ROLES OF CYTOPLASMIC DEACETYLASE HDAC6 IN ONCOGENIC TUMORIGENESIS

by

Yi-Shan Lee

Molecular Cancer Biology Program
Duke University

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Tso-Pang Yao, Supervisor

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Ann Marie Pendergast

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Christopher M. Counter

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Dennis J. Thiele

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Jen-Tsan Ashley Chi

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Reversible acetylation has emerged as an important post-translational modification that rivals phosphorylation in regulating chromatin structure and gene expression. Acetylation of histone is associated with transcriptional activation while deacetylation is linked to transcriptional repression. Moreover, histone deacetylase inhibitors induce growth arrest, differentiation and apoptosis of cancer cells and therefore appear to be promising anti-tumor agents. While transcriptional deregulation is thought to be the main mechanism underlying their therapeutic effects, the exact mechanisms and targets by which HDAC inhibitors, which are mostly non-specific, achieve their anti-tumor effects remain poorly understood. In other words, it is not known which and how each HDAC members are involved in supporting tumor growth.

In this thesis, I have showed that HDAC6, a cytoplasmic localized and cytoskeleton-associated deacetylase, is required for efficient oncogenic transformation and tumor formation. I have found that HDAC6 expression is induced upon oncogenic Ras-induced transformation in both human somatic cells and murine fibroblasts. Conversely, murine fibroblasts deficient in HDAC6 are more resistant to both oncogenic Ras and ErbB2-dependent transformation, indicating a critical role for HDAC6 in oncogene-induced transformation. Supporting this hypothesis, inactivation of HDAC6 in several human cancer cell lines effectively impairs anchorage-independent growth in
vitro and their ability to form tumors in immunocompromised mice. I have demonstrated that the impairment of anchorage independent growth in HDAC6 deficient cells is associated with increased anoikis and mechanistically a defect in activation of the AKT and ERK kinase cascades. Additionally, HDAC6 null mice are more resistant to two-stage chemical carcinogen-induced skin tumors. Finally, I have demonstrated that the tumor-promoting effect of HDAC6 is probably mediated through the molecular chaperon Hsp90. While Hsp90 is known to be deacetylated by HDAC6 and has been implicated in stabilizing Raf-1 and ErbB2, I have found that suppression of HDAC6 impairs the stability of Raf-1 and the association between Hsp90 and ErbB2.

In conclusion, my work provides the first experimental evidence that of all the HDAC members, the cytoplasmic deacetylase HDAC6 is required for efficient oncogenic transformation, indicating that reversible acetylation plays a critical role in regulating cellular functions of non-histone non-nuclear cytoplasmic proteins that contributes to malignant transformation. Furthermore, this work identifies HDAC6 as an important component underlying the anti-tumor effects of HDAC inhibitors.
Dedication

I would like to dedicate my thesis work to my family. First of all, I want to thank my parents, Hsin-Yi and Man-Chu for their love and full, unconditional support in the course of my study. My two lovely sons Wei-Xi and Yun-Xi are the driving forces that keep me moving forward even when things were not going very well. I would like to thank my husband, Kian-Huat who is my best friend and partner of the past 11 years. We have been taken similar routes, and hence he is the only one who truly understands and feels my struggles. He has accompanied me through difficult times when experiments failed, and he has given me courage and support to keep going. I would also like to thank my brother Yu-Xue and sisters Ru-Yun and Ming-Shan for their encouragement and support.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemias</td>
</tr>
<tr>
<td>BUZ</td>
<td>Binder of ubiquitin zing finger</td>
</tr>
<tr>
<td>CBP</td>
<td>p300/CREB-binding protein</td>
</tr>
<tr>
<td>DMB</td>
<td>Dynein motor binding</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>ErbB2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GNATs</td>
<td>GCN5 related N-acetyltransferases</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial Cells</td>
</tr>
<tr>
<td>HSF-1</td>
<td>Heat shock factor -1</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MOZ</td>
<td>Monocytic leukemia zinc finger protein</td>
</tr>
<tr>
<td>MYST</td>
<td>MOZ, Ybf2/Sas3, Sas2 and Tip60K</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>polyHEMA</td>
<td>Poly(hydroxyethyl methacrylic) acid</td>
</tr>
<tr>
<td>PrECs</td>
<td>Human prostate epithelial cells</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma 2 viral oncogene homolog</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5-Acetyltransferase</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian vacuolating virus 40 large T antigen</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
</tbody>
</table>
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First, I would like to thank my mentor, Dr. Tso-Pang Yao for his advice and encouragement throughout my graduate study. With his dedication to pioneering the sciences, there is not one single boring project. Rather, there is constantly exciting and novel findings developing in the Yao Lab. Pang also gives his students a lot of freedom to explore our own projects, which helps us become independent researchers. As a graduate student with two children and a crazy schedule, I would like to specially thank him for his understanding and support throughout my course of study.

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1. Introduction

1.1 Overview

Oncogenic transformation at the cellular level is a complicated process involving altered signal transductions and acquisition of capabilities to sustain unlimited proliferation, evade apoptosis, and invade surrounding tissues (Hanahan and Weinberg 2000). At the molecular level, posttranslational modifications play important roles in tuning activities, stability, and regulating functions of cellular proteins executing the signal transduction pathways. For example, upon activation by its growth factor ligands, growth factor receptors such as epidermal growth factor receptors become activated as a result of dimerization. The receptors then become autophosphorylated at several specific tyrosine residues in the C terminal domains. These phosphorylated tyrosine residues serve as docking sites for downstream effectors with phosphotyrosine binding SH2 domains such as growth factor receptor-bound protein 2 (GRB2). This in turn recruits the GRB2 binding partner SOS, the activating guanine nucleotide exchange factor of Ras to the plasma membrane where Ras resides and leads to Ras activation. Activated Ras activates the kinase activity of Raf and initiates signaling cascades involved in phosphorylation and activation of downstream kinases including MEK and extracellular signal-regulated kinase (ERK). Activated ERK in turn phosphorylates several substrates
including 40S ribosomal protein kinase (RSR) and C-myc, which are important factors regulating both translation and transcription (McKay and Morrison 2007).

While phosphorylation modulated by kinases and phosphatases has been the major focus of research and drug target in cancers for the past few decades (Manning, Whyte et al. 2002; Noble, Endicott et al. 2004; Ptacek and Snyder 2006; Scapin 2006), other posttranslational modifications such as nitrosylation (Lane, Hao et al. 2001), ubiquitination (Freiman and Tjian 2003; Gao and Karin 2005; Brooks and Gu 2006; Wullaert, Heyninck et al. 2006), sumoylation (Geiss-Friedlander and Melchior 2007) and acetylation (Kouzarides 2000; Cohen and Yao 2004) have also recently received great interest. Our lab has been particularly interested in the biological and pathological processes that reversible acetylation regulates.

### 1.2 Reversible Acetylation

Different from co-translational N-terminal acetylation, protein acetylation is one type of reversible post-translational modification involving addition of an acetyl moiety to the ε-amino group of lysine residues (Figure 1). Two classes of enzymes with opposing activity, histone acetyltransferases (HAT) and histone deacetylases (HDAC) are responsible for addition and removal of this modification. As the name implies, histones are the first identified (Vidali, Gershey et al. 1968) and the most well-known and characterized acetylated proteins. As the building blocks of chromatin, the histones
are versatile, structurally highly-organized proteins that are involved in compressing and unraveling nucleosomal DNA so it can be easily and precisely accessed for replication, transcription, and repair when needed. Histones can be post-translationally modified at amino-termini by acetylation, phosphorylation, and methylation, which could either positively or negatively regulate the access of underlying DNA to chromatin associated proteins. The combination of these modifications is termed the histone code, which is considered to encode important life-sustaining or modifying information analogous to the genetic code (Turner 1993; Jenuwein and Allis 2001). Reversible acetylation of histones in particular, is one of the major determinants of the histone code by modifying the chromatin structure and regulating the access of genetic code to numerous DNA binding proteins including polymerases, transcription factors and other associated proteins (Wade, Pruss et al. 1997; Hansen, Tse et al. 1998). Specifically, acetylation on histone tails leads to neutralization of the positive charges so that their ability to bind negatively charged DNA is diminished, resulting in a decrease in affinity between nucleosomal DNA and nucleosomal proteins, and ultimately an opener conformation of chromatin for gene expression and regulation (Wade, Pruss et al. 1997; Hansen, Tse et al. 1998). In short, histone acetylation is thought to be a molecular signature of transcriptional activation (Wade, Pruss et al. 1997; Yang and Seto
Reversible acetylation, a post-translational modification of the ε-amino group of specific lysine residue regulated by opposing enzymes histone acetyltransferase (HAT) and deacetylase (HDAC)

Besides histones, many non-histone DNA or RNA binding proteins, including many transcription factors and coregulators such as p53 (Gu and Roeder 1997), Rb (Chan, Krstic-Demonacos et al. 2001), are also acetylated. Moreover, many cytosolic
proteins such as metabolic enzymes, cytoskeleton and structure proteins, and protein kinases and phosphatases are also acetylated (Table 1) (Xu, Parmigiani et al. 2007; Yang and Gregoire 2007; Yang and Seto 2007). This indicates a general regulatory role of reversible acetylation in cell biology other than just epigenetic control (Xu, Parmigiani et al. 2007; Yang and Gregoire 2007; Yang and Seto 2007).
<table>
<thead>
<tr>
<th>Table 1: Acetylated proteins (partial list)</th>
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<tbody>
<tr>
<td><strong>Histones</strong></td>
</tr>
<tr>
<td><strong>DNA binding transcription factors</strong></td>
</tr>
<tr>
<td><strong>Transcription coregulators</strong></td>
</tr>
<tr>
<td><strong>General transcription factors</strong></td>
</tr>
<tr>
<td><strong>RNA processing</strong></td>
</tr>
<tr>
<td><strong>DNA repair</strong></td>
</tr>
<tr>
<td><strong>Recombination and replication</strong></td>
</tr>
<tr>
<td><strong>Viral proteins</strong></td>
</tr>
<tr>
<td><strong>Steroid receptor</strong></td>
</tr>
<tr>
<td><strong>HATs &amp; HDACs</strong></td>
</tr>
<tr>
<td><strong>Translation</strong></td>
</tr>
<tr>
<td><strong>Inflammation mediator</strong></td>
</tr>
<tr>
<td><strong>Chaperones</strong></td>
</tr>
<tr>
<td><strong>Metabolic enzymes</strong></td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
</tr>
<tr>
<td><strong>Apoptosis</strong></td>
</tr>
<tr>
<td><strong>Cytoskeleton</strong></td>
</tr>
</tbody>
</table>
As mentioned earlier, acetylation modulates direct histone-DNA affinity and therefore is critical for chromatin structure and gene expression. However, acetylation of non-histone proteins such as transcription factor p53 has also been found to modulate gene expression, disclosing the previously understudied fact that acetylation of non-histone proteins is as critical as histone proteins in normal cellular functions (Glozak, Sengupta et al. 2005). Specifically, acetylation of p53 by histone acetyltransferase CBP/p300 at K305, K370, K372, K373, K381 and K382 and by another histone acetyltransferase p300/CBP-associated factor (PCAF) at K320 increases its ability to bind DNA and transactivate target genes that contribute to cell cycle arrest or apoptosis, depending on the cell type and the type of DNA damage (Gu and Roeder 1997; Sakaguchi, Herrera et al. 1998; Liu, Scolnick et al. 1999; Wang, Tsay et al. 2003; Luo, Li et al. 2004). Moreover, activation of p53 by different types of damage leads to acetylation of p53 at different sites. For example, radiomimetic DNA-alkylating agents adozelesin and
bizelesin induce DNA damage and acetylation of K320 while topoisomerase inhibition by etoposide induced acetylation of K373 (Knights, Catania et al. 2006). Furthermore, the target genes activated differs depending on the amino acid residue on which p53 is acetylated. For instance, acetylation of p53 at K320 is associated with activation of cyclin-dependent kinase inhibitor p21, leading to cell cycle arrest; acetylation at K373 is associated with the activation of the proapoptotic protein, Bax, leading to apoptosis (Knights, Catania et al. 2006).

Moreover, the ε-amino groups of lysine residues serve not only as acetylation sites but are also subject to other modifications such as methylation, ubiquitination and sumoylation. Therefore, acetylation could potentially preclude other modifications and vice versa. For example, acetylation of p53 prevents it from being ubiquitinated by the ubiquitin E3 ligase, Mdm2, and targeted for proteasomal degradation, thereby leading to stabilization of p53 protein. On the other hand, Mdm2 recruits HDAC1 to deacetylate p53, thereby facilitating Mdm2-mediated ubiquitination and degradation of p53 (Ito, Kawaguchi et al. 2002). Acetylation of a lysine residue could also cross-talk with other type of modifications such as phosphorylation. For example, acetylation of p53 at K320 negatively regulates the phosphorylation of sites associated with nuclear accumulation of p53, while acetylation of p53 at K323 positively regulates the phosphorylation of sites
involved in nuclear retention of p53 and in p53 binding to promoters of proapoptotic genes (Knights, Catania et al. 2006).

1.3 Histone Acetyltransferases

HATs or acetyltransferases (ATs) are capable of transferring the acetyl group from acetyl coenzyme A (acetyl CoA) to ε-amino group of lysine residues. They are a diverse set of enzymes and divided to nuclear (A type) and cytoplasmic (B type) based on the localization, with majority being type A and only Hat1 belongs to type B. However, Hat1 has also been detected in the nucleus and is capable of acetylating histones (Parthun 2007). HATs are also grouped into five families based on their catalytic domains: GCN5 related N-acetyltransferases (GNATs); the MOZ, Ybf2/Sas3,Sas2 and Tip60 (MYST)- related HATs, p300/CREB-binding protein (CBP) HATs; the general transcription factor HATs including the TFIID subunit TBP-associated factor-1 (TAF1); and the nuclear hormone cofactor HATs SRC1 and SRC3 (Table 2)(Roth, Denu et al. 2001; Lee and Workman 2007).
<table>
<thead>
<tr>
<th>HAT family</th>
<th>Catalytic subunit</th>
<th>HAT complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNAT</td>
<td>Gcn5 (Sc)</td>
<td>SAGA, SLIK, ADA, HAT-A2</td>
</tr>
<tr>
<td></td>
<td>GCN5L(Hs)</td>
<td>STAGA, TFTC</td>
</tr>
<tr>
<td></td>
<td>PCAF(Hs)</td>
<td>PCAF</td>
</tr>
<tr>
<td></td>
<td>Hat1 (Sc)</td>
<td>HATB</td>
</tr>
<tr>
<td></td>
<td>Elp3 (Sc)</td>
<td>Elongator</td>
</tr>
<tr>
<td></td>
<td>Hpa2 (Sc)</td>
<td>Hpa2</td>
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<tr>
<td>MYST</td>
<td>Esa1(Sc)</td>
<td>NuA4</td>
</tr>
<tr>
<td></td>
<td>Ybf2/Sas3(Sc)</td>
<td>NuA3</td>
</tr>
<tr>
<td></td>
<td>Sas2(Sc)</td>
<td>SAS</td>
</tr>
<tr>
<td></td>
<td>TIP60 (Hs)</td>
<td>TIP60</td>
</tr>
<tr>
<td></td>
<td>HBO1(Hs)</td>
<td>HBO1</td>
</tr>
<tr>
<td></td>
<td>MOZ/MORF(Hs)</td>
<td>MOZ/MORF</td>
</tr>
<tr>
<td>P300/CBP</td>
<td>P300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBP</td>
<td></td>
</tr>
<tr>
<td>General</td>
<td>TAFII250</td>
<td>TFIID</td>
</tr>
<tr>
<td>transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td>factor</td>
<td>TFIII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nut1</td>
<td>Mediator</td>
</tr>
<tr>
<td>nuclear</td>
<td>SRC1</td>
<td></td>
</tr>
<tr>
<td>hormone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cofactor</td>
<td>SRC3</td>
<td></td>
</tr>
</tbody>
</table>

1. Adapted and modified from Roth, Denu et al. 2001; Lee and Workman 2007
2. Abbreviations: CBP: p300/CREB-binding protein; Esa1: Essential Sas family acetyltransferase 1; GNATs: GCN5 related N-acetyltransferases; HBO1: HAT bound to ORC1; Hs: Homo sapiens; MOZ: Monocytic leukemia zinc finger protein; MYST: MOZ, Ybf2/Sas3,Sas2 and Tip60; NuA3: Nucleosomal Acetyltransferase of histone H3; NuA4: Nucleosomal acetyltransferase for H4; PCAF: p300/CBP-associated factor; SAGA: Spt-Ada-Gcn5-Acetyltransferase; Sc: Saccharomyces cerevisiae; SRC: Steroid receptor coactivator; SRC3 also known as p300/CBP interacting protein (p/CIP), /AIB1/ACTR/RAC3/TRAM1; TAF1: TFIID subunit TBP-associated factor-1; TIP60: TAT-interactive protein with a mass of 60
Most HATs lose their acetyltransferase activities when overexpressed in vitro, indicating they might work as multi-protein complexes. In fact, many HATs associate with other HATs and co-activators (Table 2). For example, yeast Ccn5, the founding member of HATs, associate with Ada2, Ada3, and Ahc1 in the ADA HAT complex, while in the SAGA complex, it associates with Ada1, Ada2, Ada3, Ada5/Spt20, Sot3, Spt7, Spt8, Tra1, TAFII90, TAFII6/68, TAFII60, TAFII23/25, TAFII17/20, and Sin4. In addition, several co-activators and adapters of transcription have intrinsic HAT activity such as steroid receptor coactivator 1 (SRC1), a human transcriptional coactivator for steroid/nuclear receptors (Spencer, Jenster et al. 1997).

Several HATs null mice are embryonic lethal (Tanaka, Naruse et al. 1997; Yao, Oh et al. 1998; Kung, Rebel et al. 2000; Xu, Edmondson et al. 2000; Yamauchi, Yamauchi et al. 2000), indicating critical roles of HATs in development, differentiation and proliferation (Table 3). It is therefore not surprising that HATs are associated with human malignancies. In fact, several chromosomal translocations associated with human leukemias involve HATs. In one type of recurrent chromosomal translocation associated with acute myeloid leukemias (AML), CBP is fused to MOZ, a human homolog of yeast acetyltransferase Sas3, leading to synthesis of a protein with two HAT domains and possible inappropriate recruitment of CBP or MOZ cofactors and HAT activities (Borrow, Stanton et al. 1996). In another leukemia-associated recurrent
chromosomal inversion, MOZ is found to fuse to another nuclear receptor co-activator with intrinsic HAT activity, TIF2 (also known as NcoA1 and GRIP1) (Carapeti, Aguiar et al. 1998; Carapeti, Aguiar et al. 1999), which also interacts with CBP and p300. In yet another leukemia associated chromosomal abnormality, CBP or p300 is fused to MLL (mixed lineage leukemia) (Sobulo, Borrow et al. 1997) which encodes a histone methyltransferase (Milne, Briggs et al. 2002). MLL itself is a large multi-domain protein which interacts with several other proteins including hSNF, a component of the chromatin remodeling complex Swi/Snf in human cells (Muchardt and Yaniv 1999). AIB, also known as the nuclear hormone cofactor ACTR with intrinsic HAT activity is overexpressed in some breast and ovarian cancers (Anzick, Kononen et al. 1997). Finally, hypoacetylation of histone H4-K16 coupled with trimethylation of histone H4-K20 is associated with diminished recruitment of MYST family of HATs and is considered a characteristic of cancer cells (Fraga, Ballestar et al. 2005).
### Table 3: Comparison of HAT deficient mice phenotypes

<table>
<thead>
<tr>
<th>HAT</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P300</td>
<td>+/+</td>
<td>Normal</td>
<td>Yao, Oh et al. 1998</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>Decreased viability in utero</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>Embryonic lethal (9-11.5 dpc) with decreased proliferation, heart defect and open neural tube</td>
<td></td>
</tr>
<tr>
<td>CBP</td>
<td>+/+</td>
<td>Normal</td>
<td>Tanaka, Naruse et al. 1997; Kung, Rebel et al. 2000</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>Defects in skeletal formation and hematopoietic differentiation, increased hematologic malignancies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>Embryonic lethal, similar to p300 null mice</td>
<td></td>
</tr>
<tr>
<td>PCAF</td>
<td>+/+</td>
<td>Normal</td>
<td>Xu, Edmondson et al. 2000; Yamauchi, Yamauchi et al. 2000</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>GCN5+PCAF</td>
<td>+/+</td>
<td>Normal</td>
<td>Xu, Edmondson et al. 2000; Yamauchi, Yamauchi et al. 2000</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>Embryonic lethal prior to 6.5 dpc</td>
<td></td>
</tr>
</tbody>
</table>
1.4 Histone Deacetylases

In contrast to HATs, HDACs remove the acetyl groups from substrates. They are an ancient enzyme family that could be found in plants, animals and fungi, as well as archaeabacteria and eubacteria (Leipe and Landsman 1997). There are at least 18 human HDACs and they are grouped into four classes and two families based on their sequence homology to known yeast enzymes and dependence to cofactors (Figure 2) (Verdin, Dequiedt et al. 2003; Gregoretti, Lee et al. 2004). Class I HDACs, which include HDAC1-3 and 8, are homologous to the yeast transcriptional repressor yRPD3. Class II HDACs are similar to yHDA1 (Grozinger, Hassig et al. 1999) and are further sub-divided to Class IIa (HDAC4, 5, 7, 9) and Class IIb (HDAC6 and 10) with duplication of catalytic domain in the latter. Class III HDACs are similar to ySIR2 and include SIRT1-7 (Frye 1999; Frye 2000). HDAC11 (Gao, Cueto et al. 2002) is the only member of Class IV HDACs which has characteristics of both class I and class II HDACs (Gregoretti, Lee et al. 2004). Of the HDAC members, the Class I, II and IV HDACs are considered the classical HDAC subfamilies. They share sequence and structural homology within the catalytic domains and require zinc for deacetylase activity. The Class III HDACs comprise the silent information regulator 2 (Sir2)-related protein (sirtuin) family and show no sequence or structural homology to the classical HDAC family. They use
nicotinamide adenine dinucleotide (NAD+) as a cofactor, and their activities could be inhibited by nicotinamide but not common HDAC inhibitors.

Figure 2: Phylogenetic tree analysis of the classical HDAC family.

The Class I mammalian HDACs are homologous to the yeast prototype Rpd3. The Class II HDACs share homology with the yeast Hda1. The Class II can be further subdivided based on sequence homology. Class IIa includes HDAC4, HDAC5, HDAC7, and HDAC9. Class IIb includes HDAC6 and HDAC10. HDAC11 appears to belong to a new class of HDACs. Adapted and Modified from (Verdin, Dequiedt et al. 2003)

Class I HDACs are primarily nuclear localized and ubiquitously expressed while the Class II HDACs are either primarily cytoplasmic or are shuttled between the nucleus and cytoplasm and have restricted tissue expression. Both Class I and Class II HDACs are often found as components of multiproteins complexes. All classical HDACs share a
similar deacetylase core which is about 390 amino acids region (Finnin, Donigian et al. 1999). This deacetylase core also shares similarity to archeal prokaryotic acetoin utilization protein (acuC) and acetylpolyamine amidohydrolase which recognize and remove acetyl group by cleaving the amide bond to increase the positive charge of the substrate (Leipe and Landsman 1997). The structure of an HDAC homologue in archebacteria *Aquifex aeolicus* has been solved and reveals an active site in the deacetylase core which consists of a tubular pocket, a zinc binding site, and two Asp-His charge relay systems (Finnin, Donigian et al. 1999). This active site has features of both metalloprotease and serine proteases. Both Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA), two HDAC inhibitors commonly used in research bind to or block this active site and inhibit the deacetylase activity (Finnin, Donigian et al. 1999).

While histone acetylation unravels the higher-ordered chromatin structure to allow gene expression, histone deacetylation is associated with transcriptional repression. Recruitment of HDACs to gene promoters leads to decreased histone acetylation and hence reformation of more compact chromatin structure, which precludes its access by transcription machinery. Interestingly, while HDACs are generally thought to associate with transcriptional repression, genetic experiments in yeast have shown that hos2 deacetylase activity actually contributes to gene activation (Wang, Kurdistani et al. 2002).
Similar to HATs, several HDAC null mice are either embryonic lethal or have defects that predispose them to perinatal or postnatal death (Table 4). These observations point to an equally important role of HDACs, as with the HATs, in embryonic development, cellular differentiation, and proliferation. In short, both acetylation and deacetylation are critical for normal cellular functions and animal survival.
Table 4: Comparison of HDAC deficient mice phenotype

<table>
<thead>
<tr>
<th>HDAC</th>
<th>Genotype</th>
<th>phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>+/-</td>
<td>normal</td>
<td>Lagger, O'Carroll et al. 2002</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>embryonic lethal E10.5 due to severe proliferation defects and retardation in development</td>
<td></td>
</tr>
<tr>
<td>HDAC2</td>
<td>+/-</td>
<td>normal</td>
<td>Trivedi, Luo et al. 2007</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>partial perinatal lethality due to heart defect</td>
<td></td>
</tr>
<tr>
<td>HDAC4</td>
<td>+/-</td>
<td>normal</td>
<td>Vega, Matsuda et al. 2004</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>die before weaning, skeletal defects due to ectopic chondrocyte hypertrophy</td>
<td></td>
</tr>
<tr>
<td>HDAC5</td>
<td>+/-</td>
<td>Normal</td>
<td>Renthal, Maze et al. 2007</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>predispose to cardiac hypertrophy and hypersensitive to chronic cocaine or stress</td>
<td></td>
</tr>
<tr>
<td>HDAC6</td>
<td>+/-</td>
<td>normal</td>
<td>Gao, Hubbert et al. 2007; Zhang, Kwon et al. 2008</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td>HDAC7</td>
<td>+/-</td>
<td>normal</td>
<td>Chang, Young et al. 2006</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>embryonic lethal (11 dpc) due to vascular malformation</td>
<td></td>
</tr>
<tr>
<td>HDAC9</td>
<td>+/-</td>
<td>normal</td>
<td>Zhang, McKinsey et al. 2002</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>predispose to cardiac hypertrophy</td>
<td></td>
</tr>
</tbody>
</table>
Overexpression of HDACs with concurrent decrease of CDK inhibitor p21 is observed in several human cancers. For example, HDAC1 has been found to be overexpressed in prostate cancers (Halkidou, Gaughan et al. 2004). Overexpression of HDAC2 has also been detected in gastric carcinomas, colorectal carcinomas, cervical dysplasias and endometrial stromal sarcomas (Zhu, Martin et al. 2004; Huang, Laban et al. 2005; Song, Noh et al. 2005; Hrzenjak, Moinfar et al. 2006). Moreover, expression of HDAC2 is induced in intestinal mucosa with loss of function of tumor suppressor Adenomatous polyposis coli (APC). Mechanistically, up-regulation of HDAC2 prevents cellular apoptosis and contributes to adenoma formation in the APC mutant mice (Zhu, Martin et al. 2004). In fact, HDAC2 plays a critical role in determining the sensitivity of colon cancer cells to HDAC inhibitors because certain sporadic colorectal carcinoma cells expressing a truncated, non-functional HDAC2 due to frame shift mutation are resistant to apoptosis induced by HDAC inhibitors (Ropero, Fraga et al. 2006). Increased expression of HDAC3 is also shown in human colon tumor and contributes to differentiation and maturation of colon epithelial cell (Wilson, Byun et al. 2006).

Apart from overexpression of protein per se, aberrant recruitment of HDACs is also common in human cancers. This is particularly true in hematological malignancies. Normal hematopoiesis entails constant, step-wise differentiation which is governed by tightly-regulated gene expression at each distinct step. On the other hand, inappropriate
recruitment of HDACs to the regulatory region of genes involved in differentiation, as found in many leukemic and lymphoma cells, can cause repression of these genes, and lead to rampant proliferation of immature progenitors. The frequent incidence of chromosomal translocation in these cancer cells is believed to render certain oncogenic DNA-binding fusion proteins that aberrantly recruit HDACs to promoters, leading to repression of appropriate gene expression required for differentiation and contributing to pathogenesis of lymphoma and leukemia. For example, chromosomal translocation t(8;21) resulting in production of AML1-ETO, a chimeric protein consisting of transcriptional factor acute myeloid leukemia-1 (AML-1, also known as runt-related transcription factor, RUNX1) and co-repressor eight-twenty-one (ETO) is frequently observed in acute myelocytic leukemia. ETO is a transcriptional corepressor that directly binds to nuclear HDACs and other co-repressors such as mSin3a, SMRT and N-coR to regulate hematopoiesis (Amann, Nip et al. 2001). ETO also interacts with pro myelocytic leukemia zinc finger (PLZF) (Melnick, Carlile et al. 2000) and BCL6 (Chevallier, Corcoran et al. 2004), both are members of BTB/POZ family of repressor proteins that recruit HDACs and co-repressors. During t(8;21) translocation, HDACs are inappropriately recruited to the close vicinity of AML-1 by the fusion partner ETO, which leads to repression of the AML-1 target genes including tumor suppressor
p14ARF and results in loss of senescence and extended life span of myeloid progenitor cells (Linggi, Muller-Tidow et al. 2002).

While many HATs contain bromodomains which recognize acetylated lysine residues, there are no obvious consensus motifs at the acetylation sites of acetylated substrates for HATs or HDACs (Mujtaba, Zeng et al. 2007). Since some acetylated proteins such as histone and microtubule are polymer substrates, the region specificity is important for the biological functions that acetylation regulates. This specificity could be achieved by several means. Firstly, HAT and HDAC enzymes often form multi-protein complexes via the non-catalytic subunits to help determine the substrate specificity and enzymatic activity of the catalytic subunits. For example, HDAC3 complexes containing co-repressor SMRT and N-CoR specifically bind to unliganded retinoid acid receptors and prevent target gene transcription in the absence of retinoid acid (Wen, Perissi et al. 2000; Guenther, Barak et al. 2001). Secondly, certain DNA-recognizing transcription factor can recruit HATs and HDACs to specific DNA sequences of the chromosome to achieve sequence specific actions. For example, the transcription heterodimer Max-Mad represses target gene transcription by recruitment of HDAC1 via an mSin3 scaffold protein, whereas the Max-Myc heterodimer, which does not recruit HDACs is associated with transcriptional activation (Hassig, Fleischer et al. 1997).
1.5 Histone Deacetylase Inhibitor

The first generations of HDAC inhibitors such as sodium butyrate are natural products that can morphologically reverse the transformed phenotype of the cells (Ginsburg, Salomon et al. 1973; Altenburg, Via et al. 1976; Boffa, Vidali et al. 1978). They were later identified as HDAC inhibitors based on their ability to hyperacetylate the histone. The HDAC inhibitors are found to be potent agents that can cause cell cycle arrest, differentiation and apoptosis in cancer cells and quickly emerge as potential anticancer therapeutics. Several new generations of HDAC inhibitors have been developed and many are now in phase I or phase II clinical trials for different human cancers (Byrd, Marcucci et al. 2005; Atmaca, Al-Batran et al. 2007; Gojo, Jiemjit et al. 2007; Xu, Parmigiani et al. 2007). Encouragingly, Suberoylanilide hydroxamic acid (SAHA) has recently been approved for clinical use in cutaneous T-cell lymphoma (Gallinari, Di Marco et al. 2007). While various HDAC inhibitors appear to be structurally diverse and work at different range of concentrations, crystallography studies of inhibitor-enzyme interaction reveal that they share some common features including a metal-binding domains, a linker domains and a surface recognition domain (Miller, Witter et al. 2003). Based on their chemical structure, the HDAC inhibitors are divided into five classes: short chain fatty acid, hydroxamates, benzamides, cyclic peptides and electrophilic ketones (Table 5). These HDAC inhibitors have different
specificity towards different class of HDACs. For example, the butyrate target Class I and IIa HDACs while TSA are pan-HDAC inhibitors that target both Class I and Class II HDAC. While most HDAC inhibitors target several HDACs, tubacin was identified as a HDAC6-specific tubulin deacetylase inhibitor (Haggarty, Koeller et al. 2003). These differential inhibitory activities make them powerful tools in delineating the different biology of each HDAC members.
Table 5: Molecular characteristics of HDAC inhibitors

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Range</th>
<th>target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxamate</td>
<td>Trichostatin A (TSA)</td>
<td>nM</td>
<td>Class I, II</td>
</tr>
<tr>
<td></td>
<td>SAHA (Zolinza, Vorinostat)</td>
<td>μM</td>
<td>Classes I, II</td>
</tr>
<tr>
<td></td>
<td>LBH589</td>
<td>nM</td>
<td>Classes I, II</td>
</tr>
<tr>
<td></td>
<td>LAQ824</td>
<td>nM</td>
<td>Classes I, II</td>
</tr>
<tr>
<td></td>
<td>tubacin</td>
<td>μM</td>
<td>HDAC6</td>
</tr>
<tr>
<td>Cyclic tetrapeptide</td>
<td>Depsipeptide FK228</td>
<td>nM</td>
<td>Class I</td>
</tr>
<tr>
<td></td>
<td>TrapoxinA</td>
<td>nM</td>
<td>Class I, IIa</td>
</tr>
<tr>
<td></td>
<td>Apicidin</td>
<td>nM</td>
<td>HDAC1 and 3</td>
</tr>
<tr>
<td></td>
<td>CHAPs</td>
<td>nM</td>
<td>Class I</td>
</tr>
<tr>
<td>Benzamide</td>
<td>MS275</td>
<td>μM</td>
<td>HDAC1,2,3</td>
</tr>
<tr>
<td></td>
<td>CI-994 (tacedinaline)</td>
<td>μM</td>
<td>N/A</td>
</tr>
<tr>
<td>Short-chain fatty acid</td>
<td>Butyrate</td>
<td>mM</td>
<td>Class I, IIa</td>
</tr>
<tr>
<td></td>
<td>Valproic acid</td>
<td>mM</td>
<td>Class I, IIa</td>
</tr>
<tr>
<td></td>
<td>AN-9</td>
<td>μM</td>
<td>N/A</td>
</tr>
<tr>
<td>Electrophilic ketone</td>
<td>Trifluoromethylketone</td>
<td>μM</td>
<td>N/A</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Depudecin</td>
<td>μM</td>
<td>Class I</td>
</tr>
</tbody>
</table>

Adapted and modified from (Bolden, Peart et al. 2006)
Abbreviation: CHAPs: Cyclic tetrapeptide hydroxamic acid analogues; SAHA: Suberoylanilide hydroxamic acid

1.5.1 Biological Effect of HDAC Inhibitors

1.5.1.1 HDAC Inhibitors Induce Cell Cycle Arrest

Most HDAC inhibitors except tubacin cause G1 arrest at low concentration and G1 and G2/M arrest at high concentration (Richon, Sandhoff et al. 2000; Haggarty,
Koeller et al. 2003). The cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) is one of the best studied targets induced by various HDAC inhibitors in several cancer cell types. Several nuclear HDACs can repress p21 expression while HDAC inhibitors contribute to growth arrest by causing p21 gene specific histone hyperacetylation and transcriptional upregulation of this growth inhibitory gene (Archer, Meng et al. 1998; Richon, Sandhoff et al. 2000; Sasakawa, Naoe et al. 2002).

1.5.1.2 HDAC Inhibitors Induce Growth Arrest

HDACs have also been shown to repress the expression of growth inhibitory receptor such as transforming growth factor beta type I receptor and type II receptor thereby rendering cancer cells insensitive to growth restraining signals. Conversely, HDAC inhibitors such as TSA and sodium butyrate treatment induce or restore the expression of transforming growth factor beta type I and II receptors and lead to growth inhibition (Osada, Tatematsu et al. 2001; Ammanamanchi and Brattain 2004).

Other than loss of expression of growth-restraining genes, another common feature of neoplastic growth is inhibition of differentiation. For example, silencing of GATA6 by hypoacetylation of its promoter is associated with dedifferentiation and morphological transformation of ovarian epithelial cells. Interestingly, TSA treatment restores expression of GATA factors and their target genes (Caslini, Capo-chichi et al. 2006).
1.5.1.3 HDAC Inhibitors Induce Apoptosis

Like many other chemical inhibitors, another anti-tumorigenic mechanism of HDAC inhibitors is by inducing apoptosis or autophagic cell death. The HDAC inhibitors are particularly unique because they selectively induce cell death with biochemical and morphological characteristics of apoptosis in tumor cells but not the normal counterparts (Bolden, Peart et al. 2006). It has been shown that HDAC inhibitors affect the expression of key effectors molecules in both extrinsic and intrinsic apoptosis pathways. This involve upregulation of various TNF receptor super family members and their ligands (Ininga, Monestiroli et al. 2005), upregulation of proapoptotic proteins such as Bim, Bmf, Bax, Bak and Bik (Ruefli, Ausserlechner et al. 2001), downregulation of anti-apoptotic proteins such as Bcl-2, Bcl-XL and downregulation of inhibitor of apoptosis (IAP)(Zhang, Gillespie et al. 2004). HDAC inhibitors also induce release of cytochrome c from mitochondria, activation of caspase-9, alter the cleavage of Bid and induce degradation of survivin (Xu, Parmigiani et al. 2007).

1.5.1.4 HDAC Inhibitors Curb Angiogenesis

Angiogenesis is a rate-limiting step in solid tumor growth. Not only do tumor blood vessels provide oxygen and nutrient to maintain tumor cell growth, they also serve as routes for waste disposal and frequently are conduits for tumor invasion and
metastasis. To survive hypoxic environment, tumor cells frequently evolve to upregulate hypoxia inducible factors (HIF), a transcriptional factor which becomes activated in low oxygen level to promote expression of VEGF and angiogenesis. Several HDAC inhibitors including TSA, vorinostat, FK228, butyrate and LAQ824 were found to repress HIF-1 and VEGF expression and hence angiogenesis (Deroanne, Bonjean et al. 2002; Liang, Kong et al. 2006). Other molecules playing important roles in neoangiogenesis whose expression have been shown to be attenuated by HDAC inhibitors including basic fibroblast growth factor (bFGF), angiopoietin, tunica intima endothelial kinase 2 (TIE2) and endothelial nitric oxide synthase (eNos) (Qian, Kato et al. 2006). In short, HDAC inhibitors curb tumor growth by suppressing gene expression critical for angiogenesis.

1.5.1.5 HDAC Inhibitors Are Associated with Reactive Oxygen Species

HDAC inhibitors such as vorinostat, TSA, butyrate or MS-275 have been shown to induce accumulation of reactive oxygen species (ROS) in transformed cells. ROS accumulation occurs early and before disruption of mitochondria while free radical scavengers such as N-acetylcysteine decrease HDAC inhibitors-induced apoptosis (Ruefli, Ausserlechner et al. 2001), suggesting ROS-oxidation–reduction pathways might play important roles in HDAC inhibitors-induced transformed cell death (Xu, Ngo et al. 2006). HDAC inhibitor also lead to downregulation of thioredoxin, an intracellular antioxidant scavenger of ROS and inhibitor of apoptosis signal regulating kinase 1
(ASK1) which promote apoptosis (Saitoh, Nishitoh et al. 1998). HDAC inhibitors also upregulate expression of ASK1 and Trx binding protein 2 (TBP2) (Butler, Zhou et al. 2002), and the latter could inhibit thioredoxin activity (Nishiyama, Matsui et al. 1999). Taken together, HDAC inhibitor might promote apoptosis through ROS production. However, ROS accumulation may also be a consequence of apoptosis since the pan-caspase inhibitor Z-VAD-fmk blocked MS-275-induced apoptosis as well as ROS accumulation in human chronic lymphocytic leukemia cells (Lucas, Davis et al. 2004).

Even though HDAC inhibitors have emerge as promising new cancer therapeutics and many anti-tumor mechanisms have been proposed, exactly how these inhibitors work with respect to each HDAC member remains elusive. While reversible acetylation of histone and transcriptional de-repression might contribute to the mechanism of HDAC inhibitors, it is unlikely to be the whole story. In fact, according to a phylogenetic analysis of HDACs in fully sequenced free-living organisms including human, eubacteria, and archaebacteria, Gregoretti et al have found that all three classes of classic HDACs exist in eubacteria which are devoid of histones, indicating that evolution of classic HDACs precedes that of histones (Gregoretti, Lee et al. 2004). In other words, the primary targets of at least some “histone deacetylase” enzymes are indeed non-histone substrates. The therapeutic potential and contribution of these less well-studied non-histone, non-nuclear acetylated proteins in oncogenic transformation
is therefore a fascinating question. This is the main question that my thesis work has been trying to address. Specifically, my study has focused on HDAC6, which our lab has characterized to be a cytoskeleton-associated cytoplasmic deacetylase and is found to have multiple cellular functions.

1.6 HDAC6

HDAC6 is a unique deacetylase in the HDAC family. First of all, it is the only cytoplasmic-exclusive deacetylase (Hubbert, Guardiola et al. 2002). Retention of human HDAC6 in the cytoplasm relies on two domains, a nuclear export signal (NES) located N terminus to the first catalytic domain and the SE14 domain which contain 8 consecutive tetradecapeptide repeat motifs located between the second deacetylase domain and the C-terminal ubiquitin-binding motif (Figure 3) (Bertos, Gilquin et al. 2004). The NES prevents the nuclear accumulation of the protein and the SE14 domain provides a stable anchorage of the proteins in the cytoplasm. While the NES is conserved in both humans and mice, the SE14 domain is only found in humans. This suggests that HDAC6 has evolved in humans not only to be excluded from the nucleus but also actively retained in the cytoplasm, where it most likely has certain unique and indispensable functions. (Boyault, Sadoul et al. 2007)
HDAC6 have tandem deacetylase domains, Cat 1 and Cat2. It contains a sequence identified as the dynein motor binding domain (DMB). The C terminus of HDAC6 contains an ubiquitin-specific protease like zing finger, or the binder of ubiquitin zing finger domain (BUZ domain). Two sequences are associated with cytoplasmic retention of human HDAC6, a conserved nuclear export signal (NES) in mouse and human that mediate an active export of the protein from the nucleus and a SE14 domain which anchors HDAC6 in the membrane and only seen in human (Bertos, Gilquin et al. 2004).

Secondly, HDAC6 has duplicate deacetylase catalytic domains. Self-association by protein folding in human and yeast class II HDACs and dimerization/oligomerization of human class I HDACs is very common and is an ancient feature of this enzyme family. This phenomenon is uniquely required for enzymatic function of HDACs. Interestingly, HDAC6 has two catalytic domains which might have evolved to conserve this self-association (Gregoretti, Lee et al. 2004). In fact, the duplication of the catalytic domain become a signature of HDAC6 to identify orthologs in other species such as *Drosophila melanogaster* (Barlow, van Drunen et al. 2001), *Canenorhadditis elegans* and *Arabidopsis thaliana* (Pandey, Muller et al. 2002). However, the characterization of deacetylase activity of individual catalytic domain has yielded conflicting results from different investigators. Using both histone or tubulin deacetylase activity as a readout,
Grozinger et al showed that each domain function independently of each other (Grozinger, Hassig et al. 1999), whereas Zhang et al concluded that both domains or the integrity of the whole HDAC6 are required for HDAC and TDAC activity (Zhang, Li et al. 2003; Zhang, Gilquin et al. 2006). Furthermore, Zou et al and Haggarty et al found that the second catalytic domain is more critical for deacetylase activity (Haggarty, Koeller et al. 2003; Zou, Wu et al. 2006).

To date, three cytosolic acetylated proteins, tubulin (Hubbert, Guardiola et al. 2002), Hsp90 (Kovacs, Murphy et al. 2005), and cortactin (Zhang, Yuan et al. 2007) have been identified to be the substrates of reversible deacetylation mediated by HDAC6. Hyperacetylation of tubulin and cortactin due to loss of HDAC6 is associated with impaired cellular motility. Deacetylation of Hsp90 appears to regulate its association with other molecular chaperones and client proteins such as glucocorticoid receptor (Kovacs, Murphy et al. 2005), and several oncogenic kinases such as BCR-ABL and Raf-1 and is essential for maturation and functions of these proteins (Bali, Pranpat et al. 2005; Scroggins, Robzyk et al. 2007).

Thirdly, HDAC6 has an ubiquitin binding domain. This cysteine- and histidine-rich zinc finger-containing domain is termed the binder of ubiquitin zing finger domain (BUZ domain). This domain is also present in several other ubiquitin-specific proteases (Seigneurin-Berny, Verdel et al. 2001; Boyault, Gilquin et al. 2006) and is capable of
binding to both monoubiquitin and polyubiquitin (Hook, Orian et al. 2002; Boyault, Gilquin et al. 2006). In association with the dynein motor binding domain (DMB) situated between the two catalytic domains, the BUZ domain binds polyubiquitinated proteins aggregates and transports them along the microtubule tract to the aggresome, a pericentriolar structure serve as deposition center for misfolded proteins (Kawaguchi, Kovacs et al. 2003). Further clearance of these aberrant protein aggregates involve autophagy, a highly conserved cellular mechanism that is responsible for turnover and recycling of long-lived cytosolic proteins and damaged organelles. HDAC6 is also required for autophagy activation (Pandey, Nie et al. 2007). Both HDAC6-dependent aggresome formation and autophagy require deacetylase activity and have been shown to be cytoprotective for cells dealing with protein misfolding, particularly in the context of neurodegenerative diseases (Kawaguchi, Kovacs et al. 2003; Pandey, Nie et al. 2007). Additionally, the BUZ domain has been shown to be important for activating heat shock transcription factor, HSF-1 in response to protein misfolding stress (Boyault, Zhang et al. 2007). Therefore, HDAC6 appears to play a central role in dealing with cellular stress (Boyault, Sadoul et al. 2007; Matthias, Yoshida et al. 2008). In fact, several heat shock proteins become overexpressed in an adverse microenvironment that is low in pH and oxygen (as in a tumor), as well as in cells that are stressed by overproduction of
oncoproteins which are frequently mutated and highly susceptible to misfolding (such as the cancer cells) (Ciocca and Calderwood 2005).
2. Materials and Methods

Plasmids

\textit{pBabepuro-ErbB2/HER2/neu} was made by subcloning from \textit{PCI- ErbB2/HER2/neu} into SnaBI/SalI sites of \textit{pBabe-puro} empty vector (Morgenstern and Land 1990). \textit{PCI-ErbB2/HER2/neu} was kindly provided by Dr. Jeffrey Marks (Duke University).

\textit{pBabepuro-flag- H-Ras^{G12V}} was kindly provided by Dr. Christopher Counter (Duke University).

\textit{pBabe-neo-SV40 early region} was kindly provided by Dr. Christopher Counter (Duke University).

\textit{pSuperRetroPuro-RalBscram} (Lim, O'Hayer et al. 2006) was kindly provided by Dr. Christopher Counter (Duke University).

\textit{pSuperRetroPuro-HDAC6 137i} (Kawaguchi, Kovacs et al. 2003) was created according to the manufacturer’s protocol (Oligoengine) by annealing pairs of complimentary oligonucleotides bearing the 21-mer sequence AATCTAGCGAGGTAAAGAAG
(targeting 21 nucleotides starting from position 137 of HDAC6 cDNA) and subcloning into pSuperRetroPuro (Oligoengine) digested with BgII/HindIII.

*pSuperRetroGFP/Neo-HDAC6 137i* were created by subcloning the HindIII/EcoRI fragment of *pSuperRetroPuro-HDAC6 137i* into the same site of pSuperRetroGFP/Neo (Oligoengine).

*pSuperRetroPuro-tetoHDAC6 194i* was created by annealing using pairs of complimentary oligonucleotides bearing the 21-mer sequence AAGACCTAATCGTGAGACTGC (targeting 21 nucleotides starting from position 194 of HDAC6 cDNA) and subcloning into pSuperRetroPuro-teto digested with BgII/HindIII. pSuperRetroPuro-teto was kindly provided by Dr. Christopher Counter (Duke University).

*pSuperRetroGFP/Neo-tetoHDAC6 194i* were created by subcloning the HindIII/EcoRI fragment of *pSuperRetroPuro-tetoHDAC6 194i* into the same site of pSuperRetroGFP/Neo (Oligoengine).

*pBabebleo-flag-HDAC6-Mis-WT* were created by subcloning from *pBabePuro-flag-HDAC6-Mis-WT* into pBabebleo empty vector. *pBabePuro-HDAC6-Mis-WT* was created by
subcloning from \(pBJ\)-flag-\(Mis\)HDAC6-WT into pBabepuro empty vector. \(pBJ\)-flag-\(Mis\)HDAC6-WT was created by introducing silent mutations (G237C, G240A and A243C) into \(pBJ\)-flag-HDAC6 sequences that were targeted by HDAC6 137i. \(pBJ\)-flag-HDAC6 was kindly provided by Dr. Stuart L Schreiber (Harvard University).

\(pBabebleo\)-flag- \(Mis\)HDAC6-CD were created by subcloning from \(pBabepuro\)-flag-HDAC6-Mis-CD into pBabebleo empty vector. \(pBabepuro\)- \(Mis\)HDAC6-CD was created by subcloning from \(pBJ\)-flag-\(Mis\)HDAC6-CD into pBabepuro empty vector. \(pBJ\)-flag-\(Mis\)HDAC6-CD was created by introducing silent mutations (G237C, G240A and A243C) into \(pBJ\)-flag-HDAC6-CD sequences that were targeted by HDAC6 137i. \(pBJ\)-flag-HDAC6 CD which had two catalytic sites mutants (\(H216A\) or \(H611A\)) was kindly provided by Dr. Stuart L Schreiber (Harvard University).

**Cell culture**

All cells are maintained at 37 °C with 5% CO2. Mouse embryonic fibroblasts (MEF) were isolated from E14.5 wild type and HDAC6 null embryo (Gao, Hubbert et al. 2007). MEFs were maintained and propagated in Dulbecco’s Modified Eagle’s Medium (Invitrogen) and 10% fetal calf serum (Hyclone). MEFs were immortalized by stably expressing the early region of SV40 encoding large T-Ag and small t-Ag. Human
ovarian cancer cell lines SKVO3, human breast cancer cell lines SKBR3, MCF7 and human epidermoid carcinoma cell line A431 were obtained from Duke Cell Culture Facility and maintained in RMPI1640 or Dulbecco’s Modified Eagle’s Medium (Invitrogen) (for A431) supplemented with 10% fetal bovine serum. Human embryonic kidney (HEK) cells were kindly provided by Dr. Christopher Counter (Duke University) and maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum. Human mammary epithelial Cells (HMECs) (Kendall, Linardic et al. 2005; Ancrile, Lim et al. 2007) were kindly provided by Dr. S. DiSean Kendall (Duke University) and Dr. Christopher Counter (Duke University). HMECs were maintained in MEGM serum free media supplemented with SingleQuots Kit (Lonza) as recommended. Human prostate epithelial cells (PrECs) (Berger, Febbo et al. 2004) were kindly provided by Dr. Phillip Febbo and were maintained in PrEGM serum free media supplemented with BulletKit (Lonza) as recommended. 293TS cells, a clone of the telomerase positive, Ad5 E1 and SV40 T-antigen transformed HEK cell lines were kindly provided by Dr. Christopher Counter (Duke University) and maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum.

To create stable human cell lines, the indicated cell lines were infected with amphotrophic retroviruses derived from aforementioned pBabe or pSuper plasmids or
with appropriate vector controls. Viruses were generated by transiently transfecting 293TS cells at 40% confluence with 4μg pBabe or pSuper plasmids and 4μg of the pCL-10A1 packaging plasmid using 15 μl FUGENE 6 (Roche Applied Science) diluted in 300 μl serum free Dulbecco’s Modified Eagle’s Medium (Invitrogen). To create stable murine cell lines, the indicated cell lines were infected with amphotrophic retroviruses derived from aforementioned pBabe plasmids or with appropriate vector controls. Viruses were generated by transiently transfecting Phoenix cells at 40% confluence with 6μg pBabe using 15 μl FUGENE 6 (Roche Applied Science) diluted in 300 μl serum free Dulbecco’s Modified Eagle’s Medium (Invitrogen). Approximately 24 hours after transfection, media were replaced with 7 ml of Dulbecco’s Modified Eagle’s Medium/ 10% fetal bovine serum (Invitrogen). Approximately 36 to 48 hours after transfection, media were passed through 0.45 μm filter, supplemented with polybrene to final 8 μg/μl and infect cells of interested by incubating the cells seeded in a 10 cm plate at 40-50% confluence. Approximately 24 hours after infection, the media containing viruses were replaced by full media for cells of interest. Selection of polyclonal population was achieved by treating the cells with either 2 μg/ml puromycin (Sigma) in human cells, 4 μg/ml puromycin (Sigma) in murine cells, 1mg/ml of G418 (Invitrogen) or 4.5 μg/ml Blasticidin (Invitrogen) beginning 36-48 hours after infection.
**Immunoblotting**

Cells were lysed in freshly made cold lysis buffer (20 mM Tris-HCl at pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, 1% Triton X-100) supplemented with phosphatase inhibitors cocktails (Sigma), protease inhibitors cocktails (Sigma), 0.2 mM Na$_3$VO$_4$ and 1mM NaF for 10 minutes. The lysate were then centrifuged with tabletop microcentrifuge at 12000 rpm for 15 minutes at 4 °C. The supernatant were collected and the protein concentration were determined using Bradford assay (Biorad). 4XSDS loading buffer (Biorad) and 20x reducing reagent (Biorad) were added and the samples were boiled for 10 minutes. The samples were then separated on 8% or 10% SDS-PAGE mini-gel ran at 150-200 volts for about 1.5 hours at room temperature. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (millipore) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 1.5 mA/ cm$^2$ for 2 hours. The membranes were blocked in 2% non-fat milk (Carnation) in PBST (Phosphate Buffered Saline, pH 7.4-0.02% Tween 20) buffer for 30 minutes at room temperature. The membranes then were immunoblotted with the following antibodies diluted according to the manufacture’s recommendation in 10ml PBST or 1% milk-PBST plus 0.02% sodium azide overnight at 4 °C or 2 hours at room temperature while rocking: anti-α-tubulin (Sigma), anti-acetylated-α -tubulin (Sigma, 6-11-B-1), anti-β-actin (Sigma, AC-15), anti-HDAC6 antibody(Santa Cruz, H300), anti-mouse HDAC6 antibody (Gao,
Hubbert et al. 2007), anti-Akt (Cell Signaling Technology, H300), anti-phospho(Ser 473)-Akt (Cell Signaling Technology, Catalog #9271), anti-ERK1/2 (K-23, Santa Cruz), anti-phospho(Thr 202/Tyr 204)-p42/44 ERK (Santa Cruz, E10), anti-MEK 1/2 (Cell Signaling Technology, L38C12 ), anti-phospho (Ser217/221)-MEK ½ (Cell Signaling Technology, Catalog #9106), anti-c-ErbB2/c-Neu (Oncogene, Ab3), anti-Pan-Ras (Oncogene, Ab4), anti-c-Raf (Upstate, Catalog#04-412), anti-Large T antigen (Santa Cruz, pAb 101). The membranes were washed with more than 30 ml of PBST three times, 10 minutes each at room temperature, followed by goat anti-rabbit (1:5000) or goat-anti-mouse (1:5000) horseradish peroxidase-conjugated antibodies (Promega) diluted in 10 ml PBST for 30 minutes at room temperature while rocking. The membranes were washed with more than 30 ml of PBST for three times, 10 minutes each at room temperature. Proteins were detected with ECL reagent (Amersham Pharmacia Biotech) in accordance with the manufacturer’s protocol.

To examine activation of MAPK pathway and PI3K pathway under adherent culture conditions, confluent cells (10cm plate) were serum starved by washing with PBS for three time and culture in 0.1% or serum free RPMI-1640 for overnight, then stimulated with 100 μg/ml EGF for indicated time. For termination of stimulation, the cells were cooled on ice and collected and washed with PBS for three times by centrifuge
with table top centrifuge at 1000 rpm for 5 minutes at 4 °C. The cells were lyzed as described previously.

**Immunoprecipitation**

Cells were lysed in freshly made cold lysis buffer (20 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40) supplemented with 1mM PMSF, 1mM dithiotheretiol, 1μg/ml leupetin, 2 μg/ml aprotinin, 0.2 mM Na$_3$VO$_4$ and 1mM NaF for 10 minutes. The lysates were then centrifuged with tabletop microcentrifuge at 12000 rpm for 15 minutes at 4 °C. The supernatants were collected and the protein concentrations were determined using Bradford assay (Biorad). 2mg of proteins in 200-500 μl were incubated with 3μg of mouse anti-c-ErbB2/c-Neu (Calbiochem, Ab5) in 1.5 ml microcentrifuge tubes for 3 hours at 4 °C while rotating. 50 μl of protein G Sepharose beads (Roche) 1:1 slurry in lysis buffer were then added to the lysate/antibodies mix and rotated for additional one hour. The lysate/antibodies/beads mixtures were collected by gentle centrifugation and washed with 1ml lysis buffer for four times at 4 °C. After removing any trace amount of the wash buffer in the final wash, 30 μl of 2x SDS sample buffer was added to each sample which was then boiled for 10 minutes. The samples were then subjected to SDS-PAGE and immunoblotting as previously described.
**Immunostaining and fluorescence microscopy**

SKBR3 cells were plated on cover slips in 6 well plates for one day. The cells were fixed in 1ml of 4% formaldehyde in PBS for 15 minutes at room temperature, washed twice with 2ml of PBS and then permeabilized with 2ml of PBS with 0.2% Triton X-100 for 5 minutes. After blocking in PBS with 0.1% TritonX-100 containing 10% heated normal goat serum, cells were incubated with the anti-c-ErbB2/c-Neu (Calbiochem, Ab2) in 1:100 dilution in PBS with 0.1% TritonX-100 for overnight at 4 °C. Following three 5 minutes washes with 2 ml of PBS with 0.1% TritonX-100, the coverslips were incubated with FITC conjugated rabbit anti-mouse antibody (Jackson Immuno Research Laboratories) while protected from light for 20 minutes, followed by three 5 minutes washes with 2 ml of PBS with 0.1% TritonX-100. The cover slips were mounted onto slides using a mounting solution of 90% glycerol with 1mg/ml p-phenylenediamine (Sigma). Laser-scanning confocal microscopy was performed on a Zeiss LSM410 with krypton–argon and helium–neon lasers.
Soft-Agar Assay

Five thousand cells per 3 cm plate were suspended in soft agar as described (Cifone and Fidler 1980) and colonies greater than 30 cells were scored after 3–4 weeks. Assays were done in triplicate and three times independently.

Cell proliferation assay in monolayer culture

For SKOV3 ovarian cancer cells, five thousand cells per 24-well plate were seeded and maintained in RPMI-1640 with 10% fetal bovine serum in quadruplet. Fresh media were replaced every other day. Viable cells which are Trypan blue negatives were counted daily with hematocytometer for 5 days. For MEF transduced with SV40 early antigen and human HER2, 2500 cells per 12-well plate were seeded and maintained in DMEM with 10% fetal calf serum in triplet. Fresh media were replaced every other day. Viable cells which are Trypan blue negatives were counted daily with hematocytometer for 3 days. For low serum cell proliferation, MEF transduced with SV40 early antigen and human HER2, 2500 cells per 12 well plate were seeded and maintained in DMEM with 10% fetal calf serum in triplet. The next day after seeding, the cells were washed with sterile PBS for three times and then replenished with DMEM with 0.3% fetal calf serum. Fresh low serum media were replaced every other day.
Anoikis Assay

Anoikis were induced by culturing the cells in Petri dishes coated with Poly(hydroxyethyl methacrylic) acid, or polyHEMA as described previously (Frisch and Francis 1994). polyHEMA (Sigma) was first dissolved in 95% ethanol to a final concentration of 120mg/ml. Before coating the plate, polyHEMA solution was further diluted to 12 mg/ml with 95% ethanol. polyHEMA-coated plate was prepared by drying 1.5 ml of 12mg/ml polyHEMA solution in 6 cm Petri dish in 37 °C incubator for more than 48 hours. The coated plates were washed with 2 ml of PBS for three times before use.

To examine activation of MAPK pathway and PI3K pathway under anchorage-independent conditions, confluent cells in 10cm plate were washed with PBS for three times, then serum starved by culture in 0.1% or serum free RPMI-1640 for overnight, trypsinized with 0.05% Trypsin with EDTA (Invitrogen), washed with PBS for twice, resuspended in serum free RPMI-1640 and transferred to three 6cm Poly(hydroxyethyl methacrylic) acid, or polyHEMA-coated Petri dishes for one hour, then stimulated with 50 μg/ml EGF for indicated time. For termination of stimulation, the cells were cooled on ice and collected and washed with PBS for three times by centrifuge with table top centrifuge at 1000 rpm for 5 minutes at 4 °C. The cells were lysed as discribed in Immunoblotting.
For anoikis induction, subconfluent cells were trypsinized, washed and transferred to polyHEMA-coated Petri dishes at 25000 cells/ml in serum free RPMI-1640 media for indicated time. Cells were collected and washed with PBS for three times by centrifuge with table top centrifuge at 1000 rpm for 5 minutes at 4 °C. Apoptotic cells were then examined using Annexin V Apoptosis Detection Kit (BD Pharmingen) and cells negative for both 7-AAD and Annexin V were considered to be resistant to anoikis.

Xenograft tumorigenesis Assay

All xenograft experiments using immunocompromised SCID/Beige mice (Charles River Laboratory) were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. For xenograft tumorigenesis assay, ten million cells of the indicated cell lines were mixed with Matrigel and injected subcutaneously into both flanks of two SCID/Beige mice. Tumor volumes were determined approximately twice per week and calculated as 1/2 length² × width in unit of mm³.

Reculturing Harvested Tumor Xenografts

Freshly excised tumors were rinsed once with PBS to eliminate blood and mouse tissue, immediately minced with sterile razor blade under sterile condition and trypsinized for 2 hr at 37°C. The tumor tissues were then passed through 18-gauge
needles, washed, and plated in RPMI/10% FBS plus puromycin at least 4 days to eliminate all murine cells before immunoblot analysis was performed.

Two-stage skin carcinogenesis

For chemical carcinogenesis, the backs of 5 male control (wild type) SvEv/Black Swiss mice and 10 male experimental (HDAC6 null) (Gao, Hubbert et al. 2007) SvEv/Black Swiss mice were shaved, and the following day, 150 μL of 125 μg/mL DMBA (Sigma) in DMSO were applied topically, followed 1 week later by twice-weekly topical applications of 150 μL of 10⁻⁴ M TPA (Sigma) in DMSO for 20 weeks. Tumor number and size were recorded weekly. Student’s t-test was used to compare tumor growth in the various models. The differences between means were considered significant if P < 0.05.

Immunohistochemistry

Excised tumors were fixed in 10% neutral buffered formalin for overnight, followed by transfer to 75% alcohol. The fixed tumors were then embedded in paraffin and sectioned (5μm). Ki67 (α-Ki67 antibody; Vector Laboratories), and CD31 (α-PECAM-1 Ab; Santa Cruz Biotechnology) immunohistochemistry and the TUNEL assay (ApopTag+ Peroxidase in situ Detection Kit; Chemicon International) were performed on
the indicated tumor sections with an ABC staining system (Vector Laboratories) using avidin-biotinylated-peroxidase detection method according to manufacturer’s protocol. The sections were then mounted and observed by light microscopy.

**RT–PCR**

Total RNA was isolated from indicated cells using RNeasy Mini Kit (Qiagen), DNase-treated using DNA-free kit (Ambion) according to manufacturer’s protocol. 1 μg RNA was used for cDNA synthesis reaction using Iscript Reverse transcriptase kit (BioRad). 10% of cDNA was used per RT-PCR reaction. RT-PCR Program was 94° C 20 s, 48° C 20 s, 72° C 20 s for 40 cycles. RT-PCR primer sequences are as follows: HDAC6: forward, 5'-TCA GGT CTA CTG TGG TCG TT, reverse, 5'-TCT TCA CAT CTA GGA GAGCC; G-actin: forward 5'-ACCCAGGCATTGCTGACAGGATGC, reverse, 5'CCATCTAGAAGCATTTCGGTGGACG
3. The Cytoplasmic Deacetylase HDAC6 is Required for Efficient Oncogenic Tumorigenesis \textit{in vitro} and \textit{in vivo}

\subsection*{3.1 Introduction}

HDACs are a family of enzymes that were initially characterized as histone deacetylases. Reversible histone acetylation on lysine residues is a dynamic and highly regulated post-translational modification which plays a central role in gene regulation and chromatin remodeling (Wade, Pruss et al. 1997). Accordingly, HDACs have been extensively and almost exclusively studied for their roles in transcriptional regulation and chromatin remodeling. HDACs have drawn intense research interest as inhibitors for these enzymes display potent anti-tumor activities and induce cancer growth arrest or cell death (Drummond, Noble et al. 2005). Many HDAC inhibitors are at various stages of clinical trials for cancer patients (Byrd, Marcucci et al. 2005; Atmaca, Al-Batran et al. 2007; Gojo, Jiemjit et al. 2007) and suberoylanilide hydroxamic acid (SAHA) has been approved for the clinical use in advanced, refractory cutaneous T-cell lymphoma (Gallinari, Di Marco et al. 2007). Despite the potent activity of these compounds, the fundamental question of how HDAC inhibitors achieve their anti-tumor effect remains poorly understood. This is due in part to the existence of at least eleven classical HDAC family members in the human genome (Verdin, Dequiedt et al. 2003). To date, it is not known which of the classical HDAC family members are the critical targets that underlie
the anti-tumor activity of HDAC inhibitors. This knowledge not only would pave the way for the development of more selective and less toxic inhibitors by targeting specific HDAC members, but it would also elucidate the biological pathways critical for HDAC inhibitors to treat tumors, thereby facilitating rational design for future cancer therapy.

The well-established role for HDACs in histone acetylation and gene transcription has led to a general assumption that HDAC inhibitors achieve their therapeutic effects by affecting specific transcriptional programs important for proliferation and apoptosis (Archer, Meng et al. 1998). However, the model that HDAC inhibitors work solely by affecting gene transcription is likely an over-simplified one. Recent studies have clearly demonstrated that some HDAC members are localized to the cytoplasm and regulate acetylation of non-nuclear proteins (Kawaguchi, Kovacs et al. 2003; Bali, Pranpat et al. 2005; Glozak, Sengupta et al. 2005; Gao, Hubbert et al. 2007; Zhang, Yuan et al. 2007). Whether the non-genomic functions regulated by HDACs are important in oncogenesis, however, remains unknown.

Among the expanding non-genomic functions regulated by HDACs, those controlled by HDAC6 are best characterized. Unlike the extensively studied nuclear HDACs involved in gene regulation, HDAC6 localizes exclusively to the cytoplasm where it associates with microtubule and actin cytoskeleton (Hubbert, Guardiola et al. 2002; Gao, Hubbert et al. 2007). Studies have shown that HDAC6 affects cytoskeleton-dependent processes, including actin remodeling, fluid phase endocytosis
(macropinocytosis), dynamics of cell adhesion and motility (Hubbert, Guardiola et al. 2002; Gao, Hubbert et al. 2007; Tran, Marmo et al. 2007; Zhang, Yuan et al. 2007). HDAC6 has also been shown to regulate microtubule-dependent transport and processing of toxic misfolded proteins, thereby protecting cells from the toxicity of protein aggregates, a common cause of neurodegenerative diseases (Kawaguchi, Kovacs et al. 2003). Consistent with its function in the cytosol, the activities of HDAC6 are independent of histones but instead involve cytoplasmic substrates, such as tubulin and Hsp90 (Hubbert, Guardiola et al. 2002; Haggarty, Koeller et al. 2003; Kovacs, Murphy et al. 2005). Acetylation modulated by HDAC6 has been shown to regulate the formation of the molecular chaperones complex and maturation of Hsp90 client proteins, including glucocorticoid receptor (Kovacs, Murphy et al. 2005) and several oncogenic kinases (Bali, Pranpat et al. 2005; Scroggins, Robzyk et al. 2007). Taken together, HDAC6 appears to be involved in the regulation of several critical cellular functions intimately linked to cancer. A specific role for HDAC6 in oncogenic transformation, however, is not yet established. In this chapter, I explore whether HDAC6 plays a role in oncogene-driven transformation.

3.2 Results

3.2.1 HDAC6 Expression is Up-regulated in Oncogene-driven Transformation
To explore a possible role for HDAC6 in malignant transformation, I examined whether HDAC6 expression is induced in transformed cells versus their non-transformed counterparts. Specifically, primary human cells from various origins were transformed into tumor cells by serial introduction of human telomerase (hTERT), SV40 early region, which contains large T antigen and small t antigen, and finally oncogenic Ras (Hahn, Counter et al. 1999). As shown in Figure 4, introduction of SV40 early region alone induces HDAC6 proteins level by 2-3 folds in human mammary epithelial cells (HMEC) and human embryonic kidney cells (HEK). Subsequent introduction of oncogenic Ras\textsuperscript{G12V} further induces HDAC6 proteins level by about 2 fold in HEK and prostate epithelial cells (PrECs). Similarly, introduction of oncogenic Ras\textsuperscript{G12V} mutant also modestly induces HDAC6 in mouse embryo fibroblast (MEFs), when compared to control MEFs infected with empty vector. RT-PCR analysis showed that HDAC6 mRNA was up-regulated in PrEC and HEK (Figure 4 and data not shown). This up-regulation of HDAC6 in response to oncogenic transformation suggests a potential regulatory role of HDAC6 in the malignant transformation processes. Since HDAC6 is well-characterized as a tubulin deacetylase, I examined whether tubulin acetylation is affected when HDAC6 is induced in malignant transformation processes. However, the levels of acetylated tubulin are similar in both transformed and untransformed PrEC, suggesting that the potential roles HDAC6 play in the oncogenesis might not be mediated by reversible acetylation of tubulin.
Figure 4: Elevated HDAC6 expression in transformed cells.
(Upper panel) Detection of HDAC6 by immunoblot and RT-PCR in transformed HMEC, HEK, PrEC and MEF cells compared to their non-transformed counterparts. Actin serves as a loading control. The band intensity of the HDAC6 levels was normalized to the actin levels in each lane. (Lower panel) Detection of acetylated α-tubulin and α-tubulin by immunoblot in transformed PrEC compared to their non-transformed counterparts.

3.2.2 HDAC6 Is Required for Malignant Growth of Transformed Cells in vitro

To directly assess a role of HDAC6 in oncogenic transformation, we utilized mouse embryonic fibroblasts (MEFs) derived from wild type or HDAC6 null embryos. We and others have generated HDAC6 knockout mice (Gao, Hubbert et al. 2007, Zhang, 2008 #187) which are viable and phenotypically normal. These MEFs provide a well-established and genetically defined model to examine the requirement of HDAC6 in
malignant transformation. To this end, wild type and HDAC6 null MEFs were transduced with retrovirus expressing SV40 early region and subsequently assayed for anchorage independent growth in soft agar, a standard and stringent assay for malignant transformation in vitro. As shown in Figure 5, wild type MEFs gave rise to large number of colonies after retroviral transduction of Large T, small t antigens and Ras\textsuperscript{G12V} oncogenes. In contrast, transduction of the same oncogenes at similar protein expression levels into HDAC6 null MEFs results in more than 10 fold fewer colonies (Figure 5), revealing that HDAC6 is required for Ras-induced oncogenic transformation. To investigate whether HDAC6 plays a broader role in oncogene-induced transformation, we also examined ErbB2-dependent transformation in wild type and HDAC6 deficient MEFs. As shown in Figure 5, the transforming activity of ErbB2 is also impaired in HDAC6 null MEFs as shown by a reduction in soft-agar colony formation. Together, these results show that HDAC6 is required for efficient oncogene-induced transformation.
Figure 5: HDAC6 is required for anchorage-independent growth of oncogenes-driven transformed cells.

(Upper panel) Detection of mouse HDAC6, Ras and tubulin by immunoblot in the transformed wild type or HDAC6 null MEFs stably expressing early region of SV40 and oncogenic Ras\(^{G12V}\). (Lower panel) Detection of mouse HDAC6, ErbB2/HER2/neu, Large T antigen, acetylated \(\alpha\)-tubulin and tubulin by immunoblot in the transformed wild type or HDAC6 null MEFs stably expressing early region of SV40 and human ErbB2/HER2/neu. \(\alpha\)-Tubulin serves as a loading control. Photographs demonstrating anchorage independent growth of indicated polyclonal aforementioned MEFs. The bottom labels showed the average and standard deviation of the percent of the colonies growing in an anchorage independent fashion compared to wild type transformed MEF (normalized to 100%) as calculated from triplicate plates. Data are from two independent assays.
After establishing that HDAC6 is required for oncogenic transformation in primary murine fibroblasts, we asked if HDAC6 is critical to promote the transformed phenotype of established human cancer cell lines. Human cancer cell lines are used because they are derived from human tissues and therefore more clinically relevant. To this end, we stably expressed two sets of small hairpin RNA targeting different regions of HDAC6 mRNA in three well characterized lines: ovarian cancer cell line SKOV3, and breast cancer cell lines SKBR3 and MCF-7. These specific HDAC6 siRNA but not a scramble shRNA efficiently reduced HDAC6 levels and caused an increase in the level of acetylated α-tubulin in all three cancer cell lines (Figure 6A). The control and HDAC6 knockdown tumor cell lines were then subjected to a soft-agar growth assay. As shown in Figure 6B, knockdown of HDAC6 by either of the two shRNA significantly inhibits the anchorage-independent growth of these cancer cells by 5 to 30-fold. Therefore, HDAC6 is not only important for oncogenic transformation of the primary cell, but it is also required for promoting the transformed phenotypes of established cancer cell lines.
Figure 6: Deacetylase activity of HDAC6 is required for anchorage-independent growth in human cancer cells.
A. Detection of HDAC6, acetylated α-tubulin and α-tubulin by immunoblot in the indicated three different human cancer cells stably expressing a scramble control sequence, two different HDAC6 shRNA (HDAC6 KD1 and HDAC6 KD2), a HDAC6 shRNA plus a vector (+vector), HDAC6 shRNA in the presence of siRNA resistant wild type HDAC6 (+HD6 WT), HDAC6 shRNA in the presence of siRNA resistant catalytically inactive HDAC6 (+HD6CD). α-Tubulin serves as a loading control.

B. Photographs demonstrating anchorage independent growth of indicated aforementioned polyclonal human cancer cells. The bottom labels show the average and standard deviation of the percent of the colonies growing in an anchorage independent fashion compared to scramble control cells (normalized to 100%) as calculated from triplicate plates. Data are from two independent assays.

3.2.3 Deacetylase Activity of HDAC6 Is Required for Anchorage-Independent Growth of Cancer Cells

We next determined whether deacetylase activity of HDAC6 is required for anchorage-independent growth of cancer cells. To this end, we reconstituted the HDAC6 knockdown cancer cell lines with shRNA-resistant wild type or catalytically inactive mutant HDAC6 (Figure 6A) and determined their ability to grow in soft agar. As shown in Figure 6A, reconstitution of shRNA resistant wild type but not catalytically inactive HDAC6 caused a decrease in the level of acetylated α-tubulin in all three cancer cell lines with HDAC6 knockdown. Moreover, reintroduction of wild type HDAC6 effectively restored colony formation of HDAC6 knockdown cancer cells (Figure 6B), demonstrating that the requirement of HDAC6 for anchorage-independent growth is specific. In contrast, HDAC6 knockdown cell lines reconstituted with a catalytically inactive mutant HDAC6 remain defective in forming colony in soft agar. These results
show that deacetylase activity of HDAC6 is required to support malignant growth of cancer cells in vitro.

### 3.2.4 HDAC6 is Required for Robust Tumorigenic Growth of Human Cancer Cells in vivo

The analyses so far support the important role of HDAC6 in oncogenic phenotype in vitro. To determine whether HDAC6 is critical for tumorigenic growth of cancer cells in vivo, SKOV3 stably expressing either an HDAC6 specific shRNA or a scramble control was subcutaneously injected into immunocompromised SCID-Beige mice, and tumor growth was followed over time. As shown in Figure 7, tumorigenic growth of HDAC6 knockdown cells was significantly retarded when compared to that of control cancer cells by a two-fold increase in latency and significant reduction in tumor volume (~4 fold at 28 days). This effect is specific as HDAC6 knockdown cells reconstituted with wild type HDAC6 but not the catalytically inactive mutant regained robust tumorigenic growth comparable to the parental cell lines (Figure 7). Analyses of tumor samples revealed that tumor derived from HDAC6 knockdown cells showed approximately two-fold reduction in Ki-67 staining, a marker for mitotic cells, indicating a reduction in tumor proliferation caused by a loss of HDAC6. Consistent with this conclusion, re-expression of wild type but not catalytically inactive HDAC6 mutant fully restored the number of Ki-67 positive cells in tumors sections (Figure 8). A similar requirement of HDAC6 for xenograft tumor growth was found in another cancer cell
line A431 (Figure 9). Importantly, tumors that eventually grew from HDAC6 knockdown cells restored HDAC6 expression (Figure 10), suggesting a strong selective pressure against tumor cells deficient in HDAC6.
Figure 7: HDAC6 is critical for tumorigenic growth of human ovarian cancer cells SKOV3 in vivo.
A. (Top) Representative subcutaneous flank tumor in mice and resected tumors 28 days after SKOV3 cells expressing the described construct were injected in mice. (Bottom) Tumor volume (mm³) and standard deviation versus time (days) of SKOV3 cells stably expressing a scramble control sequence (scram), HDAC6 shRNA (HD6KD), HDAC6 shRNA in the presence vector (HD6KD+V), HDAC6 shRNA in the presence of si-RNA resistant wild type HDAC6 (HD6KD+HD6WT), HDAC6 shRNA in the presence of si-RNA resistant enzymatically inactive HDAC6 (HD6KD+HD6CD) injected into the flanks of immunocompromised mice.

B. Tumor volume (mm³) and standard deviation versus time (days) of indicated aforementioned polyclonal SKOV3 injected into the flanks of immunocompromised mice.
Figure 8: HDAC6 is required for tumorigenic growth of human ovarian cancer cells SKOV3 in vivo.

Representative histological sections with Ki-67 staining of the aforementioned tumors from mice injected with the indicated cells. The scale bar indicates 50 µm. The graph shows the average number and standard deviation of Ki-67 positive cells from 10 different fields of 2 different tumor sections.
Figure 9: HDAC6 is critical for tumorigenic growth of human epidermoid carcinoma cells A431 in vivo.
A. Representative subcutaneous flank tumor in mice (top) and resected tumors (bottom) 63 days after A431 cells expressing the described construct were injected in mice

B. Tumor volume (mm$^3$) and standard deviation versus time (days) of A431 cells stably expressing a scramble control sequence (scram), HDAC6 shRNA (HD6KD), HDAC6 shRNA in the presence vector (HD6KD+V), HDAC6 shRNA in the presence of si-RNA resistant wild type HDAC6 (HD6KD+HD6WT), HDAC6 shRNA in the presence of si-RNA resistant enzymatically inactive HDAC6 (HD6KD+HD6CD), injected into the flanks of immunocompromised mice.

![Figure 10: Re-expression of HDAC6 in HDAC6 knockdown cells in xenograft.](image)

Detection of HDAC6, acetylated $\alpha$-tubulin, and tubulin by immunoblot in the SKOV3 human ovarian cancer cells stably expressing either a scramble control sequence or HDAC6 shRNA and the same cells recultured from xenograft tumors. $\alpha$-Tubulin serves as a loading control.

**3.2.5 HDAC6 Null Mice Are Resistant to Carcinogen-Induced Skin Tumors**

Finally, we determined whether HDAC6 is required for carcinogen-induced spontaneous tumorigenesis. Topical application of the carcinogen 7,12-
Dimethylbenzanthracene (DMBA), followed by repetitive application of 12-O-tetradecanoylphorbol-13-acetate (TPA) induces papillomas with a high level of Ras mutations (Quintanilla, Brown et al. 1986). We treated wild type and HDAC6 null mice with topical DMBA and then TPA for 20 weeks and monitored for tumor growth. Consistent with previously published results (Reiners and Singh 1997; Scott, Moore et al. 2003), skin tumors appeared within 9 weeks after initiation of treatment in wild type mice with an average of 6 tumors per mouse by termination of the experiment (Figure 11). Significantly, in HDAC6 null mice, the appearance of tumors was delayed by 2 weeks with an average number of 3 tumors per mouse at the termination of the experiment. Furthermore, the average volume of the tumors at week 20 was three-fold smaller in HDAC6 null mice when compared to the wild-type mice ($P < 0.05$) (Figure 11). We conclude that loss of HDAC6 impedes spontaneous formation of carcinogen-induced tumors.
Figure 11: HDAC6 null mice are resistant to carcinogen-induced skin tumors.
A. Reduction of spontaneous tumors in \textit{HDAC6} null mice. Representative mice of the indicated genotype at 20 weeks (Arrow head: tumor). Percentage of wild type and \textit{HDAC6} null mice with tumors versus time after initial application of DMBA (weeks).

B. Reduction in the number of tumors in \textit{HDAC6} null mice. Mean number of tumors per wild type and \textit{HDAC6} null mouse versus time after initial application of DMBA (weeks). (*) $P < 0.05$

C. Reduction in tumor volume in \textit{HDAC6} null mice. Mean tumor volume per wild type and \textit{HDAC6} null mouse versus time after initial application of DMBA (weeks). (*) $P < 0.05$

### 3.3 Discussion

In this chapter, I have presented evidence that HDAC6 is required for efficient oncogenic transformation, anchorage-independent growth, and tumor growth. Inactivation of HDAC6 by genetic ablation or specific siRNA renders cells more resistant to oncogene-driven transformation markedly reduces tumor cell growth \textit{in vitro} and \textit{in vivo}. Supporting these observations, we found that \textit{HDAC6} null mice are more resistant to chemical carcinogen-induced skin tumor formation. Importantly, we found that deacetylase activity is required for HDAC6 to support malignant tumorigenic growth, suggesting that pharmacological inhibition of HDAC6 enzymatic activity could potentially confer an anti-tumor effect. Our study provides the first experimental evidence that a specific HDAC member is required for autonomous cancer cell growth of solid tumors both \textit{in vitro} and \textit{in vivo} and indicates that HDAC6 could be a therapeutic target for cancer treatment.
Although the analysis of HDAC6 in human cancer remains scarce, HDAC6 expression has been shown to be up-regulated in primary oral squamous cell carcinoma tissue and cell lines. Elevated levels of HDAC6 were also documented in primary acute myeloid leukemia blasts, several myeloblastic cell lines and some human breast cancers (Yoshida, Omoto et al. 2004; Bradbury, Khanim et al. 2005; Saji, Kawakami et al. 2005; Sakuma, Uzawa et al. 2006). Interestingly, in breast cancer cell lines, HDAC6 was identified as an estrogen regulated gene. HDAC6 was proposed to play a role in estrogen-induced breast tumor cell motility and invasiveness (Yoshida, Omoto et al. 2004; Saji, Kawakami et al. 2005). In fact, HDAC6 is required for estrogen-induced proliferation in MCF7 cells (Itoh, Suzuki et al. 2007). The potential importance of HDAC6 in ER-dependent tumor phenotype is supported by a clinical study of breast cancer patients, which revealed that HDAC6 levels positively correlate with a favorable response to anti-estrogen tamoxifen treatment (Saji, Kawakami et al. 2005). These findings are consistent with our conclusion that HDAC6 plays an important role in maintaining tumor growth and are suggestive of a potential utility for targeting HDAC6 in breast cancer.

For decades, protein phosphorylation/dephosphorylation controlled by oncogenic kinases has been the primary target for therapeutic manipulation in cancer treatment. The efficacy of HDAC inhibitors in model systems and clinical trials points to the potential importance of reversible protein acetylation and HDAC in oncogenic
signaling. The anti-tumor activity of HDAC inhibitors has been mostly attributed to their effects on gene expression and chromatin dynamics. However, the identification of non-nuclear HDAC members and a large number of non-nuclear acetylated proteins (Kim, Lee et al. 2006) has raised a critical question as to whether a non-genomic mechanism plays an important role underlying the therapeutic effect of HDAC inhibitors.

Overall, the data presented in this chapter indicated that HDAC6, a cytoplasmic deacetylase family member, is required for full and autonomous tumorigenic growth. This finding highlights the potential importance of non-genomic targets in the anti-tumor activity of HDAC inhibitors. These conclusions also strongly indicate that, similar to protein phosphorylation, reversible protein acetylation occurring outside the nucleus could play an important role in cancer biology.
4. HDAC6 Regulates Tumor Cell Growth and Survival

4.1 Introduction

In this chapter, I characterized the cell biology underlying the facilitative role of HDAC6 in oncogenesis, with particular emphasis on proliferation and survival which are the two most important features in tumor growth. I also explored the molecular mechanisms that mediated the tumor promoting effect of HDAC6 with particular emphasis on the molecular chaperone Hsp90 that is also critical in oncogenesis.

Hsp90 is abnormally up-regulated in many human tumors, and specific Hsp90 inhibitors such as 17-allylamino-17-demethoxygeldanamycin (17-AGG) induce destabilization of Hsp90 client proteins such as ErbB2 and Raf-1 and show anti-tumor activity against human breast cancer in murine xenograft models (Solit, Basso et al. 2003). While Hsp90 has emerged as a promising target in cancer treatment, little is known about how its activity is regulated to facilitate oncogenesis. Interestingly, evidence suggests that malignant transformation is accompanied by a dramatic reorganization of Hsp90 into a stable chaperone complex with its co-chaperones (Kamal, Thao et al. 2003). These observations suggest that Hsp90 is uniquely regulated in neoplastic cells. Understanding this process could provide valuable information for developing a mean to disrupt such regulation and thereby impair tumorigenesis driven by Hsp90 client oncoproteins.
One potential regulation that dictates Hsp90 activity is reversible acetylation. It has been reported that Hsp90 is an acetylated protein, and that the acetylation status of Hsp90 might govern its capacity for binding to client proteins (Yu, Guo et al. 2002). Remarkably, deacetylase inhibitor treatment in SKBR3 breast cancer cells increases Hsp90 acetylation with concurrent reduction in the levels of ErbB2 and Raf-1, two oncoproteins which are well-known to be chaperoned by Hsp90 (Yu, Guo et al. 2002). This observation indicates that Hsp90 acetylation might be important for regulating the stability and/or activity of certain oncogenic client proteins critical for cancer formation. To characterize the regulation and importance of Hsp90 acetylation, our lab has identified HDAC6, a member of the histone deacetylase family, as a bona fide deacetylase for Hsp90 (Kovacs, Murphy et al. 2005). We have found that HDAC6 can associate with Hsp90. Over-expression of HDAC6 leads to Hsp90 deacetylation whereas, reduction of HDAC6 by RNA interference results in an increase in Hsp90 acetylation. Functionally, using the glucocorticoid receptor (GR) as a model Hsp90 client protein, we have demonstrated that acetylation of Hsp90 decreases its interaction with the essential co-chaperone, p23, which in turn, leads to dissociation of GR from Hsp90 and failure of GR maturation and function (Kovacs, Murphy et al. 2005). These observations provide strong evidence that acetylation negatively regulates Hsp90 chaperone activity and that by deacetylating Hsp90, HDAC6 positively regulates the chaperone function of Hsp90.
4.2 Results

4.2.1 HDAC6 is Required for Robust Growth of Cancer Cells

In order to form colonies in soft agar, cells need to both grow and escape anoikis, a specific form of cell death caused by the lack of proper adhesion to basement membrane or extracellular matrix. To directly assess if HDAC6 affects tumor cell growth, control and HDAC6 knockdown SKOV3 cancer cells were analyzed in monolayer culture. As shown in Figure 12, a two-fold reduction in cell number was observed in HDAC6 knockdown cells comparable to a scramble control cell line. This is consistent with an approximate two-fold reduction in proliferation marker Ki-67 staining in tumor derived from HDAC6 knockdown cells previously shown in Figure 8. Together, these results show that HDAC6 is required for robust tumor cell growth.
Figure 12: HDAC6 is required for robust tumor cell growth in monolayer culture.

Cellular growth in monolayer culture of SKOV3 cells stably expressing a scramble control sequence, or HDAC6 shRNA in RPMI160 with 10% fetal bovine serum.

While the growth rates of primary wild type and HDAC6 null MEF were similar when they were first isolated, partial transformation of these two cells by transduction of SV40 early region that encodes large T antigen and small t antigen revealed that growth of HDAC6 null MEF was significantly slower than MEF isolated from their wild type littermates when grown in full (10%) serum. Such discrepancy in growth rate was even more dramatic with transduction of HER2 (Figure 13).
Figure 13: HDAC6 is required for robust cell growth in monolayer culture (10% serum) in transformed cells.

Cellular growth in monolayer culture in full media of MEFs from wild type and HDAC6 null embryos immortalized with transduction of SV40 early region without or with further transformation with human HER2.

When grown in 0.3% serum, overexpression of HER2 confer the ability of wild type MEFs to continue dividing in low serum, whereas the HDAC6 null MEFs either stop growing or die (Figure 14).
Figure 14: HDAC6 is required for robust cell growth in monolayer culture (0.3% serum) in transformed cells.

Cellular growth in monolayer culture in low serum (0.3% fetal calf serum) media of MEFs from wild type and HDAC6 null embryos immortalized with transduction of SV40 early region without or with further transformation with human HER2.
Figure 15: HDAC6 modulates MAPK signal transduction.

Detection of activation of MAPK and PI3K by phospho-AKT, total AKT, phospho-MEK, phospho-ERK, and total ERK by immunoblot in the MCF7 human breast cancer cells stably expressing a scramble control sequence (scram) or two different HDAC6 shRNA (HDAC6 KD1, KD2) stimulated with 100 μg/ml of EGF fro indicated time after serum starved for overnight.

The ability to sustain deregulated signaling is one of the hallmarks of cancer cells. In light of our observation that loss of HDAC6 renders cells more resistant to both Ras and Her2 oncogene-driven transformation, I examined the downstream effectors in the growth factor receptor/Ras/MAPK signal pathway. Loss of HDAC6 results in a
marked reduction in the activation MAPK signaling pathway as shown by an attenuation of phosphorylation of both MEK and ERK (Figure 15), both of which are downstream effectors of growth factor receptor/Ras/MAPK signal pathway. Together, the results in this section support that HDAC6 is required for robust cellular growth in cancer cells.

4.2.2 HDAC6 Contributes to Anoikis Resistance in Cancer Cells

Resistance to anoikis is another important cellular mechanism underlying anchorage-independent growth and is a hallmark of malignant transformation (Douma, Van Laar et al. 2004). Given the possible effect of HDAC6 on cell adhesion (Tran, Marmo et al. 2007), we examined whether HDAC6 status affects anoikis. To this end, we grew control and HDAC6 knockdown SKVO3 cells on culture plates coated with polyHEMA. PolyHEMA prevents cells from adhering to the plates and therefore allows one to test the ability of cells to survive in an anchorage-independent fashion (Frisch and Francis 1994). As shown in Figure 16, while scramble control cells retained the resistance to anoikis induction and survived, HDAC6 knockdown SKVO3 cells were significantly more susceptible to anoikis. This result indicates that HDAC6 contributes to anoikis resistance in cancer cells.
Resistance to anoikis of human ovarian cancer cells SKOV3 stably expressing a scramble control sequence (black) or two different HDAC6 shRNA (gray) plated on polyHEMA coated plates for indicated time as described in Material and Methods. Apoptotic cells were then examined and cells negative for both 7-AAD and Annexin V were considered to be resistant to anoikis. Each experiment was done in duplicate and results shown are representative of 2 independent experiments.

It has been shown that activation of PI3K/AKT and MAPK/ERK signaling pathways are critical for anoikis resistance in cancer cells (Reddig and Juliano 2005). To examine whether HDAC6 modulates these pathways during anchorage-independent growth, we compared the phosphorylation status of AKT and ERK1/2 in scramble control cells or HDAC6 knockdown cells grown in the polyHEMA-coated plates. As shown in Figure 17, HDAC6 knockdown cells showed a significant decrease (compared
to scramble control cells) in phosphorylation of both AKT and ERK1/2 in response to
growth factor stimulation, indicating that HDAC6 is required for growth factor induced
activation of MAPK and PI3K signaling cascades that could contribute to anchorage-
independent growth. Interestingly, as shown previously in Figure 15, with adherent
cultured cells, only the activation of MAPK/ERK but not PI3/AKT signaling pathway
was significantly affected in HDAC6 knockdown cells as shown by a significant
decrease in phosphorylation of MEK and ERK1/2 but not AKT in response to growth
factor stimulation, compared to vector control cells. These results suggest the important
role of HDAC6 in regulating the pro-survival AKT pathway particularly in evading
anoikis.
Detection of phospho-AKT, total AKT, phospho-ERK, and total ERK by immunoblot in the SKOV3 human breast cancer cells stably expressing a scramble control sequence (scram) or HDAC6 shRNA (HDAC6 KD) plated on polyHEMA coated plates, stimulated with 50 μg/ml of EGF for indicated time after serum starved for overnight.

4.2.3 HDAC6 Regulates Association of Hsp90 and ErbB2 in Cancer Cells

As mentioned before, Hsp90 activity is regulated, in part, at the level of chaperone complex formation. Although most Hsp90s exist as free dimers in primary and untransformed cells, a dramatic reconfiguration of Hsp90 into a stable chaperone complex was observed in cancer cells over-expressing Hsp90 client oncoproteins, such as ErbB2 (Kamal, Thao et al. 2003). In these tumor cells, Hsp90 was found to form stable complexes with co-chaperones such as p23 and p50cdc37, presumably resulting in an
increase in chaperone activity toward client proteins, such as ErbB2, thereby stabilizing them and contributing to malignant transformation. In contrast, when exposed to Hsp90 inhibitors, such as geldanamycin, Hsp90 forms a different complex with co-chaperones such as Hsp40, Hsp70 and p60\(^{\text{Hop}}\), and targets client proteins for proteasomal degradation (Isaacs, Xu et al. 2003). As discussed previously, HDAC6-mediated reversible acetylation of Hsp90 might play an important role in modulating specific complex formation between Hsp90 and its co-chaperones (Kovacs, Murphy et al. 2005). The complexes formed in response to Hsp90 acetylation status may thus dictate the subsequent fate of ErbB2. I hypothesize that in cancer cells, HDAC6 works by deacetylating Hsp90, causing it to preferentially complex with client protein ErbB2, resulting in active and stable ErbB2 that promotes malignant transformation. Conversely, inhibition of HDAC6 would cause Hsp90 to be hyperacetylated, leading to the dissociation and possible degradation of ErbB2, reverting oncogenic phenotype.

To test this hypothesis, I examined the nature of the Hsp90 molecular chaperone complex and its association with ErbB2 in cancer cells expressing shRNA against HDAC6 or a vector control. ErbB2 was immunoprecipitated from ErbB2-overexpressing SKBR3 breast cancer cell lines expressing shRNA against HDAC6 or a vector control, followed by immunoblotting for the presence of Hsp90 as well as important co-chaperones such as Hsp70 by specific antibodies. If acetylation of Hsp90 indeed regulates ErbB2 and co-chaperone interaction in breast cancer cells, it would be expected
that a readily detectable co-immunoprecipitation of Hsp90 with ErbB2 in breast cancer cells would be observed while this complex would not form stably in HDAC6 deficient cells. Instead, Hsp90 might preferentially associate with Hsp40, Hsp70 and p60\textsuperscript{Hop}. To further confirm the specificity of this result, SKBR3 breast tumor cell lines can be treated with TSA, a pan-HDAC inhibitors capable of inhibiting deacetylase activity of HDAC6 and results can be examined to reveal whether the Hsp90-ErbB2 chaperone complex is similarly disrupted. Moreover, treatment of geldanamycin, an Hsp90 inhibitor that is known to disrupt Hsp90-ErbB2 interaction can serve as a positive control (Xu, Mimnaugh et al. 2001). This series of experiments should reveal whether the Hsp90-ErbB2 interaction requires HDAC6.

As shown in Figure 18, consistent with other investigators’ results (Xu, Mimnaugh et al. 2001), inhibition of Hsp90 function with geladanamycin treatment caused a dissociation of Hsp90 from and an increased association of Hsp70 to ErbB2. A similar trend of dissociation of Hsp90 from ErbB2 was observed with either HDAC6 knockdown or TSA treatment. Association of Hsp70 to ErbB2, however, was not observed with either HDAC6 knockdown or TSA treatment in the same experiment. These results indicate that HDAC6 regulates association of Hsp90 but not Hsp70 with client proteins-ErbB2 in cancer cells.
Figure 18: HDAC6 regulates association of Hsp90 and client proteins-ErbB2 in cancer cells.

Detection of Hsp90 and Hsp70 associated with ErbB2 in SKBR3 human breast cancer cells stably expressing a vector control sequence (vector) or HDAC6 shRNA (HDAC6 KD) by using ani-ErbB2 antibody to immunoprecipitate ErbB2 and then immunoblot for Hsp90 and Hsp70 in the absence or presence of 5μM TSA or 3μM geldanamycin for 4 hours.

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**4.2.4 Stability of ErbB2 is not Affected by Loss of HDAC6 in SKBR3 Cancer Cell Lines**

Hsp90 inhibitors cause polyubiquitination and subsequent depletion of mature and nascent ErbB2 proteins in breast cancer cells (Mimnaugh, Chavany et al. 1996; Xu,
Mimnaugh et al. 2002), leading to the hypothesis that Hsp90 controls ErbB2 stability. Interestingly, HDAC inhibitors can also cause destabilization of ErbB2 (Yu, Guo et al. 2002). After characterizing the role of HDAC6 in Hsp90-ErbB2 and chaperone complex formation, I further examined whether loss of HDAC6 would impair the ability of Hsp90 to chaperone ErbB2, leading to ErbB2 destabilization. To test this hypothesis, I performed immunoblotting of ErbB2 protein levels from the vector control breast cancer cell lines SKBR3 and their HDAC6 knockdown counterparts. As shown in Figure 18, the protein level of ErbB2 proteins is similar in both vector control and HDAC6 knockdown breast cancer cell lines SKBR3. This indicated that stability of ErbB2 is not affected by loss of HDAC6.

4.2.5 Membrane Targeting of ErbB2 is not Affected by Loss of HDAC6 in SKBR3 Cancer Cell Lines

Fully matured and functional ErbB2 is located in the plasma membrane. Hsp90 inhibitor treatment prevents ErbB2 translocation to the plasma membrane (Chavany, Mimnaugh et al. 1996) suggesting this maturation process is Hsp90-dependent. Since only properly folded ErbB2 proteins can be targeted to the plasma membrane, loss of HDAC6 may therefore impair Hsp90-dependent ErbB2 maturation and result in a decrease in membrane-bound ErbB2. To test this, I performed immunofluorescence to assess the targeting of ErbB2 to cell membrane. Specifically, I used the anti-ErbB2 antibody that only recognizes the extracellular domain of ErbB2. If HDAC6 is required
for proper maturation of ErbB2 to the membrane, a loss or attenuation of membrane ErbB2 and/or an accumulation of intracellular ErbB2 in HDAC6-deficient breast cancer cells would be expected. As shown in Figure 19, membrane staining is the dominant staining pattern in both vector control and HDAC6 deficient SKBR3 breast cancer cell lines. Also, the signal intensity is detected at equal density in both cell types. This indicated that plasma membrane localization and maturation of ErbB2 is not affected by loss of HDAC6.

![Vector control and HDAC6 KD images](image)

**Figure 19: HDAC6 does not affect the subcellular localization of ErbB2 in cancer cells.**

Detection of plasma-membrane localized ErbB2 by immunofluorescence with anti-erbB2 antibody that only recognize the extracellular domain of ErbB2 in the SKOV3 human breast cancer cells stably expressing a vector control sequence (vector control) or HDAC6 shRNA (HDAC6 KD).
4.2.6 HDAC6 Contributes to Stability of Some Hsp90 Client Proteins

Hsp90 inhibitors cause polyubiquitination and subsequent depletion of client proteins such as Raf-1, which also plays a critical role in growth factor receptor-MAPK signaling pathway. Interestingly, HDAC inhibitors can also cause destabilization of these client proteins (Yu, Guo et al. 2002). Even though stability of ErbB2 did not seem to be affected by loss of HDAC6 as discussed earlier, it is possible that the protein level of other client proteins were affected. Therefore, I examined whether loss of HDAC6 would impair Hsp90 ability to chaperone Raf-1, leading to Raf-1 destabilization. To test this hypothesis, I performed immunoblotting of Raf-1 protein levels from MCF7 human breast cancer cells and SKOV3 human ovarian cancer cells stably expressing a scramble control sequence (scram) or HDAC6 shRNA (HDAC6 KD). In both cell types, the proteins level of Raf-1 is attenuated with loss of HDAC6. This is consistent with results from other investigators in other cell types (Yu, Guo et al. 2002; Bali, Pranpat et al. 2005).
4.3 Discussion

In this chapter, I have demonstrated that HDAC6 is required for robust cellular growth and anoikis resistance of cancer cells which contributes to oncogenic transformation \textit{in vitro} and tumorigenic growth \textit{in vivo}. Signaling wise, HDAC6...
contributes to both growth factors activated PI3K/AKT and ERK1/2 signaling pathways, both of which are important in preventing anoikis and promoting cell growth but impaired in HDAC6 deficient cells.

While the specific molecular mechanism through which HDAC6 achieves such tumor promoting effect remains to be identified, the recent characterization of HDAC6 suggests several potential mechanisms by which HDAC6 could affect tumor formation. At least three HDAC6 substrates have been identified: α tubulin (Hubbert, Guardiola et al. 2002), Hsp90 (Kovacs, Murphy et al. 2005), and cortactin (Zhang, Yuan et al. 2007). Moreover, HDAC6 has also shown to involved in several actin-dependent processes such as actin remodeling, fluid phase endocytosis (macropinocytosis) and dynamics of cell adhesion (Hubbert, Guardiola et al. 2002; Gao, Hubbert et al. 2007; Tran, Marmo et al. 2007; Zhang, Yuan et al. 2007). HDAC6 has also been shown to be a sensor and effector in management of clearance of misfolded protein aggregates involved microtubule-dependent transport, ubiquitin binding, aggresome formation, autophagy induction and HSF-1 activation (Kawaguchi, Kovacs et al. 2003; Boyault, Zhang et al. 2007; Pandey, Nie et al. 2007).

4.3.1 Tubulin and Actin Cytoskeleton

Composed of tubulin heterodimers, microtubules are key components of cellular cytoskeletons critical for cell division, cell motility, intracellular trafficking, and cell morphology. Microtubules have long been the target of chemotherapeutic agents for the
past few decades. Vinca alkaloids and taxane are among the most successful and widely used chemotherapeutic agents. While vinca alkaloids are thought to induce depolymerization of microtubules, taxane induces tubulin polymerization and forms extremely stable and nonfunctional microtubules. Both agents lead to mitotic arrest and apoptosis (Jordan and Wilson 2004). As one of many regulatory mechanisms, α–tubulin could be post-translationally modified by polyglutamylation, polyglycylation, phosphorylation, acetylation and tyrosination. Inactivation of HDAC6 leads to microtubule hyperacetylation (Hubbert, Guardiola et al. 2002; Matsuyama, Shimazu et al. 2002; Zhang, Li et al. 2003), resulting in altered dynamics in microtubule stability, focal adhesion turnover and reduced motility (Hubbert, Guardiola et al. 2002; Matsuyama, Shimazu et al. 2002; Haggarty, Koeller et al. 2003; Zhang, Li et al. 2003; Gao, Hubbert et al. 2007; Tran, Marmo et al. 2007). Interestingly, taxane also potently induces tubulin acetylation (Piperno, LeDizet et al. 1987).

HDAC6 has been shown to be involved in several actin-dependent processes such as actin remodeling, fluid phase endocytosis (macropinocytosis) and dynamics of cell adhesion (Hubbert, Guardiola et al. 2002; Gao, Hubbert et al. 2007; Tran, Marmo et al. 2007; Zhang, Yuan et al. 2007). In particular, focal adhesions might be linked to the anchorage-dependent growth (Tran, Marmo et al. 2007). In this regard, the involvement of HDAC6 in anoikis caused by a loss of proper adhesion is of particular interest. Transformed cells often acquire resistance to anoikis so they can detach from the
basement membrane, invade and metastasize to distant organs (Geiger and Peeper 2007). Using various human cancer cell lines, we showed that HDAC6 activity is required for cancer cells to efficiently escape anoikis and grow in soft agar. While the specific molecular mechanism through which HDAC6 achieves such an anti-apoptotic effect remains to be identified, our analysis suggests it could involve the PI3K/AKT and ERK1/2 signaling pathways, both of which are important in preventing anoikis but impaired in HDAC6 deficient cells (Figure 17).

Interestingly, HDAC6 has also been shown to be involved in another actin-based cell adhesion like structure related to cancer invasion (ie. podosome formation) (Destaing, Saltel et al. 2003). Podosomes are most prominent in macrophages, osteoclasts and dendritic cells which are cells derived from monocytes but also have been observed in epithelial, endothelial, and even smooth muscle cells. They are required for cellular processes such as migration for monocytic cells and adhesion, migration and invasion of endothelial cells during the formation of blood vessels. They share the same molecular components as invadopodia which are protrusions at the ventral surface of invasive tumor cells where degradation of the underlying extracellular matrix take places (Linder 2007). During differentiation of osteoclasts, the patterning of podosome changes from a dynamic podosome ring in immature osteoclasts to a stable peripheral podosome belt in mature osteoclast (Destaing, Saltel et al. 2003). This transition correlated with a decrease of Rho activity and increase in acetylation of microtubule. On the other hand, activation
of Rho or mDia, a Rho effector causes deacetylation of microtubule and podosome belt disruption. Importantly, the mDia, one member of the forming-homology family has been shown to coordinate the microtubule and actin cytoskeleton (Ishizaki, Morishima et al. 2001; Palazzo, Cook et al. 2001). Moreover, there is a direct association of mDia2 and HDAC6 (Destaing, Saltel et al. 2005). These results suggest a Rho-mDia2-HDAC6 pathway that controls podosome patterns through reversible acetylation of microtubule in osteoclasts.

4.3.2 Hsp90

Hsp90 is a key molecular chaperone that is responsible for the folding of newly synthesized polypeptides and refolding of damaged proteins to insure correct conformation critical to client protein stability, activity, and function. Compared to the general chaperone Hsp70, Hsp90 only binds to a more limited selection of client proteins, many of which are oncogenic proteins such as ErbB2, Raf-1, BCR-Abl and AR. It is shown that Hsp90 is overexpressed in stressed conditions including cancers (Ciocca and Calderwood 2005). In transformed cells, many oncogenic proteins are either overexpressed or mutated or chimeric that requires Hsp90 to maintain their stability and functions. Inhibition of Hsp90 by inhibitors leads to degradation of these client proteins (Isaacs, Xu et al. 2003).

Hsp90 is post-translationally modified by phosphorylation, acetylation, ubiquitination and s-nitrosylation. We and others have shown that Hsp90 requires
HDAC6 for its full activity (Bali, Pranpat et al. 2005; Kovacs, Murphy et al. 2005; Scroggins, Robzyk et al. 2007). Inactivation of HDAC6 in cancer cells could affect oncogenic signaling thereby inhibiting tumor growth. Indeed, hyperacetylation of Hsp90 has been associated with a defect in oncogenic kinase stability and signaling (Bali, Pranpat et al. 2005). In this chapter, I have shown that interaction between Hsp90 and one of its client proteins-ErbB2 is attenuated with loss of HDAC6. Consistent with other investigators’ observation with Hsp90 inhibitors (Schulte, Blagosklonny et al. 1995), HDAC inhibitors (Yu, Guo et al. 2002) or HDAC6 knockdown (Bali, Pranpat et al. 2005), the protein level of one of Hsp90 client proteins, Raf-1, is also attenuated with loss of HDAC6.

However, the stability of ErbB2 does not seem to be affected with loss of HDAC6. In fact, this is also the case for GR. This could be due to the long half-life of ErbB2. This possibility could be addressed by a pulse-chase metabolic labeling of ErbB2 to assess the half-life of nascent ErbB2 in the presence or absence of HDAC6. It is also possible that there will be no marked change in half-life following inactivation of HDAC6. Such an observation might reflect the important activity of HDAC6 in regulating misfolded protein degradation (Kawaguchi, Kovacs et al. 2003). Therefore, loss of HDAC6 could impair the degradation of misfolded ErbB2. This possibility, however, would indicate that ErbB2 produced in HDAC6 deficient cells would likely be compromised in their maturation and activity. The membrane targeting of ErbB2 did not
seem to be affected with loss of HDAC6. To address the maturation of ErbB2, a kinase assay by immunoblotting with phosphospecific antibody against activated ErbB2 or immunoprecipitation of ErbB2 protein followed by immunoblot with phosphotyrosine antibody to access the activation of ErbB2 will be needed. Also, the misfolding of ErbB2 might be examined by testing the solubility of ErbB2 to mild detergent with or without HDAC6. If HDAC6 modulates the degradation of misfolded ErbB2, it is predicted that ErbB2 will be less soluble with loss of HDAC6.

4.3.3 Cortactin

Cortactin was first identified as a Src substrate that binds to F-actin and localizes to the cell cortex, including membrane ruffles and lamellipodia (Wu, Reynolds et al. 1991; Wu and Parsons 1993). It can stimulate actin polymerization by direct interaction with the Arp2/3 complex. The cortactin gene, also termed EMS1, (chromosome eleven, band q13, mammary tumor and squamous cell carcinoma-associated gene1), has been mapped to chromosome 11q13, a region frequently amplified in multiple types of carcinoma (Schuuring 1995). Amplification of EMS1 has been shown to correlate with lymph node metastasis and unfavorable clinical outcome (Schuuring 1995).

Experimentally, overexpression of cortactin has been shown to promote cellular motility, invasion (Patel, Schechter et al. 1998), anoikis resistance (Timpson, Wilson et al. 2007), and metastasis (Li, Tondravi et al. 2001). This is consistent with the finding that cortactin is also enriched in the “invadopodia” from invasive tumor cells, which are
cellular protrusions associated with degradation of extracellular matrix. In HDAC6 deficient cells, hyperacetylated cortactin showed reduced activity in stimulating actin polymerization, suggesting that HDAC6 might be required for cortactin to promote tumor formation as well (Zhang, Yuan et al. 2007).

4.3.4 Cytoprotective Roles of HDAC6

Another potential oncogenic role of HDAC6 is mediated by its cytoprotective functions in conformational disease that involve disposition and clearance of toxic, misfolded protein aggregates. We have previously shown that HDAC6 protects cell from toxic protein aggregates, at least in part, by facilitating the sequestration of these aggregates into an aggresome, a specialized inclusion body (Kawaguchi, Kovacs et al. 2003). Subsequent removal of aggregates by autophagy machinery (which serves as an alternative pathway to the proteasome in eliminating protein waste) also requires HDAC6 (Iwata, Riley et al. 2005; Pandey, Nie et al. 2007). Although toxic misfolded proteins have been primarily studied in neurodegenerative disease, tumor cells are likely prone to produce excessive amounts of misfolded proteins due to their high rates of metabolism (Warburg 1930), protein synthesis, and production of mutated proteins (Neckers and Neckers 2002). This might be particularly true for tumors with secretory function, such as a multiple myeloma, as secretory proteins are highly susceptible to misfolding. Put together, we suggest that loss of HDAC6 inhibits cancer cell growth by causing accumulation of toxic misfolded proteins. Indeed, combined use of proteasome
inhibitors and HDAC6-selective inhibitors has synergistic cytotoxic effects on cancer cell lines (Kawaguchi, Kovacs et al. 2003; Hideshima, Bradner et al. 2005).

HDAC6 also modulates activation of heat shock factor-1 (HSF-1) (Boyault, Zhang et al. 2007), a master transcription factor required for activation of heat shock proteins when proteasome functions are compromised and ubiquitinated proteins accumulate. HSF-1 associated with Hsp90 and this repressive complex is to be disrupted before HSF-1 becomes activated when the cells are under stress. This activation of HSF-1 is dependent on HDAC6 and one of the HDAC6 interacting proteins, p97/VCP (Boyault, Zhang et al. 2007).

In fact, the resistance to oncogene-driven transformation in vitro and two step carcinogen-induced tumor formation in vivo in HDAC6 null mice is reminiscent to HSF-1 null mice (Dai, Whitesell et al. 2007). Both HDAC6 and HSF-1 null MEFs showed a resistance to DMBA-TPA two-stage carcinogen-induce skin tumor formation, oncogenic Ras driven anchorage-independent growth, and growth factor receptor driven anchorage-independent growth (ErbB2 in our study and PDGFR in HSF-1 study). Both cells also showed a blunting in MAPK signaling and both HDAC6 and HSF-1 are required for maintenance of transformed phenotypes of established cancer cells.

Taken together, HDAC6 appears to play a critical role in coordinating cell responses to various cytotoxic events and in promoting cell survival under stressful conditions, including oncogenic transformation.
5. Conclusion and Future Directions

5.1 Conclusion

Reversible acetylation has emerged as one type of post-translational modification that might be as important as phosphorylation (Wade, Pruss et al. 1997; Cohen and Yao 2004; Yang and Gregoire 2007). Study of biological and pathological functions that reversible acetylation regulate for non-histone non-nuclear proteins has been the main research focus of our lab. HDAC6, one unique member of the deacetylase family is of particular interest in that it is exclusively cytoplasmic, has duplicate catalytic domains and ubiquitin binding domains (Hubbert, Guardiola et al. 2002; Kawaguchi, Kovacs et al. 2003; Gao, Hubbert et al. 2007). My research has focused on the role of this cytoplasmic deacetylase in oncogenic transformation. I first demonstrated an upregulation of HDAC6 in both mRNA and protein levels using various Ras-transformed human somatic cells and murine fibroblasts. I then further demonstrated that HDAC6 is an important modifier for Ras and ErbB2-driven oncogenic growth using an in vitro transformation of MEF isolated from HDAC6 null mice and wild type littermate. I have also shown HDAC6 is required for transformed growth of various established human cancer cell lines. These in vitro results were vigorously tested in vivo by tumor xenograft in immunocompromised mice and two step carcinogen-induced skin tumors using HDAC6 null mice and wild type littermates. Importantly, this in vitro
and *in vivo* transformed growth requires the deacetylase activity of HDAC6. These results provide the first experimental evidence that a cytoplasmic deacetylase is an important modifier of oncogene-driven transformation.

Mechanistically, I found that HDAC6 contributes both to robust growth and resistance to anoikis in the cancer cells. In response to growth factor stimulation, blunting of activation of MAPK/ERK signaling pathway in normal monolayer culture and both activation of PI3K/AKT and MAPK/ERK pathways in anchorage-independent growth conditions are observed with loss of HDAC6. At molecular level, HDAC6 contributes to the association between Hsp90 and oncoprotein ErbB2. However, the membrane localization and the steady state protein level of ErbB2 is not affected with loss of HDAC6. The level of other Hsp90 client oncoproteins such as Raf-1 is decreased with loss of HDAC6. Therefore, dysfunction of Hsp90 appears to be partially responsible for the some of the phenotypes observed in HDAC6 deficient cells. Thus I concluded that reversible acetylation of non-histone proteins mediated by HDAC6 contribute to oncogene driven transformation.
5.2 Future Directions

5.2.1 Application of HDAC6 Specific Inhibitors as Pharmacological Therapeutics in Cancer Treatment

HDAC inhibitors have been shown to induce cellular differentiation, growth arrest and apoptosis in cancer cells (Glozak and Seto 2007). Several HDAC inhibitors are currently under various stages of clinical trials as anticancer pharmacological therapeutics (Xu, Parmigiani et al. 2007). We have identified that HDAC6 modulates several critical processes in malignant transformation and deacetylase activity is required for such tumor promoting effects. Since HDAC6 knockout mice are viable and develop normally (Gao, Hubbert et al. 2007; Zhang, Kwon et al. 2008), and that HDAC6 can be pharmacologically inhibited by specific small molecule inhibitors (Haggarty, Koeller et al. 2003), HDAC6 serves as an ideal novel target for cancer treatment with low toxicity. So far the only HDAC6 specific inhibitor-tubacin works at millimolar concentration; effort is being made to search for more potent HDAC6 inhibitors (Suzuki, Kouketsu et al. 2006; Itoh, Suzuki et al. 2007).

We and others have shown that through both deacetylase activity and binding to dynein motor and ubiquitinated proteins, HDAC6 plays a central role in the management of proteins misfolding stress (Boyault, Sadoul et al. 2007; Matthias, Yoshida et al. 2008) by regulating aggresome formation (Kawaguchi, Kovacs et al. 2003), autophagy induction (Iwata, Riley et al. 2005; Pandey, Nie et al. 2007), and HSF-1
activation (Boyault, Zhang et al. 2007). This will predict a possible indispensable role of HDAC6 in promoting survival of tumor cells (such as multiple myeloma), which are prone to producing an excess amount of misfolded proteins. Indeed, combined use of proteasome inhibitors and HDAC6-selective inhibitors has synergistic cytotoxic effects on cancer cell lines (Kawaguchi, Kovacs et al. 2003; Hideshima, Bradner et al. 2005). Further work needs to be done to address if other types of cancers are also susceptible to HDAC6 inhibitors.

5.2.2 Determination of the Molecular Effectors for Tumor Promoting Effects of HDAC6

To date, at least three substrates of HDAC6, tubulin (Hubbert, Guardiola et al. 2002; Matsuyama, Shimazu et al. 2002; Zhang, Li et al. 2003), Hsp90 (Bali, Pranpat et al. 2005; Kovacs, Murphy et al. 2005; Scroggins, Robzyk et al. 2007), and cortactin (Zhang, Yuan et al. 2007) have been identified. These substrates all play critical roles in regulating cellular functions that are important in oncogenic transformation such as motility, adhesion, and stability of oncogenic proteins. It is interesting to understand which proteins are the major effectors that are responsible for promoting oncogenic transformation. To address this question, a rescue experiment with mutation to mimic acetylated (glutamine and alanine) or unacetylated (arginine) lysine at acetylation sites (Scroggins, Robzyk et al. 2007) targeted by HDAC6 on tubulin, Hsp90, and cortactin should be performed in oncogene-driven in vitro transformation. If deacetylation at that
particular site of one of the substrates proteins dictates the molecular and cellular events for tumor promoting function of HDAC6, the unacetylation mutants (but not the acetylation mimic mutants) would rescue the growth defect in HDAC6 deficient cells. However, this could be technically difficult for Hsp90 and tubulin since both are extremely abundant in the cells and ectopic expression of mutants might not be enough to compete out the endogenous proteins. Moreover, given that there are multiple acetylation sites and HDAC6 is not the only deacetylase for these substrates (Scroggins, Robzyk et al. 2007), a negative result cannot exclude the possibility that a particular substrate is responsible for the tumor promoting functions of HDAC6. It is also likely that the tumor promoting function of HDAC6 relies on all three substrates.

As a master regulator of cellular responses to stress, it is likely that HDAC6 have more substrates that yet to be identified. In light of finding new substrates of HDAC6, in particular ones that are critical to oncogenesis, a systemic proteomics approach using in vitro transformed cells or the carcinogen-induced tumors with wild type or HDAC6 deficient cells and animals should be conducted to identify proteins that are hyperacetylated in HDAC6 deficient cells compared to wild type counterparts.

5.2.3 Investigations of Potential Roles of HDAC6 in Metastasis in vivo

I have shown that HDAC6 contributes to anoikis resistance in cancer cells which is the cellular basis for tumor metastasis and invasion. It would be interesting to determine if HDAC6 contributes to metastasis in vivo. This can be examined using the
established metastatic cancer model, murine 4T metastatic breast cancer cells (Aslakson and Miller 1992), and human MDA-MB-231 metastatic breast cancer cells (Kelly, Moeller et al. 2006). These cells have been shown to be capable of invading locally and metastasizing to distal organs via hematogenous spread after orthotopic injection into mammary fat pads of syngeneic or immunocompromised mice (Kelly, Moeller et al. 2006). With the advance in bioimaging technologies, these cells could be engineered to carry markers such as GFP or luciferases that can be traced \textit{ex vivo} (Kelly, Moeller et al. 2006). By comparing the invasion and metastasis potentials of 4T1 or MDA-MB-231 that expressing shRNA against HDAC6 or scramble control, the contributions of HDAC6 in cancer invasion and metastasis could be easily addressed. This is important because tumor metastasis is the leading cause of treatment failure and cancer death with current therapeutic modality. If HDAC6 contributes significantly to cancer invasion and metastasis, then pharmacological inhibition of HDAC6 could serve as a potential treatment or prevention for invasion and metastasis of cancer.
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