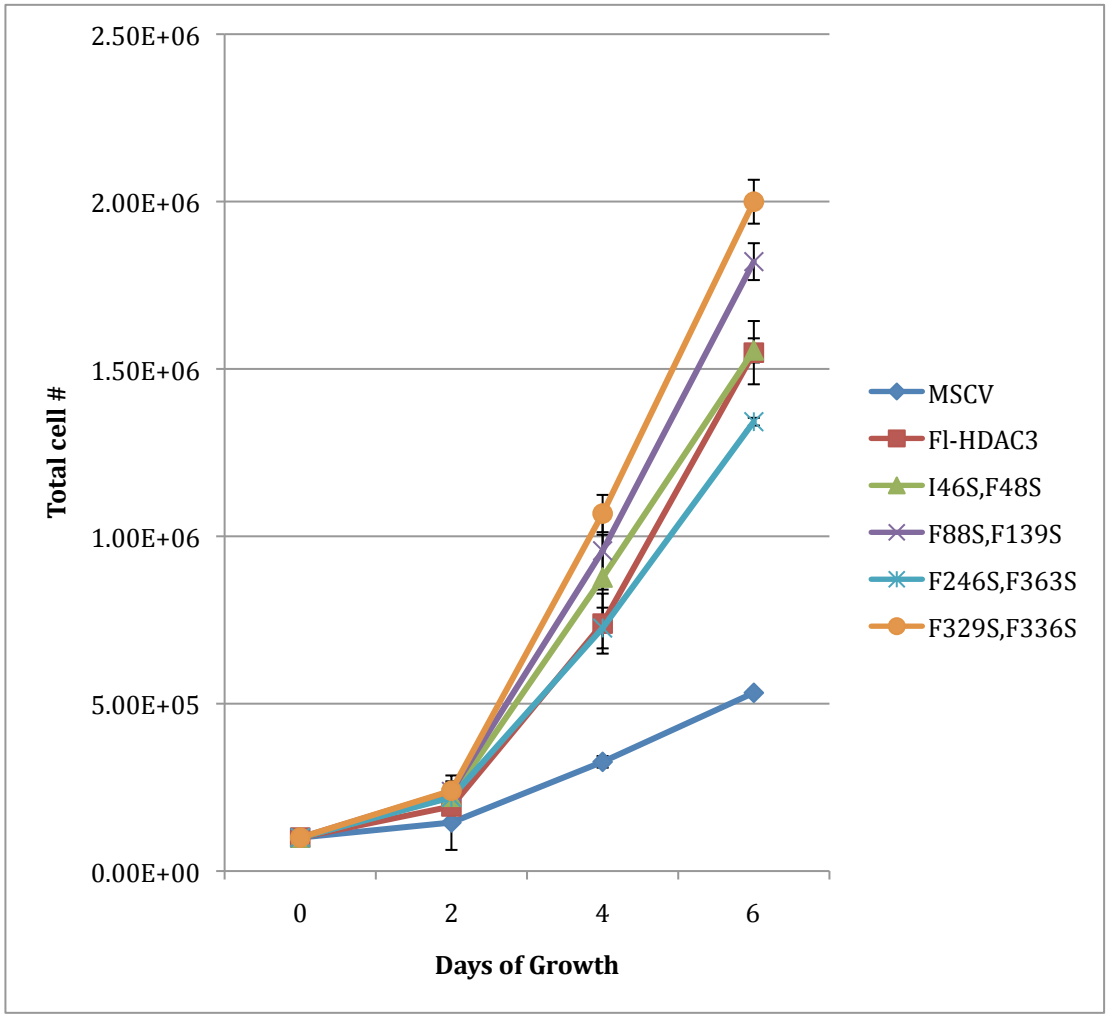


Figure 16. Protein surface hydrophobic regions are not necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), and mutants intended to eliminate binding to hydrophobic regions (I26S,F48S G88S,F139S F246S,F363S F329S,F336S). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

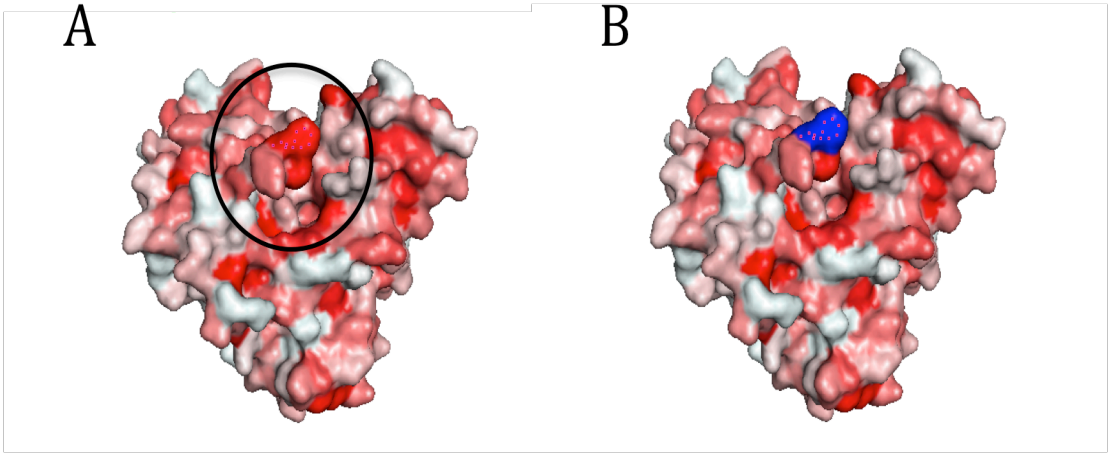


Figure 17. "Lid" amino acid of HDAC3 not present in HDAC2. HDAC3 amino acids colored based on a spectrum of white being hydrophilic to red being hydrophobic. **A.** "Lid" and active site of HDAC3. **B.** Amino acid F199 deleted to make the "lid" of HDAC3 look more like that of HDAC2 shown in blue.

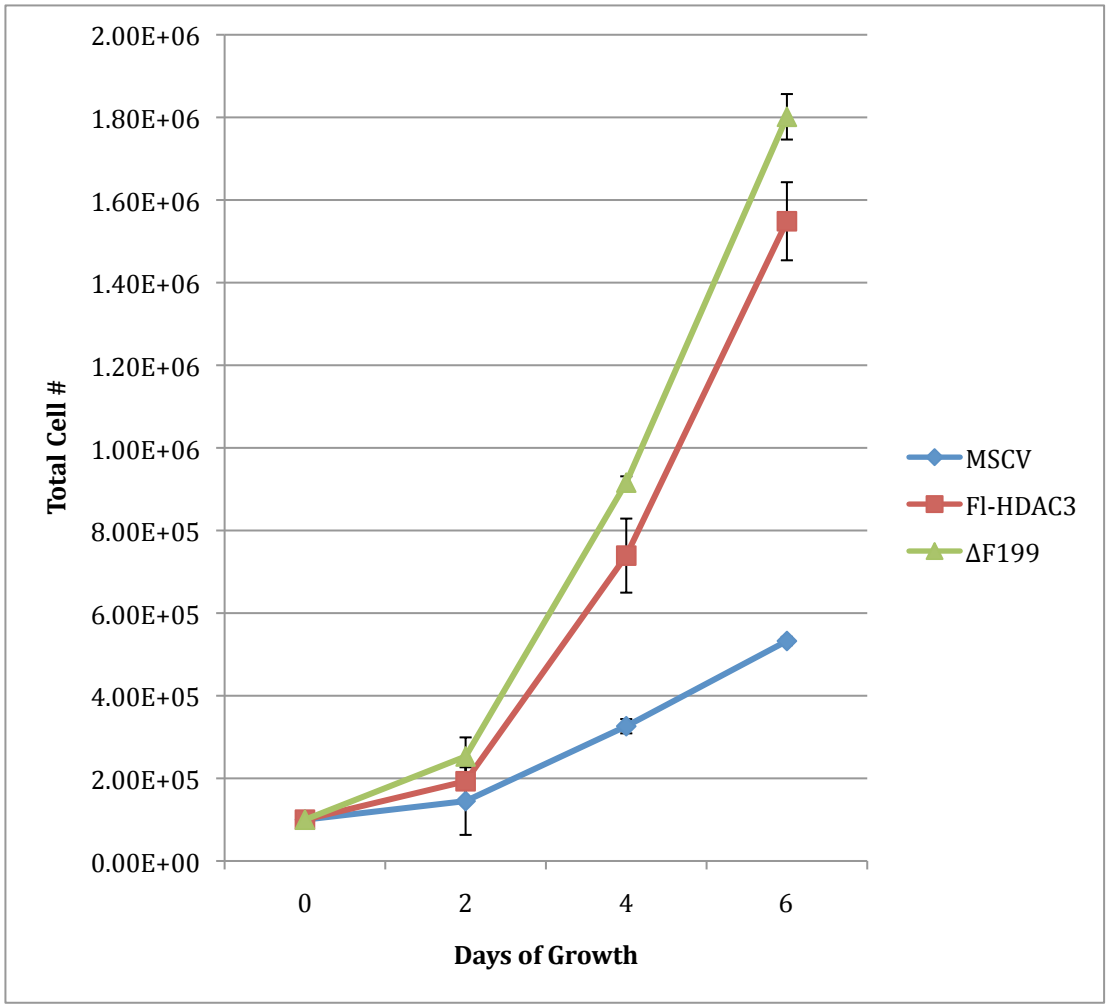


Figure 18. An intact "lid" of HDAC3 is not necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), and a mutant intended to make the "lid" of HDAC3 look more like that of HDAC2 (ΔF199). Following an initial plating at 1 x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

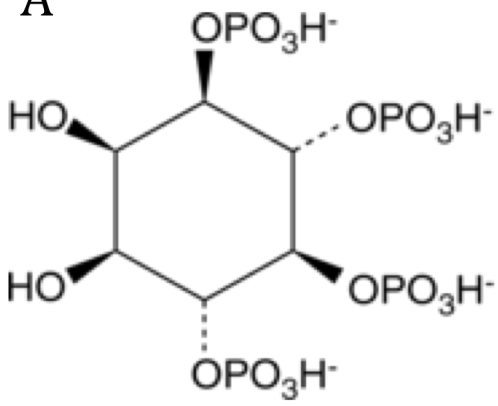
Binding of the SMRT/N-CoR deacetylase activating domain to HDAC3 is necessary for a normal growth rate

The recent publication of an HDAC3 crystal structure prompted further analysis of HDAC3 interactions (83). HDAC3 is seen in complex with not only the deacetylase activating domain (DAD) of SMRT/N-CoR but also an inositol tetraphosphate molecule [Ins(1,4,5,6)P_4] (Fig. 19A). This crystal structure provides a more accurate view of the HDAC3 three-dimensional structure than the HDAC3 homology model. Five HDAC3 amino acids were noted to contribute hydrogen bonds and salt bridges to Ins(1,4,5,6)P_4 (83) (Fig. 19B). Four combinations of HDAC3 point mutants resulted in loss of *in vitro* deacetylase activity and interaction with the SMRT-DAD (83). The published point mutants were recreated to explore if interaction with Ins(1,4,5,6)P_4 and subsequently the DAD of SMRT/N-CoR was necessary for the mechanism by which HDAC3 regulates cellular proliferation (Fig. 20). Amino acids in HDAC3: single loop 6 (leucine 265), double loop 6 (arginine 264 and leucine 265), loop 1 (histidine 17, glycine 21, lysine 25), and double loop 1+6 (histidine 17, glycine 21, lysine 25, arginine 264 leucine 265, and arginine 301) were simultaneously mutated to their corresponding amino acids in HDAC8 (Table 4).

Table 4. Crystal structure mutants

Location Name	Mutation(s)
Single Loop 6	L265M
Double Loop 6	R264P,L265M
Loop 1	H17C,G21A,K25I
Double Loop 1+6	H17C,G21A,K25I,R264P,L265M,R301A

A



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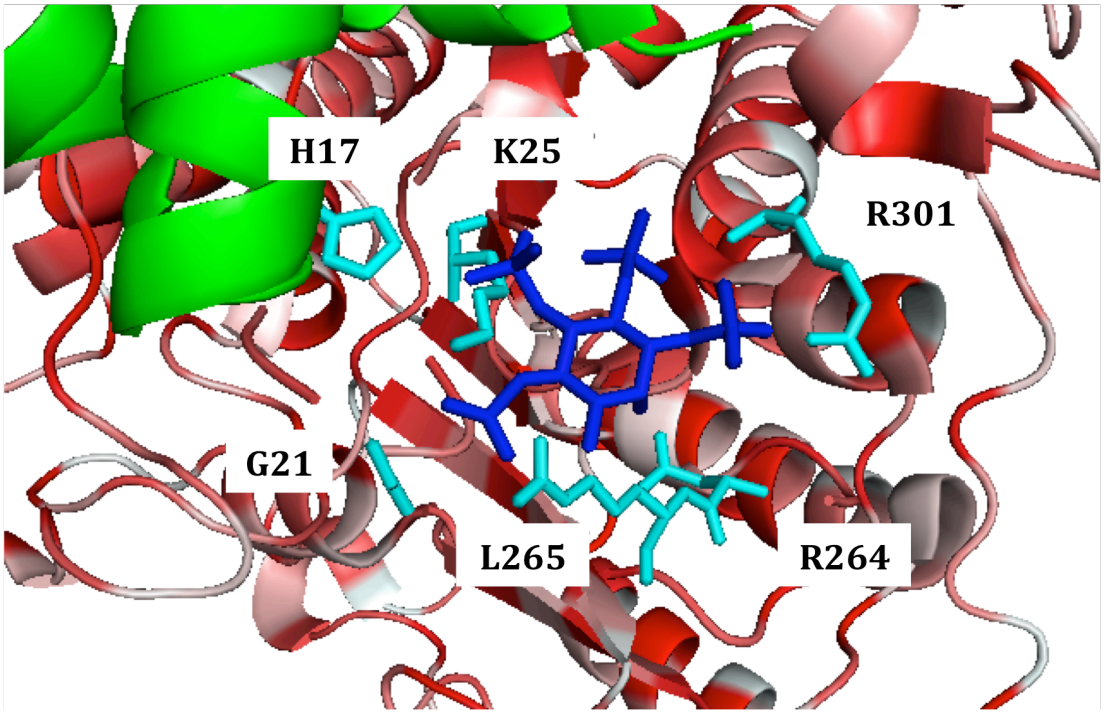

Figure 19. HDAC3 amino acids interacting with Ins(1,4,5,6)P_4. A. D-myo-inositol-(1,4,5,6)-tetrakisphosphate (Ins(1,4,5,6)P_4). **B.** Amino acids H17, G21, K25, R264, L265, R301 mutated to eliminate binding to (1,4,5,6)P_4 shown in cyan. Single loop 6 (L265), double loop 6 (R264,L265), loop 1 (H17,G21,K25), and double loop 1+6 (H17,G21,K25,R264,L265,R301). DAD of SMRT/N-CoR (green), Ins(1,4,5,6)P_4 (blue), and HDAC3 (spectrum of white to red).

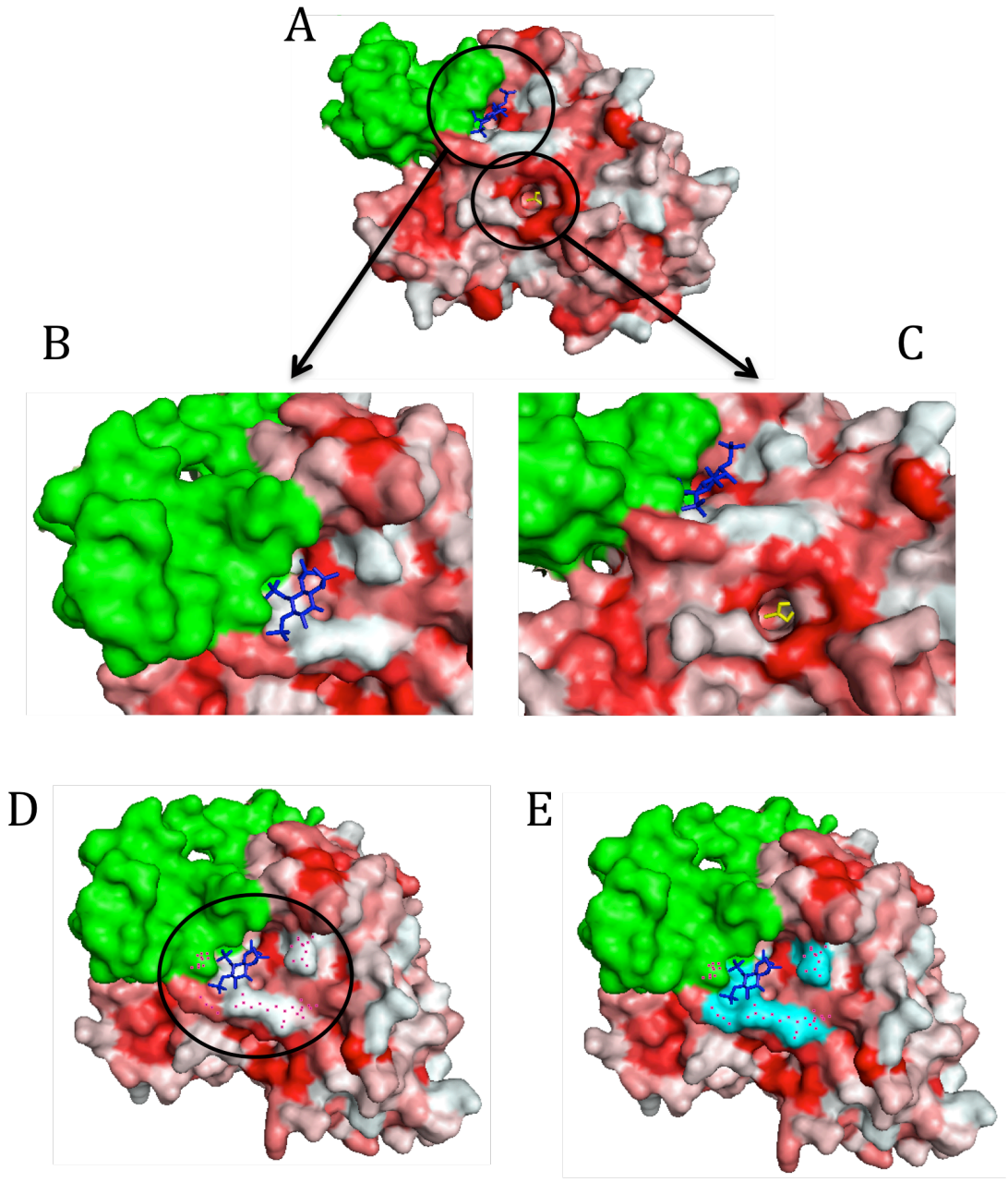

Figure 20. HDAC3 amino acids shown to interact with Ins(1,4,5,6)P_4 and mediate association with the DAD of SMRT/N-CoR. HDAC3 amino acids colored based on a spectrum of white being hydrophilic to red being hydrophobic. **A.** View of the active site with acetyl group (yellow) and the association between DAD of SMRT/N-CoR (green), Ins(1,4,5,6)P_4 (blue), and HDAC3 (spectrum of white to red). **B.** Inset of DAD, Ins(1,4,5,6)P_4, HDAC3 interface. **C.** Inset of acetyl containing active site. **D.** Ins(1,4,5,6)P4 interacting region. **E.** Amino acids H17, G21, K25, R264, L265, R301 mutated to eliminate binding to (1,4,5,6)P_4 shown in cyan.

Surprisingly, expression of these HDAC3 mutants had variable effects on the growth of *Hdac3*$^{-/-}$ NIH 3T3 cells. Both the single loop 6 mutant (L265M) and the loop 1 mutant (H17C,G21A,K25I) complemented the growth defect (Fig 21). On the other hand, both the double loop 6 mutant (R264P,L265M) and the double loop 1+6 mutant (H17C,G21A,K25I,R264P,L265M,R301A) failed to complement the growth defect (Fig. 21). These data suggest that the interaction with Ins(1,4,5,6)P_4 and subsequently the DAD of SMRT/N-CoR does affect the growth rate. That said, some of the previously published SMRT-DAD binding and *in vitro* deacetylase assay data is in conflict with this *in vivo* proliferation data. Given the Y298H data it is unlikely that single loop 6 mutant (L265M) and the loop 1 mutant (H17C,G21A,K25I) would be catalytically inactive and still complement the growth defect. This seems similar to the HDAC3 serine 424 phosphorylation situation where *in vitro* assays may not accurately represent the *in vivo* situation.

Discussion

The HDAC family of proteins is defined by the presence of a histone deacetylase domain. The majority of HDACs, including HDAC3, are catalytically active enzymes. Through the use of a well-established HDAC3 catalytically inactive mutant, I demonstrated that catalytic activity is required for the ability of HDAC3 to regulate cellular proliferation (Fig. 8). HDAC3 could regulate growth rate through deacetylation of histones or non-histone proteins. To date RelA is one of the only identified non-histone targets of HDAC3. Deacetylation of RelA,

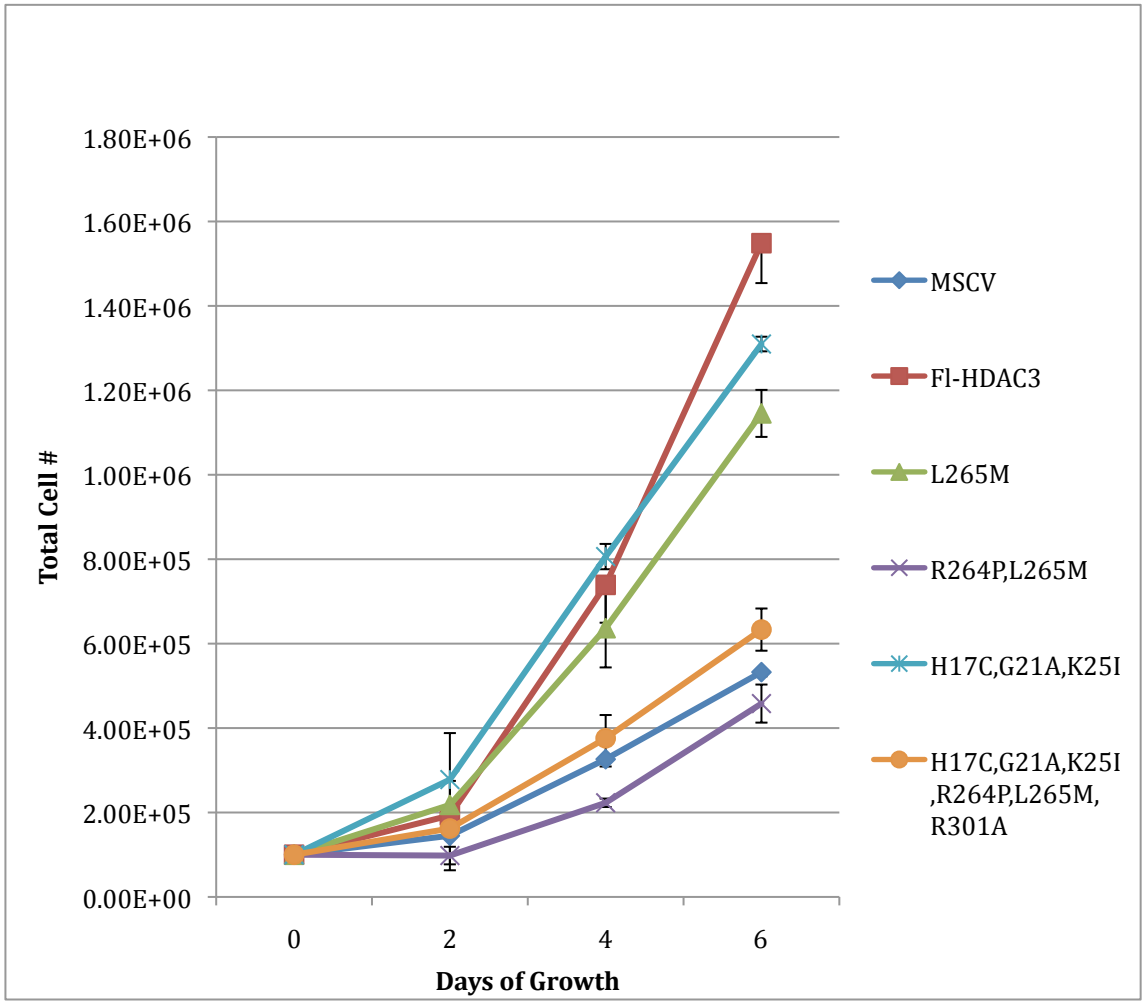

Figure 21. Binding of Ins(1,4,5,6)P_4 to HDAC3 is necessary for a normal growth rate. *Hdac3*$^{-/-}$ NIH 3T3 cells were infected with MSCV virus expressing no HDAC3 (MSCV), wild-type flag tagged HDAC3 (Fl-HDAC3), and mutants intended to eliminate binding to Ins(1,4,5,6)P4 (L265M R264P,L265M H17C,G21A,K25I H17C,G21A,K25I,R264P,L265M,R301A). Following an initial plating at 1x 10^5 cells, every two days cells were trypsinized, counted, and replated at 1 x 10^5 cells.

which binds directly to the N-terminus of HDAC3, terminates NF-κB target gene transcription through effective binding of IκBα inhibitory proteins. NF-κB regulates genes that control cell survival and proliferation. Expression of an HDAC3 mutant intended to eliminate binding to RelA complemented the growth defect (Fig. 14). This implies that RelA binding to HDAC3 is not required for the mechanism by which HDAC3 regulates cellular proliferation. At this time I am unable to confirm that the HDAC3 mutant does not bind RelA. Other HDACs, including class 1 HDACs, deacetylate a number of non-histone targets including p53, YY1, signal transducer and activator of transcription 3 (STAT3), E2F transcription factor 1 (E2F1), Ku70, and heat shock protein 90 (HSP90) (91). It seems likely that additional non-histone targets of HDAC3 exist. Deacetylation of these targets could be the source of the mechanism by which HDAC3 regulates cellular proliferation.

Even among class 1 histone deacetylases, HDAC3 is subject to unique regulation that directly impacts catalytic activity. The C-terminal 35 amino acids of HDAC3 do not share sequence similarity with any other histone deacetylase. Much attention has been paid to this unique region as potentially key to HDAC3 regulation. Phosphorylation of serine 424, within this unique region, is the lone HDAC3 phosphorylation event impacting activity (86). Phosphorylation of serine 424 was required for HDAC3 dependent *in vitro* histone deacetylase activity (86). Through mutation of this amino acid to alanine and aspartic acid I demonstrated that phosphorylation at amino acid 424 is not required for the mechanism by which HDAC3 regulates cellular proliferation (Fig. 9). Expression of HDAC3

containing an alanine mutant, which cannot be phosphorylated and does not resemble a phosphorylated amino acid, complemented the growth defect. Given the *in vitro* deacetylase data, this suggests that either the mechanism by which HDAC3 regulates growth rate is not dependent on the deacetylation of histones or HDAC3 phosphorylation at serine 424 does not alter HDAC3 activity *in vivo*. Other class 1 HDACs, HDAC1 and 2, are phosphorylated at multiple sites (92). In the case of HDAC2, phosphorylation of different amino acids can regulate activity, co-repressor binding, and chromatin distribution (93, 94). That said, phosphorylation of HDAC2 is not important for transcriptional repression (94). It seems that, similar to HDAC2, the impact of HDAC3 phosphorylation is more complex than originally appreciated.

A the time these experiments were started the full impact of changes to HDAC3, such as those seen following phosphorylation, could not be elucidated due to a lack of structural knowledge. A structural model of HDAC3 was produced through the use of protein disorder prediction software, sequence alignment, homology modeling, and measures of protein surface hydrophobicity. This model allowed prediction, based on level of hydrophobicity, of HDAC3 regions likely to bind other proteins. Expression of HDAC3 containing mutations intended to eliminate binding to four independent surface accessible hydrophobic regions complemented the growth defect (Fig. 16). This suggests that either no proteins bind these novel regions, the mutations introduced did not eliminate binding to the yet unidentified proteins, or the proteins that bind these regions are not required for the mechanism by which HDAC3 regulates cellular proliferation.

Previously, HDAC3 interacting proteins were identified using a stringent, high-throughput yeast two-hybrid system (95). These included proteins already known to interact with HDAC3 including RB, RelA, PPARγ, PML, heat shock proteins, and members of the TCP-1 ring complex. Novel proteins were also identified such as mitogen-activated protein kinase 11 (MAPK11), protein inhibitor of activated STAT 2 (PIAS2), and retinoblastoma binding protein 4 (RBBP4), among others. Although the HDAC3-binding region has not been identified for most of these proteins, many of them have established roles in the regulation of proliferation. Our approach to identifying novel binding regions can only be further enhanced by the publication of an HDAC3 crystal structure bound to the deacetylase activating domain of SMRT/N-CoR (83).

The HDAC3 crystal structure offers a more precise picture of the protein surface. In particular the “lid”, hypothesized to be important for HDAC3 catalytic site specificity, is not nearly as pronounced in the crystal structure as had been suggested by the homology model. It is thus not surprising that expression of HDAC3 containing a single amino acid deletion in the “lid” complemented the growth defect, proliferating at a rate faster than *Hdac3*$^{-/-}$ NIH 3T3 cells expressing wild-type Flag tagged HDAC3 (Fig. 18). This data highlights the idea that a model is only a model and may not provide an accurate representation of a proteins structure in all situations.

The publication of an HDAC3 crystal structure also reinforces the importance of the SMRT/N-CoR deacetylase activating domain (DAD) for HDAC3 deacetylase activity (83). Unexpectedly, expression of an HDAC3

mutant intended to eliminate binding to SMRT/N-CoR complemented the growth defect (Fig. 14). The finding that Ins(1,4,5,6)P_4 appears at the interface between HDAC3 and the SMRT/N-CoR DAD helps explain why this SMRT/N-CoR mutant, designed using the homology model, complemented the growth defect. The homology model did not represent the impact of SMRT/N-CoR binding and the presence of Ins(1,4,5,6)P_4. It is likely that this mutant did not perturb HDAC3 interaction with Ins(1,4,5,6)P_4 and thus SMRT/N-CoR. That said, expression of HDAC3 mutants specifically designed to eliminate interaction with Ins(1,4,5,6)P_4 had variable effects on growth of *Hdac3*$^{-/-}$ NIH 3T3 cells (Fig. 21). These data highlight the importance of HDAC3 amino acids 264 and 265 (loop 6) to Ins(1,4,5,6)P_4 interaction and the mechanism by which Hdac3 regulates cellular proliferation.

Small active molecules that regulate chromatin such as inositol polyphosphates (IPs), ATP, NAD^+, acetyl coenzyme A (Acetyl-CoA), and S-adenosyl methionine (SAM) are generated through normal cell metabolism (12, 96). These small molecules and metabolites can regulate chromatin directly through modification of histones or by altering the activity of effector proteins such as nucleosome remodeling complexes. Some remodelers are even subject to covalent modification through use of metabolites. (12, 96)

Inositol polyposphates, such as Ins(1,4,5,6)P_4 seen in the HDAC3 crystal structure, are increasingly being recognized for their regulatory role in chromatin remodeling. In yeast, mutations in genes encoding polyphosphate kinases, responsible for IP biogenesis, have led to altered gene transcription and impaired

promoter remodeling due to inefficient recruitment of ATP-dependent nucleosome remodeling factors (97-99). The number of phosphate groups on an inositol ring, dictated by the activity of polyphosphate kinases, can affect IPs regulatory role. IP_6 inhibits while IP_4 and IP_5 stimulate nucleosome mobilization by remodeling factors (97). Even membrane bound inositol 4,5-bisphosphate (PIP_2) controls localization of some remodeling factors (100). The presence of Ins(1,4,5,6)P_4 at the interface of HDAC3 and SMRT/N-CoR lends further support to the idea of IPs regulating chromatin, this time through acetylation. The catalytic activity of HDAC3, made possible through the HDAC3-Ins(1,4,5,6)P_4-SMRT/N-CoR interaction, is required for HDAC3 regulation of chromatin structure and cellular proliferation.

CHAPTER IV

FUTURE DIRECTIONS

Complementation of *Hdac3*$^{-/-}$ NIH 3T3 cells through expression of HDAC3 mutants clearly represents a powerful tool to explore how HDAC3 structure affects function. Additional replicates of all of the presented complementation experiments should be performed to further solidify the results. Though I am confident in the conclusions, a significant number of equivalent experiments have not yet been performed to obtain statistical significance. In addition, proper interpretation of the complementation experiments depends on the status of the HDAC3-protein interactions targeted. Co-immunoprecipitations (co-IPs) should be performed to determine if the mutant intended to eliminate binding to RelA did in fact not bind RelA. Also, co-IP experiments should be performed to determine if the homology model based SMRT/N-CoR mutant, the serine 424 mutants, and the crystal structure mutants are able to bind SMRT/N-CoR. The status of SMRT/N-CoR interaction with the HDAC3 crystal structure mutants is of particular interest given the apparent discrepancy between the complementation data of two of the crystal structure mutants and the published SMRT-DAD data. All four of the crystal structure mutants were non-functional in *in vitro* deacetylase assays and did not bind the SMRT-DAD (83). In the context of the DAD alone (91 amino acids), all of the crystal structure mutants may eliminate binding to the deacetylase activating domain of SMRT/N-CoR through disruption of the

Ins(1,4,5,6)P_4 interaction. However, full length SMRT binds both the N and C-terminal ends of HDAC3. *In vivo*, SMRT (2514 amino acids) is a significantly larger more dynamic protein than was represented by the SMRT-DAD. The impact of full length SMRT, in combination with SMRT-DAD, should be taken into account when considering the effects of HDAC3 mutations.

Histone acetylation should also be examined in complementation experiments as another measure of HDAC3 function *in vivo*. Histone H4 lysine 5 and lysine 12 are well-established targets of HDAC3 histone deacetylase activity (35). Increased H4K5 and H4K12 acetylation was observed in *Hdac3*$^{-/-}$ NIH 3T3 cells as compared to controls (39). Immunoblotting should be performed to compare the levels of H4K5 and H4K12 acetylation in *Hdac3*$^{-/-}$ NIH 3T3 cells expressing the control MSCV, Flag tagged wild-type HDAC3, and HDAC3 mutations. H4K5 and H4K12 acetylation should return to their normally low levels following complementation of the growth defect. This would further address the discrepancy between the complementation data of two of the crystal structure mutants and the published SMRT-DAD data, particularly as it relates to the *in vitro* deacetylase assays.

The HDAC3/SMRT-DAD crystal structure represents a more accurate depiction of the true structure of HDAC3 and breaks new ground with the identification of Ins(1,4,5,6)P_4. The presence of any molecule, let alone an inositol polyphosphate, bridging the interaction between HDAC3 and SMRT/N-CoR had not been considered in the HDAC field. The HDAC3 surface visualized in the crystal structure is different than that visualized in the homology model.

With the more accurate HDAC3 structure, further analysis of hydrophobic regions should be performed. If HDAC3 regulates proliferation through deacetylation of a non-histone protein, such as RelA or those suggested by the yeast two-hybrid data (95), that protein is likely to interact directly with HDAC3. Hydrophobic regions on the protein surface still represent likely sites of HDAC3-protein interaction.

The presence of Ins(1,4,5,6)P_4 in the HDAC3 crystal structure opens up new avenues of potential investigation not considered at the start of these studies. Further analysis of the impact of inositol polyphosphates on HDAC3 function should be performed through the use of *Hdac3*-null and heterozygous NIH 3T3 cells. It is conceivable that there is an inositol polyphosphate feedback loop used to regulate the activity of HDAC3 (Fig. 22). An increase in HDAC3 activity, through Ins(1,4,5,6)P_4 dependent formation of the SMRT/N-CoR complex, could lead to decreased levels of Ins(1,4,5,6)P_4 through transcriptional repression of a polyphosphate kinase. This decrease in Ins(1,4,5,6)P_4 would lead to a decrease in HDAC3 activity through an inability to form a functional SMRT/N-CoR complex thereby leading to increased levels of Ins(1,4,5,6)P_4. It would be interesting to compare the levels of different inositol polyphosphates (IP_2-IP_6) in *Hdac3*$^{F/-}$ and *Hdac3*$^{-/-}$ NIH 3T3 cells through anion exchange HPLC chromatography. *Hdac3* loss could lead to an over all increase in the levels of inositol pohlyphosphates due to an increase in kinase expression, which could be evaluated through gene expression analysis, or a shift in the balance between

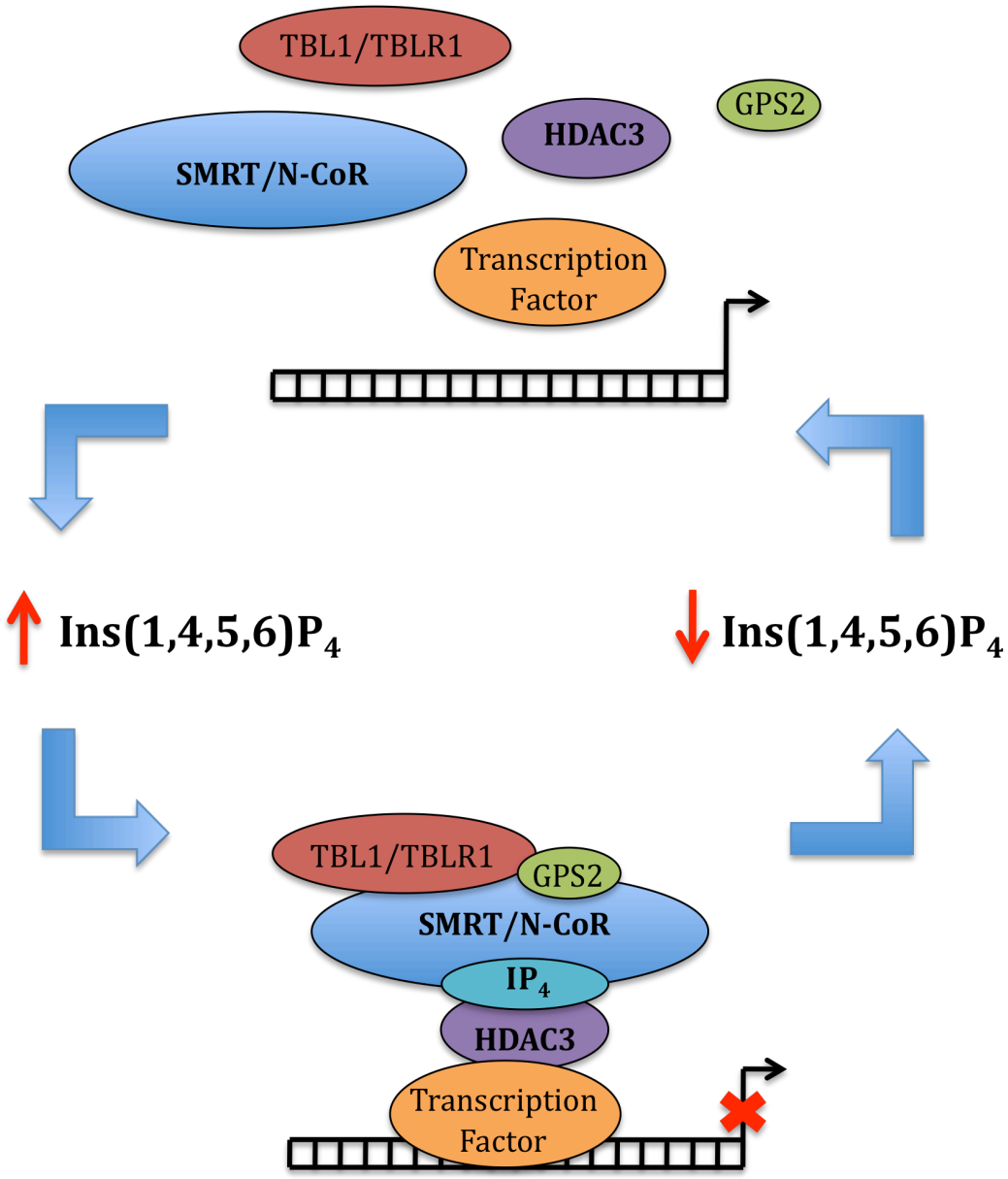

Figure 22. Potential inositol polyphosphate feedback loop that regulates HDAC3 activity. An increase in HDAC3 activity, through Ins(1,4,5,6)P_4 dependent formation of the SMRT/N-CoR complex, could lead to decreased levels of Ins(1,4,5,6)P_4 through transcriptional repression of a polyphsphate kinase. This decrease in Ins(1,4,5,6)P_4 could lead to a decrease in HDAC3 activity through an inability to form a functional SMRT/N-CoR complex thereby leading to increased levels of Ins(1,4,5,6)P_4.

individual inositol polyphosphates due to a lack of SMRT/N-CoR complex formation.

A significant number of inositol kinases are necessary to generate the all of the inositol polyphosphates. Up to six phosphates can be placed on the inositol ring in different combinations of locations. Ins(1,4,5)P_3 3-kinase is specifically responsible for phosphorylating Ins(1,4,5)P_3 to generate Ins(1,3,4,5)P_4. Nonsense mutation of Ins(1,4,5)P_3 3-kinase in mice led to severe T-cell deficiency (101, 102). A similar loss of single positive T-cells was observed following conditional deletion of *Hdac3* in mice using Lck promoter driven *cre* expression (A. Summers, unpublished data). This further supports the argument that inositol polyphosphate regulation impacts HDAC3 activity. Polyphosphate kinases, including Ins(1,4,5)P_3 3-kinase, should be knocked down in *Hdac3*$^{F/-}$ NIH 3T3 cells to determine if a similar decrease in proliferation, as that seen in the absence of *Hdac3*, would be observed. In addition, H4K5 and H4K12 acetylation should be assessed in these experiments to confirm that the effects seen in the absence of a polyphosphate kinase are directly related to loss of Hdac3 function. Binding of full length SMRT/N-CoR and SMRT-DAD to Hdac3 should also be assessed in these experiments. It is clear from our data and the HDAC3 crystal structure that the activity of HDAC3 is dependent upon Ins(1,4,5,6)P_4 bridging between SMRT/N-CoR and HDAC3.

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