MDM2–Mediated Degradation of SIRT6 Phosphorylated by AKT1 Promotes Tumorigenesis and Trastuzumab Resistance in Breast Cancer

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MDM2-Mediated Degradation of SIRT6 Phosphorylated by AKT1 Promotes Tumorigenesis and Trastuzumab Resistance in Breast Cancer

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Contents

Dedication

Dedicated to my parents, Dr.Thirumurthi and Vijayalakshmi, my husband, Balasubramaniam Sekhar and my sister, Dr. Hemamalini for their unconditional love and support, without which this dream would not have been possible. A special token of love to my son, Dakshith who has filled my life with a special meaning! Last but not the least; I will always be grateful for the blessings of god almighty “Amma”.

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Abstract

Sirtuin6 (SIRT6) is one of the members of the Sirtuin family and functions as a longevity assurance gene by promoting genomic stability. It also regulates various cancer-associated pathways and was recently established as a bonafide tumor suppressor in colon cancer. This suggests that SIRT6 is an attractive target for pharmacological activation in cancer treatment, and hence, identification of potential regulators of SIRT6 would be an important and critical contribution towards cancer treatment. Here, we show that AKT1 phosphorylates SIRT6 at Ser^{338} and induces MDM2-SIRT6 interaction, priming SIRT6 for degradation via the MDM2-dependent ubiquitin-proteasome pathway. Blocking SIRT6 Ser^{338} phosphorylation prevents its degradation by MDM2 and results in inhibition of cell proliferation and breast cancer tumorigenesis *in vivo.* In addition, knockdown of SIRT6 in trastuzumab-sensitive cells renders them resistant to trastuzumab

Where as overexpression of phosphorylation defective SIRT6 mutant restores trastuzumab sensitivity in the resistant cells. Thus, activation or re-expression of SIRT6 has potential clinical application to overcome trastuzumab resistance.

Chapter 1
Introduction

Introduction

Sirtuins

Sirtuins are a family of class III NAD^+ dependent deacetylases that are involved in lysine deacetylation of a wide variety of substrates including the histones and also in ADP-ribosylation. The first sirtuin was initially identified in yeast in 1984 as the Silent information regulator (Sir2) gene and it regulated transcriptional silencing of mating-type loci, ribosomal DNA and lifespan [1,2]. Their homologues were identified in Caenorhabditis elegans in 2001 [3] and in Drosophila melanogaster in 2004 [4]. There are seven sirtuins, Silent Information Regulator (SIRT) (SIRT1-SIRT7) in humans and mammals and they have different enzymatic activity, sub cellular localization, activity, tissue specificity and targets [5]. Over the past few years, sirtuins have gained attention as important contributors of extended lifespan in both lower and higher level organisms [6,7] and also due to their role as a nexus between caloric restriction and longevity [8-11]. Due to the fact that malfunctions of even a single sirtuin results in a number of pathophysiological complications, many pharmaceutical companies have identified agents that target sirtuins [12,13]. In addition, resveratrol, a natural flavonoid present in red grapes was shown to activate some of the Sirtuins and hence mimicking caloric restriction was seen as a cure for cardiovascular disease, type 2 diabetes and even cancer [14].

The sirtuins have a broad range of localization and substrates. The localization pattern and some of the key substrates of different sirtuins are summarized in Table 1.

Table 1: The localization and activity of sirtuins.

Sirtuins	Location	Activity	Substrates/Targets	Functions
SIRT1	Nucleus, Cytoplasm	Deacetylation	FOXO1, FOXO3,P53, NF-KB, NOTCH, etc	Cell survival, apoptosis, autophagy and metobolism
SIRT2	Cytoplasm, Mitochondria	Deacetylation	FOXO1, PEPCK, Tubulin, PAR-3	Autophagy, metabolism and cytoskeletal reorganization
SIRT3	Cytoplasm, Mitochondria	Deacetylation, ADP-rybosylation	SOD2, IDH2, PIP2, FOXO3, ALDH2, etc	Gene regulation
SIRT4	Mitochondria	ADP-ribosylation	GDH	Fatty acid metabolism, mitochondrial gene regulation
SIRT5	Mitochondria	Deacetylation, Demalonyation, Desuccinylation	CPS1	Metabolic homeostasis
SIRT6	Nucleus	Deacetylation, ADP-rybosylation	H3K9, NF-KB, H3K56, PARP-1, CtIP	Metabolism, genomic stability, DNA repair, aging
SIRT7	Nucleolus	Deacetylation		RNA polymerase 1 dependent gene transciption

Sirtuins and cancer

The role of some of the sirtuins in cancer is still under debate due to the complexity in their functions and diversity of the substrates. SIRT1 has been shown to be an oncogene and is upregulated in a number of cancers including breast cancer [15,16], colon cancer [17], prostate cancer [18], pancreatic cancer [19], Hepatocellular carcinoma [20, 21], acute myeloid leukemia [22], non-melanoma skin cancers [23,24] and in adult T-cell leukemia [25].

SIRT2 has been shown to be a tumor suppressor in gliomas and deletion in locus 19q13.2, where SIRT2 is located is often deleted in gliomas [26]. SIRT2 expression is also reduced in esophageal and gastric adenocarcinomas [27], mammary carcinoma and hepatocellular carcinoma [28] and an inactivating mutation has been identified in the catalytic domain of SIRT2 in melanomas [29]. Moreover, Histone 3 lysine 53(H3K53), the substrate of SIRT2 is hyper acetylated in cancer cells [30]. However, in acute myeloid leukemia cells, SIRT2 is upregulated and associated with increased cell proliferation and cell survival [31].

SIRT3 is a mitochondrial sirtuin that regulates oxidative stress and metabolism [32]. Although there is some evidence that SIRT3 might be a tumor suppressor, it is still debatable [33] as its levels are reduced in breast cancer [34] and heaptocellular carcinoma, but elevated in malignant and lymph node positive breast cancer [35]. Loss of SIRT3 leads to an increase in reactive oxygen species (ROS), which in turn enhances expression of HIF-dependent genes leading to a cancer phenotype [36]. On the other hand, SIRT3 overexpression inhibited the growth and induced apoptosis in hepatocellular carcinoma cells [37]. Also SIRT3 is elevated in oral cell carcinomas that are resistant to anoikis [38].

SIRT6 is one of the better studied sirtuins after SIRT1 with respect to cancer. SIRT6 is a tumor suppressor of the pancreas and colon [39]. SIRT6 overexpression has been shown to induce massive apoptosis in a number of cancer cell lines but not in normal cell lines [40]. The tumor suppressive activity of SIRT6 has been attributed to both its deacetylase activity and ADP-ribosyl transferase activity. Histone 3 lysine 56 (H3K56), a substrate of SIRT6 is hyper acetylated in a number of cancers including the breast, colon, skin, liver and thyroid [30].

SIRT7 is perceived as an oncogene as its expression is elevated in breast cancer including node-positive breast cancers and in thyroid cancer. SIRT7 is important for cancer maintenance rather than cancer initiation [41]. SIRT7 deacetylates Histone 3 lysine 18 (H3K18), leading to anchorage independent growth, loss of contact inhibition and progression to tumorigenesis [42].

Sirtuin 6

Sirtuin 6 (SIRT6) is a mammalian homologue of the yeast Sir2 protein. The **SIRT6** gene is localized to chromosome 19p13.3 in humans and consists of 8 exons that codes for a 355-amino acid protein [43]. SIRT6 is ubiquitously expressed in most of the tissues and predominantly localized in the nucleus and is associated with the chromatin [44,45]. The catalytic site of SIRT6 is present in the N-terminal extension and is essential for its activity and the nuclear localization signal exists between amino acids 345-351 at the C-terminal. The nuclear localization signal of SIRT6 is required for its proper nuclear localization [46,47]. SIRT6 nuclear localization is independent of its enzymatic activity [48,49].

SIRT6 has both deacetylase and ADP-ribosyl transferase activities that are dependent on NAD^+ levels [50]. Although SIRT6 is capable of deacetylating a number of substrates including H3K9, H3K56, it's ADP- ribosyl transferase activity is not yet well studied and it's substrates include itself and PARP1[45,51]. SIRT6 also hydrolyses long-chain fatty acyl groups from lysine residues in palmitoyl, myristoyl and butyryl acids [52].

SIRT6 is regulated positively by SIRT1 under nutritional stress by forming a complex with Forkhead Box O3 (FOXO3a) and Nuclear respiratory factor 1 (NRF1) on the NRF1-binding sites on SIRT6 promoter [53]. P53 also positively regulates SIRT6 protein levels under normal nutrient availability and leads to SIRT6 upregulation under starvation [54]. Also, miR-33b overexpression down regulates SIRT6 mRNA and protein levels [55,56].

SIRT6-deficient mice are small and exhibit severe metabolic defects including reduced levels of serum glucose and IGF-1 and they also develop aging associated abnormalities by 2-3 weeks [44]. Caloric restriction (CR) leads to increase in SIRT6 levels in a tissue-specific manner in mice that overexpress SIRT6 (MOSES mice) and the increase in SIRT6 levels was due to enhanced protein stability. The MOSES mice are protected against diet induced obesity and other metabolic defects [57]. Also SIRT6 overexpression has been shown to extend life span in male mice through inhibition of IGF-1 signaling [58]. SIRT6 binds to RelA subunit of NF-κB and gets recruited to NF-κB target gene promoters, deacetylates H3 Lysine 9 (H3K9) and suppress genes associated with aging and thus extends life span [59].

SIRT6 negatively regulates HIF1α by binding to it and hence SIRT6 deficient cells exhibit increased glycolysis and reduced oxygen consumption, similar to the Warburg effect [60]. SIRT6 inhibits AKT phosphorylation at residues Ser^{473} and Thr^{308} and the absence of SIRT6 results in enhanced activation of IGF-AKT signaling and hence leading to hypoglycemia. SIRT6 deficient mice are more sensitive to insulin and exhibit increased AKT activation in various organs even without stimulation with insulin. As a consequence of AKT hyper activation in the absence of SIRT6, increase in membrane translocation of GLUT1 and GLUT4 were seen in mice leading to enhanced glucose uptake [61]. SIRT6 interacts with and deacetylates Forkhead box protein O1 (FOXO1) leading to its nuclear exclusion and subsequent downregulation of genes involved in gluconeogenesis like Glucose-6-phosphatase (G6PC) and phospho enolpyruvate carboxykinase-1 (PCK1) [62]. Relatedly, SIRT6 also regulates gluconeogenesis by enhancing GCN5 mediated acetylation and inhibition of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [63].

$SIRT6^{-/-}$ cells exhibit chromosomal aberrations like fragmented chromosomes, detached centromeres and dysfunctional telomeres [64]. SIRT6 repairs DNA double strand breaks (DSBs) through both Non-homologues end joining (NHEJ) and homologous recombination (HR) and SIRT6 deficiency results in inefficient double strand repair and base excision DNA repair pathways [44,65-67]. SIRT6 plays a role in DSB repair by regulating C-terminal binding protein (CtBP) interacting protein (CtIP) [66] and poly ADP-ribose polymerase 1 (PARP1) [67] and by physically interacting with and mobilizing DNA-dependent protein kinase catalytic subunit (DNA-PK) to the sites of DSBs [68]. SIRT6 also recruits the chromatin remodeler SNF2H to DSBs and deacetylates H3K56. Loss of SIRT6 impairs recruitment of SNF2H and hence increases sensitivity to genotoxic stress and DNA damage [69] (Figure 1).

SIRT6 and cancer

SIRT6 has been shown to be associated with cancer through its role in regulating cellular stress and organismal aging [70] and through inhibition of the shift towards anaerobic glycolysis [39]. Characteristics of SIRT6 knockout or knockdown cells including genomic instability, hyperactive NF-κB signaling [71], aberrant glucose homeostasis [72], aneuploidy [73,74], are common characteristics of cancer cells and as expected, loss of SIRT6 is observed in a variety of cancers. SIRT6 downregulation in human hepatocytes leads to hepatocellular carcinoma (HCC) through upregulation of oncogenes such as IGF2. SIRT6 also suppresses HCC by inhibiting the extracellular signal-regulated kinase (ERK) pathway [75]. SIRT6 acts as a tumor suppressor in gliomas

by inhibiting the expression of poly(C)-binding protein (PCBP) 2 (PCBP2) by deacetylating H3K9Ac [76]. SIRT6 expression is downregulated in colon cancer cells through FOXO3a [77]. SIRT6 mRNA and protein expression were downregulated in the advanced stage of head and neck squamous cell carcinoma than in the early stage [78]. In liver cancers, c-Fos induces SIRT6 expression and in turn SIRT6 represses expression of survivin by deacetylating H3K9 and reducing NF-κB activation. Increasing SIRT6 levels at the liver cancer initiation stage impairs liver cancer development and SIRT6 expression was also found to be low in human dysplastic liver nodules [79]. SIRT6 inhibits colon and pancreatic cancers through repression of MYC dependent transcription and ribosomal gene expression and SIRT6 was shown to act as tumor suppressor by regulating cancer metabolism and glycolysis and its loss could lead to tumorigenesis even in the absence of oncogene addiction [39]. Also downregulation of SIRT6 mRNA expression and Sirt6 gene copy number is observed in various human cancers [39]. Moreover, the locus in which SIRT6 gene is located is prone to high rates of Loss of Heterozygosity (LOH) in human breast cancers [80-82].

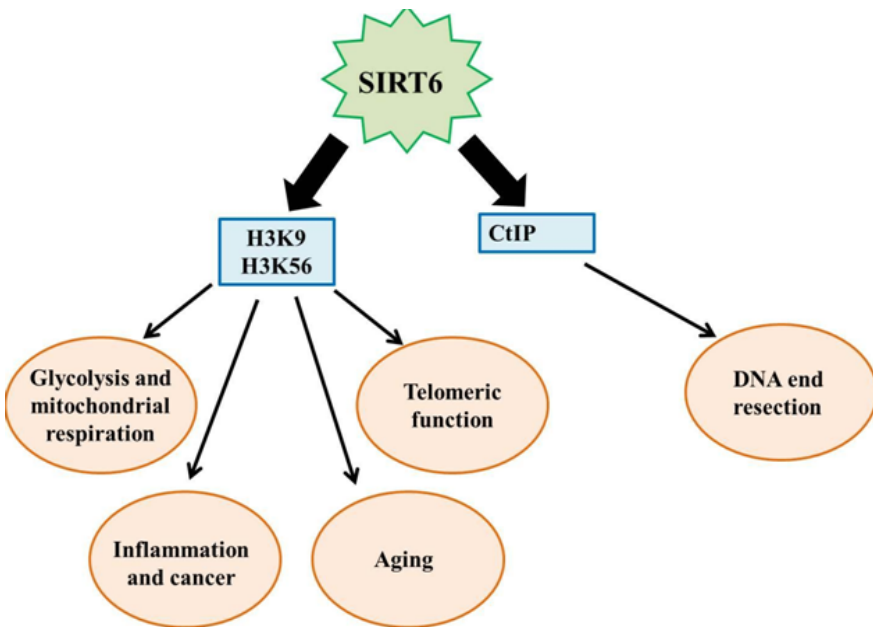

Figure 1: Summary of the functions of SIRT6.

Breast cancer and insulin-like growth factor (IGF) /PI3K/AKT signaling

Breast cancer is the second most common malignancy among women and the second leading cause of cancer associated deaths in the US. In 2014, the American Cancer Society estimates that about 296,980 new cases of breast cancer are expected to be diagnosed among US women and around 39,620 US women are expected to die from the disease.

Breast cancer is a heterogeneous disease and is classified into different subtypes according to the hormone receptor expression pattern. The relationship between breast cancer and IGF-PI3K/AKT signaling is well studied and documented. AKT is primarily activated by IGF signaling in a phosphatidylinositol 3-kinase (PI3K) dependent manner. IGF stimulation leads to dimerization of PI3K and subsequently its autophosphorylation and activation. AKT is the central downstream effector of PI3K and is activated by interaction with PtdIns [3,4,5]P3 and followed by phosphorylation on two key residues, Thr^{308} and Ser^{473} by Protein Dependent Kinase 1 (PDK1) [83]. Activated AKT in turn activates a number of downstream substrates by phosphorylating serine/threonine residues in the RXRXXS/T motif [84]. AKT signaling plays a critical role in malignant transformation through activation of numerous downstream substrates via phosphorylation including but not limited to I-κB kinase (IKK), cAMP response element-binding protein (CREB), forkhead family of transcription factors (FOXOs), glycogen synthase kinase (GSK-3), $p21^{Cip1}$ etc. While growth factors and cytokines result in the activation of the pathway, phosphatases negatively regulate the PI3K/AKT activity [85-88].

Protein kinase B (AKT) regulates a number of cellular processes including cell survival, metabolism, cell cycle, transcriptional regulation and protein synthesis [89-91].

There are three members in the AKT family, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ and they are coded by three different genes. Although these three AKT isoforms share high homology, they differ in their biological functions. Disruption of AKT1 exhibits growth retardation in mice, while AKT2 depleted mice show normal development, but have type II diabetes like syndrome and AKT3 has been shown to be required for normal brain development in mice [92-96]. AKT/PKB pathway activation is seen in a variety of cancers including the breast, ovarian, colon, pancreas etc. [97,98].

Relation between IGF/AKT signaling and SIRT6

SIRT6 negatively regulates IGF/AKT signaling at chromatin level through deacetylation of Histone 3 Lysine 9 (H3K9) [99]. SIRT6 knockout mice exhibited cardiac hypertrophy and these failing hearts showed activation of genes related to the IGF/AKT signaling pathway like IGF-1R, IGF-2R, AKT, FOXO1, mTOR, myc and GSK3. Coincidentally, chronic AKT activation exacerbates aging induced cardiac hypertrophy [100]. SIRT6 interacts with c-Jun and inhibits its transcriptional activity. Under physiological stress and pathological conditions, when SIRT6 levels are reduced in the cells, there is de-repression of c-Jun mediated transcription, thus leading to increase in transcription of the IGF-AKT signaling pathway genes that harbor c-Jun binding sites in their promoters [101].

Transgenic male mice over expressing SIRT6 show 15% increase in lifespan when compared to control mice. The observed increase in lifespan was due to the lower levels of IGF-AKT pathway related genes in the SIRT6 over expressing male mice. Insulin and insulin-like growth factor (IGF) signaling have also been uniformly shown to regulate life span and longevity ranging from worms to mice and mammals [102-104]. Mutations in insulin receptor DAF-2 or the PI3K AGE-1 in Caenorhabditis elegans extended the life span by more than 100% in these worms [105-108]. Lifespan of female Drosophila melanogaster is extended by about 85% by mutating the insulin receptor and the insulin-receptor substrate [109,110] and heterozygous Igf1r+/- mice live 26% longer than their wild type counterparts [111]. SIRT6 also inhibits phosphorylation of AKT at Ser^{473} and Thr^{308} by inhibition of various upstream molecules including IRS1, IRS2 and insulin receptor and hence the absence of SIRT6 leads to enhanced insulin signaling, activation of AKT and membrane recruitment of GLUT1 and GLUT4 leading to hypoglycemia in mice [61]. This suggests an existing converse relationship between AKT and SIRT6 in aging and in cardiac hypertrophy.

Trastuzumab resistance in HER2 over expressing breast cancers

The Epidermal Growth Factor Receptor 2 (ErbB2 or HER2 or Neu) is one of the members of the ErbB family of receptor tyrosine kinases and it is over expressed in around 25% of human breast cancers [112]. On ligand binding, HER2 dimerizes with itself or with other members of the ErbB family, which results in the phosphorylation of several tyrosine residues within the regulatory domain, hence leading to its activation. HER2 contributes to oncogenesis through activation of a number of downstream signaling pathways including the Ras-Raf-MAPK and the PI3K-AKT pathways [113,114].

Trastuzumab or Herceptin is a humanized monoclonal antibody developed by Genentech that targets the extracellular region of HER2. Trastuzumab was approved for the treatment of HER2 positive breast cancer patients as an adjuvant therapy, in combination with chemotherapy [115]. Although trastuzumab had promising initial response in the patients, a subset of patients developed primary or de novo resistance and another subset of patients who responded initially, acquired resistance during the course of treatment.

A number of factors have been shown to contribute to trastuzumab resistance. Epitope masking by MUC4 and CD44, in which the binding of trastuzumab to HER2 is disrupted, upregulation of HER2 downstream signaling pathways due to mutation or loss of PTEN, increased MAPK and PI3K signaling, overexpression of HER ligands such as TGF-α, EGF and heregulin and impaired immune-mediated response due to alteration of antibody-dependent cell-mediated cytotoxicity (ADCC).

Several efforts are being undertaken to overcome this issue of acquired resistance, including combining trastuzumab with PI3K inhibitors and c-Src inhibitors, which have been shown to be effective in reverting resistance to trastuzumab in preclinical settings [116,117]. Trastuzumab-DM1 (T-DM1) was recently developed by Genentech, in which the antibody is conjugated to a cytotoxic agent mertansine [118] via a thioether linker. Phase 1 clinical trial results with T-DM1 showed that the modified drug was effective in patients who had progressed on trastuzumab [119]. Also, a Phase II study showed that T-DM1 was effective as a single-agent therapy in metastatic breast cancer patients who had progressed in trastuzumab [120]. Thus these studies show that HER2 pathway is an effective therapeutic target and novel approaches to target this pathway or to overcome its signaling mediated effects would be valuable towards disease treatment.

Hypothesis

Although the biological functions of SIRT6 have been gradually recognized, its upstream regulation that leads to loss of SIRT6 activity or protein levels is not well understood. A recent study reported that the phosphorylation of SIRT6 on S^{338} may regulate interactions with a subset of proteins, but no biological consequences of this phosphorylation were identified [121].

Phenotypes of $SIRT6^{-/-}$ mice, including accelerated aging, cardiac hypertrophy, and reduced lifespan, are similar to those associated with hyper activation of the IGF-AKT

pathway [58,122]. Also, studies have indicated that SIRT6 negatively regulates IGF-AKT signaling by inhibiting gene transcription and AKT phosphorylation [59,99]. As PI3K/AKT signaling pathway is one of the major oncogenic signaling cascades that results in tumor growth and development [123-125], we hypothesized that IGF-AKT signaling might also regulate SIRT6. If this is the case, identification of novel regulations of SIRT6 would be valuable towards the treatment of various metabolic disorders including cancer.

Note

Chapters 2 to 8 are based on the publication:

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Chapter 2
Material and Method

Material and Method

Cell lines

All cell lines used were purchased from the American Type Culture Collection (ATCC). They included HEK293T, a HEK cell line; MCF-7, a human mammary adenocarcinoma cell line from pleural effusion; MDA-MB-231, a human mammary adenocarcinoma cell line from pleural effusion; Hs578T, a human mammary carcinoma cell line; and HBL-100, a human mammary epithelial carcinoma. The BT474 (BT474-P) cell line and its trastuzumab-resistant counterpart (BT474-TtzmR) were gifts from D. Yu at The University of Texas MD Anderson Cancer Center (Houston, TX). All cells were grown on tissue culture dishes in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin and streptomycin (100 U, 100 ng/ml) at 37°C in a humidified atmosphere with 5% CO2, unless specified otherwise. Before IGF (50 ng/ml) or EGF (50 ng/ml) treatment, the cells were serum-starved overnight. The concentrations and treatment durations of each chemical were as follows: MK2206 (2 μM, 1 hour), MG-132 (10 μM, 7 to 10 hours), and cycloheximide (1 μg/ml). Stable puromycin-resistant cell lines were maintained in DMEM/F12 medium that contained puromycin (1 μg/ml).

Antibodies

Commercial antibodies against SIRT6 (1:1000), AKT1 (1:1000), AKT2 (1:1000), **AKT3** (1:1000), and phospho-AKT Ser^{473} (1:1000) were purchased from Cell Signaling Technology; antibody against phosphorylated SIRT6 Ser^{338} (1:500) was from Abnova; and antibodies against HA (1:2000) and Flag (1:2000) were from Sigma.

Reagents

The AKT inhibitor MK2206 was purchased from Selleck Chemicals. MG-132, cycloheximide, EGF, and IGF were purchased from Sigma.

Plasmids

DNA plasmids encoding Flag-SIRT6 (plasmid 13817), HA-myr-AKT1 (plasmid 9008), and HA-myr-AKT3 (plasmid 9017) were from Addgene. Wild-type MDM2 and the MDM2 deletion mutant (MDM2- 9) were gifts from J. Chen (H. Lee Moffitt Cancer Center, Tampa, FL).

SIRT6-S338A and SIRT6-S338D point mutants were generated using the QuickChange Site-Directed Mutagenesis Kit from Stratagene using the following primers: SIRT6-S338D, 5′-GCGGCCCACCGACCCTGCCCCCCACAG-3′ (forward) and 5′-GTGGGGGGCAGGGTCGGTGGGCCGCTC-3′ (reverse). All lentiviral pLKO.1 expression and shRNA-encoding plasmids were purchased from Sigma. SIRT6 shRNA 1 clone ID: TRCN0000232532:

CCGGCTCCCTGGTCTCCAGCTTAAACTCGAGTTTAA-GCTGGAGACCAGGGAG TTTTTG; SIRT6 shRNA 2 clone ID: TRCN0000050473:CCGGTGGAAGAATGTGCCAAG-TGTACTCGAGTACACTTGG CACATTCTTCCATTTTTG. Wild-type MDM2 and MDM2 deletion mutants were gifts from J. Chen (H. Lee Moffitt Cancer Center, Tampa, FL). AKT1, AKT2, and MDM2 siRNAs were purchased from Sigma.

Immunoblotting, immunoprecipitation, and ubiquitination assays

Immunoblotting, immunoprecipitation, and ubiquitination assays were performed as previously described [124], using antibodies against SIRT6, AKT1, AKT2, phosphorylated AKT at Ser^{473} (Cell Signaling Technology), tubulin, and actin (Sigma). For glutathione S-transferase (GST) pull-down assays, GST-SIRT6 protein (10 μg) was incubated with 2 mg of MCF-7 cell extract overnight at 4°C. GST-tagged proteins were recovered by incubating the reaction mixture with 20 μl of glutathione Sepharose beads at 4°C overnight. The bead pellet was washed three times in 1X phosphate-buffered saline. The boiled samples were then subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro kinase assay

Purified GST-SIRT6 (wild-type or mutant) fragments were incubated with active AKT1 (Millipore) and 50 mM ATP (adenosine 5′-triphosphate) in a kinase buffer containing [^{32}P] ATP (5 μCi) for 30 min at 30°C. The reaction products were resolved via SDS-PAGE, and ^{32}P-labeled products were detected using autoradiography.

Identification of phosphorylation sites by mass spectrometry analysis

HeLa cell lysates were immunoprecipitated with an antibody against SIRT6 to identify the phosphorylation sites of SIRT6 in cells. *In vitro*, the phosphorylation site of SIRT6 was identified using an *in vitro* kinase assay with recombinant, active AKT1 kinase and full-length GST-SIRT6. After protein gel electrophoresis, the bands were excised and subjected to digestion with trypsin. The enriched phosphopeptides were isolated using immobilized metal affinity chromatography and analyzed by micro–liquid chromatography–tandem mass spectrometry using an UltiMate Capillary LC system (LC Packings) coupled to a QSTAR XL quadruple time-of-flight mass spectrometer (Applied Biosystems). The product ion spectra, generated by nanoscale capillary spectrometry, were searched against National Center for Biotechnology Information databases for exact matches using the ProID (Applied Biosystems) and MASCOT search programs. Carbamidomethyl cysteine was set as a fixed modification, and serine, threonine, and tyrosine phosphorylation were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

Cell growth, soft agar, and cell viability assays

Cell growth was determined by cell counting. Cells (1×10^5) were plated in triplicate in 12-well plates. They were then trypsinized at the indicated time points and counted. For the soft agar transformation assay, 2.5×10^4 cells were seeded in 1 ml of DMEM with 10% FBS and 0.4% agarose and

overlaid on 1 ml of DMEM with 10% FBS and 0.8% agarose in each well of a six-well plate. After 2 to 3 weeks, colonies larger than 2 mm in diameter were counted.

Animal studies

MDA-MB-231 cells (2×10^6) with lentiviral-stable expression of SIRT6-WT, SIRT6-S338A, or SIRT6-S338D and Sh SIRT6 or Sh Luc control cells were injected into the mammary fat pads of nude mice (five per group). Tumor size was measured every 3 days with a caliper, and tumor volume was determined using the formula $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter. All animal procedures were conducted under regulations of Division of Laboratory Animal Medicine at The University of Texas MD Anderson Cancer Center. Animal protocols (protocol number 06-87-06139) were reviewed and approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

Breast tumor tissue specimens

One hundred twenty-six formalin-fixed and paraffin-embedded infiltrating breast carcinoma patient samples were obtained from the Department of Pathology, Shanghai East Breast Disease Hospital, and People's Republic of China. Breast cancer tissue microarray containing 186 cases was purchased from Pantomics (BRC2281).

Immunohistochemical staining

A modified immunoperoxidase staining was used as described previously [126] for staining with SIRT6 (Novus, NB100-2522), phospho-AKT Ser^{473} (Cell Signaling Technology, 3787S), and phospho-SIRT6 (Bioss, bs-5634R-bio).

Statistical analysis

SAS software (version 8.1) was used for the statistical analysis (SAS Institute). A univariate analysis was used to determine the variable distributions. Categorical variables among the groups were compared using the $\chi 2$ test or Fisher's exact test if 20% of the expected values were less than 5. Continuous variables were analyzed using Student's t test. A P value <0.05 was considered statistically significant.

Chapter 3
Sirt6 is down regulated by AKT1

Sirt6 is down regulated by AKT1

Activation of AKT1 promotes SIRT6 degradation

To determine whether AKT signaling regulates SIRT6 expression, AKT1 and AKT2 were knocked down by silencing RNA [small interfering RNA (siRNA)] in MCF-7 (Figure 2a & 2b) and MDA-MB-231 (Figure 2c) human breast cancer cells. Only knockdown of AKT1, but not AKT2, resulted in significant increase in SIRT6 protein abundance. We also observed increased reduction in the endogenous SIRT6 protein abundance with overexpression of constitutively active AKT1 in MDA-MB-231 cells (Figure 3a) and exogenous SIRT6 abundance in human embryonic kidney (HEK) 293T cells (Figure 3b). Overexpression of constitutively active AKT3 did not decrease SIRT6 protein abundance (Figure 3c), indicating that AKT1 may be the dominant kinase that regulates SIRT6 abundance. Thus, we focused on AKT1 for further experiments. Adding MK2206, an AKT inhibitor, to cultures increased the abundance of SIRT6 in MCF-7, MDA-MB-231, and two additional breast cancer cell lines, HBL-100 and Hs578T (Figure 4). Treatment with growth factors, such as epidermal growth factor (EGF) and IGF activated AKT1 and decreased SIRT6 abundance in a time-dependent manner (Figure 5). Furthermore, only the expression of constitutively active, but not the dominant negative, kinase-deficient AKT1 decreased the abundance of Flag-tagged SIRT6 in HEK293T cells (Figure 6). Thus these results suggest an inverse correlation between AKT activation and SIRT6 abundance and that the kinase activity of AKT1 is required for SIRT6 degradation.

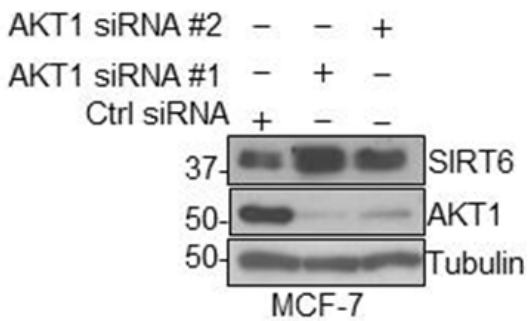

Figure 2a: Western blotting for SIRT6 and AKT1 against tubulin (loading control) in lysates from MCF-7 cells transfected with one of two siRNAs against AKT1

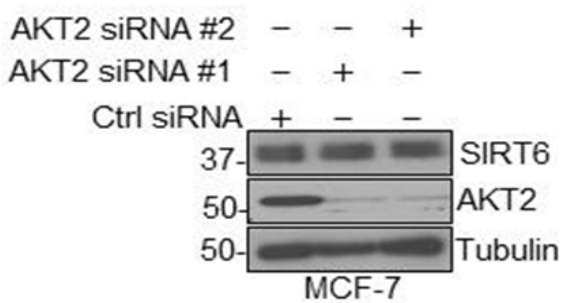

Figure 2B: Western blotting for SIRT6 and AKT2 against tubulin (loading control) in lysates from MCF-7 cells transfected with one of two siRNAs against AKT2.

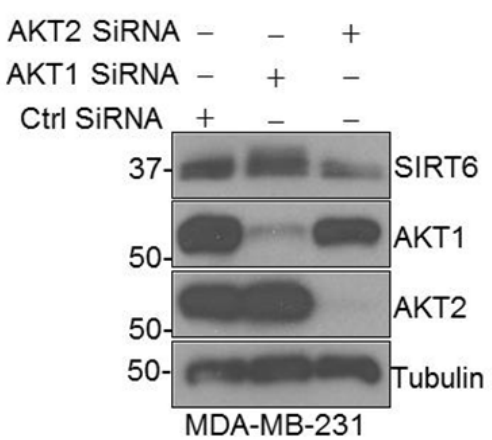

Figure 2C: Western blotting for SIRT6, AKT1 and AKT2 against tubulin (loading control) in lysates from MDA-MB-231 cells transfected with siRNAs against AKT1 and AKT2.

Figure 2: AKT activation promotes SIRT6 degradation.

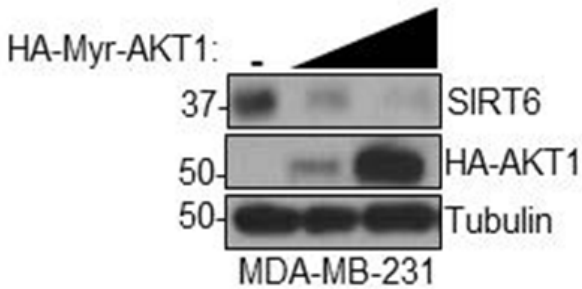

Figure 3A: Western blotting for SIRT6 or HA in lysates from MDA-MB-231 cells transfected with 2 or 6 µg of HA-tagged, constitutively active AKT1 (HA-myr-AKT1) or a control vector (−).

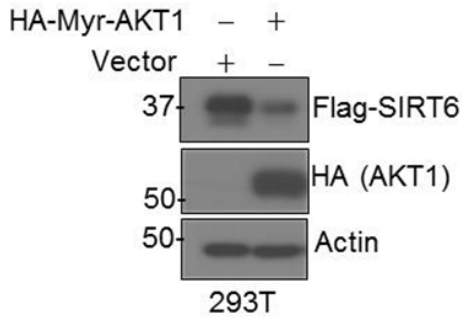

Figure 3B: Western blotting for the Flag and the HA tag in lysates from HEK293T cells 72 hours after transfection with HA-tagged constitutively active AKT1 (HA-myr-AKT1) along with Flag-tagged SIRT6.

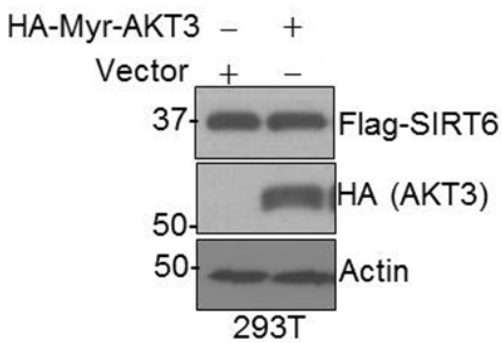

Figure 3C: Western blotting for the Flag and the HA tag in lysates from HEK293T cells 72 hours after transfection with HA-tagged constitutively active AKT3 (HA-myr-AKT3) along with Flag-tagged SIRT6.

Figure 3: AKT1 but not AKT2 and AKT3 degrades SIRT6.

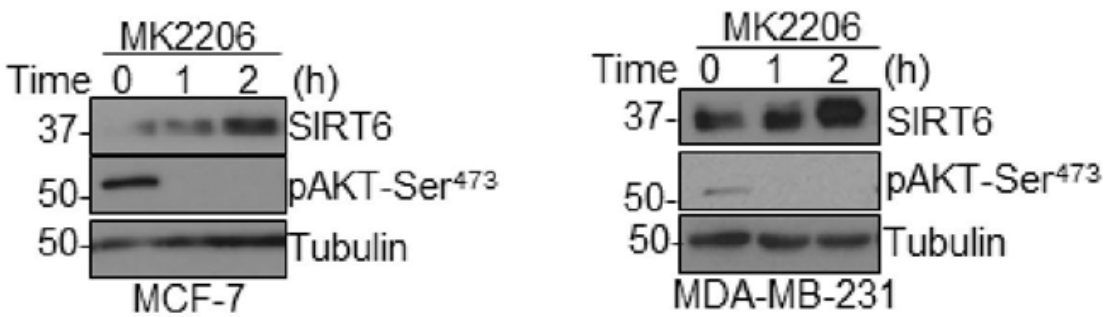

Figure 4: Inhibition of AKT with an inhibitor rescues SIRT6 levels.

Western blotting for SIRT6 or phosphorylated AKT (pAKT-Ser^{473}) in lysates from MCF-7, MDA-MB-231, HBL100 and Hs578T cells treated with 2 µM MK2206.

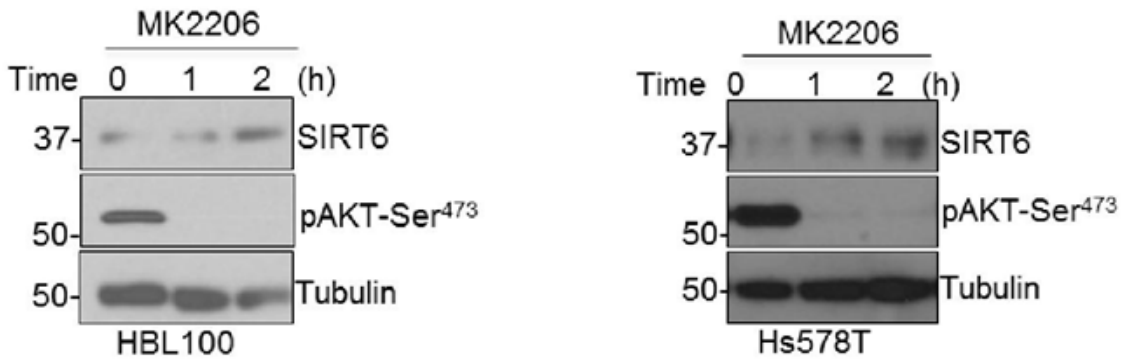

Figure 4: Inhibition of AKT with an inhibitor rescues SIRT6 levels.

Western blotting for SIRT6 or phosphorylated AKT (pAKT-Ser^{473}) in lysates from MCF-7, MDA-MB-231, HBL100 and Hs578T cells treated with 2 μM MK2206.

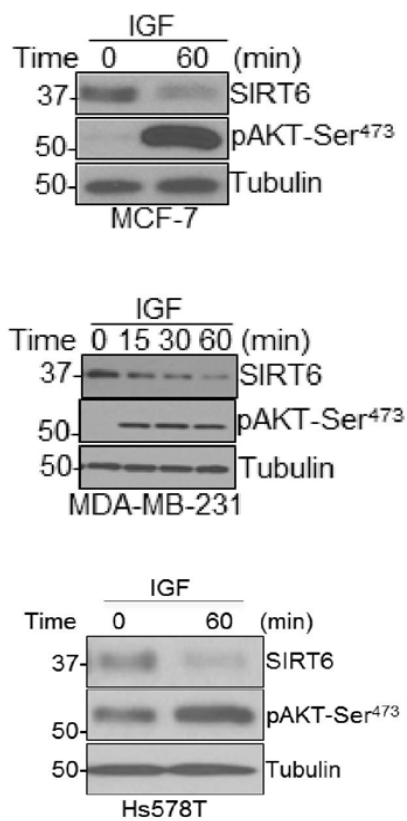

Figure 5: Activation of AKT with growth factors results in SIRT6 degradation.

Western blotting for SIRT6 or phosphorylated AKT (pAKT-Ser^{473}) in lysates from MCF-7, MDA-MB-231 and Hs578T cells treated with IGF (50 ng/ml) for the indicated time.

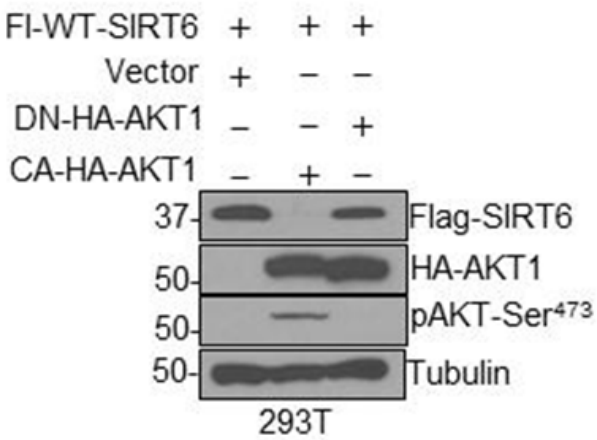

Figure 6: Kinase activity of AKT is required for SIRT6 degradation.

Western blotting in lysates from HEK293T cells transfected with HA-tagged dominant negative AKT1 (DN-HA-AKT1) or constitutively active AKT1 (HA-myr-AKT1; "CA") and Flag-tagged, wild type (WT) SIRT6.

Inverse correlation between AKT activation and SIRT6 levels in human breast cancers

To further study the observed inverse correlation between AKT activation and SIRT6 abundance, we stained for SIRT6 and AKT phosphorylated at Ser^{473} in a panel of breast cancer cell lines (Figure 7a) and 312 patient breast tumor tissue specimens (126 paraffin-embedded samples and 186 samples from tissue microarray) (Figure 7b & Table 2). In concert with the cell line data, we observed a negative correlation between SIRT6 levels and that of phospho-AKT-Ser^{473} in human breast cancers.

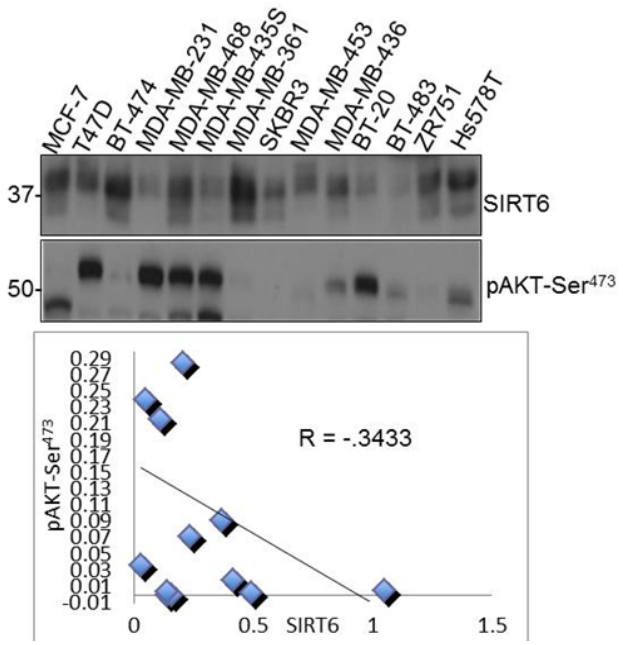

Figure 7A: Western blotting for SIRT6 and phosphorylated AKT in lysates from a panel of breast cancer cell lines.

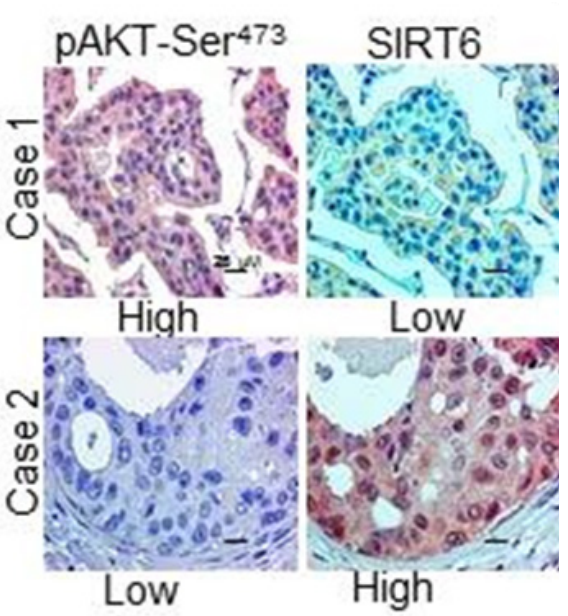

Figure 7B: Immunohistochemical analysis of the abundance of phosphorylated AKT (Ser473) and SIRT6 in tumor sections from two different breast cancer patients (cases 1 and 2). Scale bars, 25 μm.

Figure 7: Inverse correlation between AKT activation and SIRT6 levels in Breast Cancers.

AKT1 promotes SIRT6 degradation in a proteosome dependent manner

To determine whether AKT1-mediated SIRT6 suppression was because of changes in protein stability, we measured the half-life of a Flag-tagged SIRT6 in HEK293T cells that over expressed hemagglutinin (HA)–tagged, constitutively active AKT1. The half-life of SIRT6 was shorter in the presence of active AKT1 than it was in the presence of the vector (Figure 8a), prompting us to examine whether this decrease was the result of 26S proteasome–mediated degradation. Pretreating HEK293T cells with the proteasome inhibitor MG-132 or the AKT inhibitor MK2206 rescued AKT1-induced suppression of SIRT6 abundance (Figure 8b). Additionally, overexpression of AKT1 enhanced the ubiquitination of SIRT6 in the presence of MG-132, which was inhibited by either MK2206 or wortmannin, a PI3K inhibitor (Figure 8c). Together, these results suggest that SIRT6 protein abundance is suppressed in a proteasome-dependent manner, and this is dependent on the kinase activity of AKT1 Figure 7a.

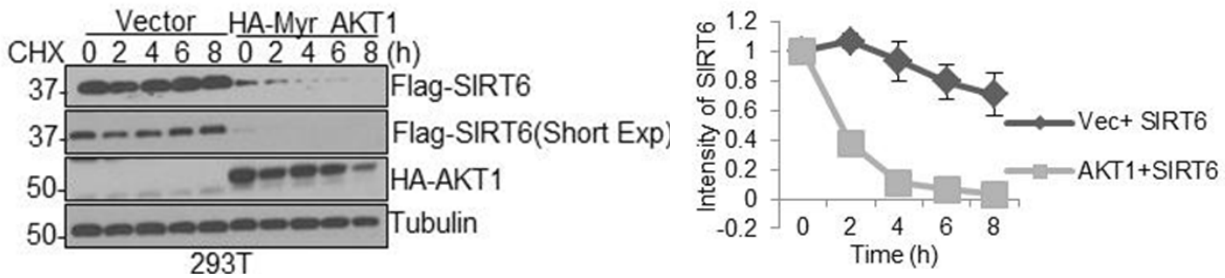

Figure 8A: Western blotting in lysates from HEK293T cells transfected with Flag-tagged SIRT6 and either vector or HA-AKT1 in the presence of cycloheximide (CHX) for up to 8 hours. Short Exp- shorter exposure time.

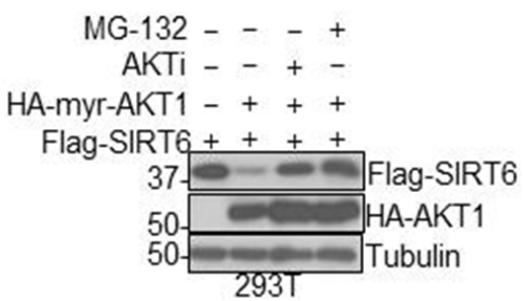

Figure 8B: Western blotting in lysates from HEK293T cells transfected with constitutively active AKT1 or Flag-tagged SIRT6 and treated with either MG-132 or MK2206 (AKTi).

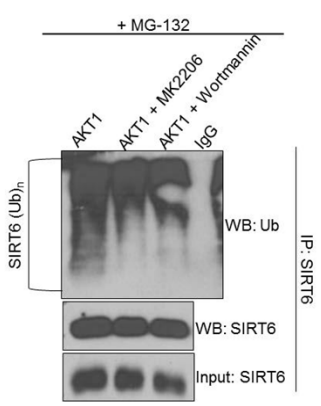

Figure 8C: Western blotting (WB) for ubiquitin and SIRT6 after immunoprecipitation (IP) for SIRT6 in lysates from HEK293T cells transfected with HA-myr-AKT1 then treated with either MK2206 or PI3K inhibitor Wortmannin for 1 hour and MG-132 for 7 hours.

Figure 8: AKT1 promotes SIRT6 degradation in a proteosome dependent manner.

Table 2: Correlation between phospho-AKT Ser^{473} and SIRT6 in breast cancer.

	SIRT6		
Phospho-AKT Ser^{473}	**Low**	**High**	**Total**
	51	164	215
	35	62	97
	86	226	312

The correlation was studied in an array of 186 human breast cancers and 126 formalin fixed and paraffin-embedded breast carcinoma patient samples, analyzed using χ^2 test (P = 0.024).

Chapter 4

Akt1 Interacts with and Phosphorylates SIRT6

Akt1 Interacts with and Phosphorylates SIRT6

AKT1 interacts with SIRT6

To explore the mechanism of how AKT1 mediates the suppression of SIRT6, we first characterized the interaction between the two proteins. Both endogenous SIRT6 (Figure 9a) and exogenous Flag-tagged SIRT6 (Figure 9b) physically associated with AKT1 in an immuno precipitation assay. In addition, endogenous AKT1 interacted with endogenous SIRT6, as shown by reciprocal immuno precipitation (Figure 9c).

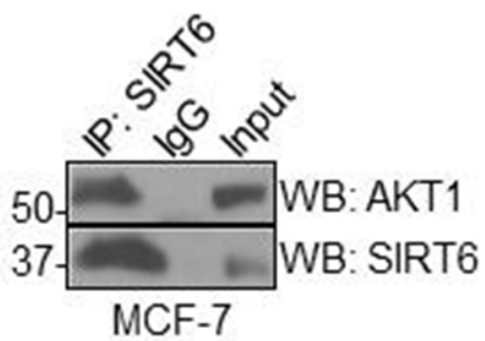

Figure 9A: Immunoprecipitation (IP) for SIRT6 followed by immunoblotting in lysates from MCF-7 cells against an IgG (immunoglobulin G) control.

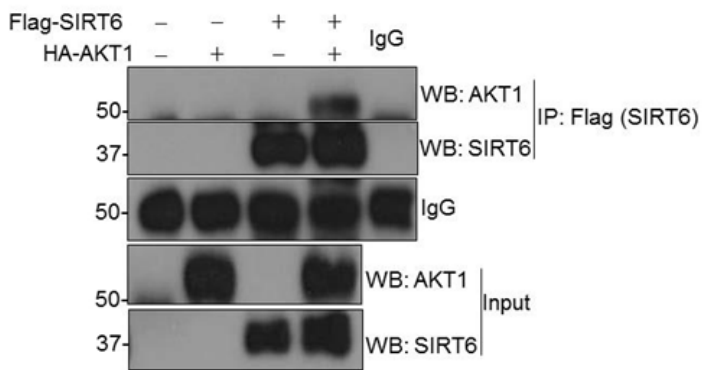

Figure 9B: Lysates of HEK293T cells that were transfected with Flag-tagged SIRT6 and HA tagged AKT1 were immunoprecipitated with a Flag antibody and immunoblotted for SIRT6 and AKT1.

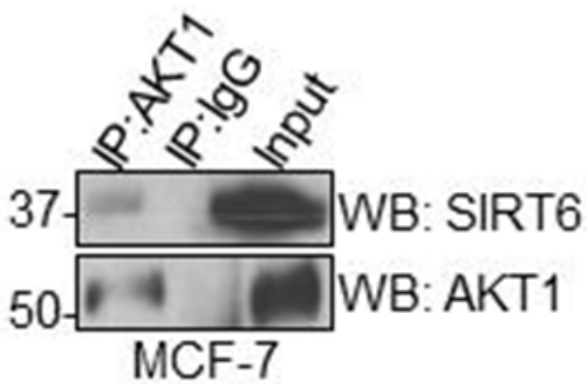

Figure 9C: Immunoprecipitation (IP) for AKT1 followed by immunoblotting in lysates from MCF-7 cells against an IgG (immunoglobulin G) control.

Figure 9: AKT1 interacts with SIRT6.

AKT1 phosphorylates SIRT6 on Ser^{338}

In order to investigate if AKT1 phosphorylates SIRT6, we performed an *in vitro* kinase assay, which showed that full-length recombinant SIRT6 could be directly phosphorylated by recombinant, functionally active AKT1 (Figure 10). To further identify the AKT1-mediated phosphorylation sites on SIRT6, we isolated SIRT6 from cells treated with EGF or IGF in the presence of MG-132 and analyzed it by mass spectrometry.

Three phosphorylation sites were identified on SIRT6: Ser^{303}, Ser^{330}, and Ser^{338} (Figures 11a-11c). To determine which site (or sites) is phosphorylated by AKT1, we mutated each one to an alanine residue and subjected all three mutants to *in vitro* kinase assays. Of these three mutants, phosphorylation was abolished in S338A (Figure 12), suggesting that AKT1 specifically phosphorylates SIRT6 at this position. To validate whether this site is phosphorylated in cells, we used a commercially available antibody that recognizes SIRT6

phosphorylated at Ser^{338} and, thus, detected Flag-tagged wild-type but not the nonphosphorylatable S338A mutant SIRT6 in MDA-MB-231 cells (Figure 13a). Moreover, in serum-starved MDA-MB-231 cells treated with IGF-1 for 1 hour in the presence of the protease inhibitor MG-132 to stabilize protein abundance, we observed an increase in SIRT6 phosphorylation at Ser338 (Figure 13b). A search of the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST) revealed that Ser^{338} of SIRT6 is highly conserved among mammals (Figure 14). It should be noted that Ser^{338} residue was also identified recently by another independent group [127]. Together, these results support that Ser^{338} of SIRT6 is an AKT1 phosphorylation site.

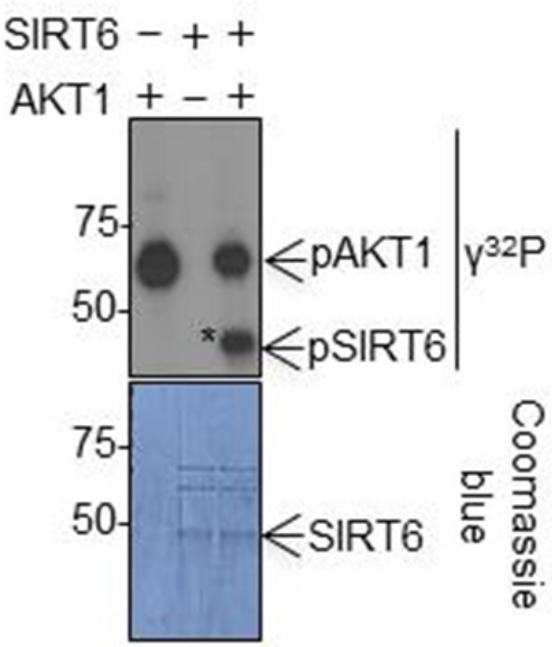

Figure 10: AKT1 phosphorylates SIRT6.

In vitro kinase assay with recombinant, active AKT1 and recombinant GST-tagged, full- length WT SIRT6.

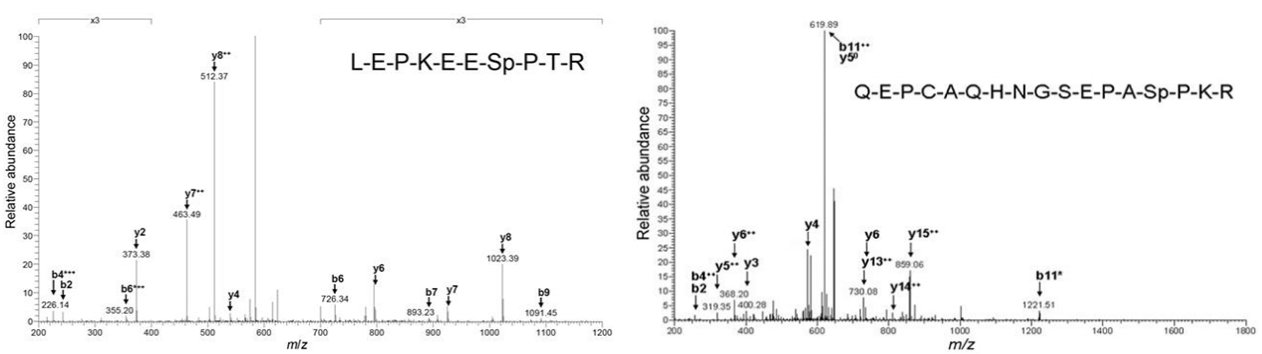

Figure 11: Three phosphorylation sites identified on SIRT6.

Mass spectrometry analysis of lysates from HeLa cells that had been serum-starved overnight, stimulated with EGF (50 ng/ml) for 30 min, and subjected to immunoprecipitation with a SIRT6 antibody identified three phosphorylation sites on SIRT6. A. Ser303 , B. Ser330, C. Ser338.

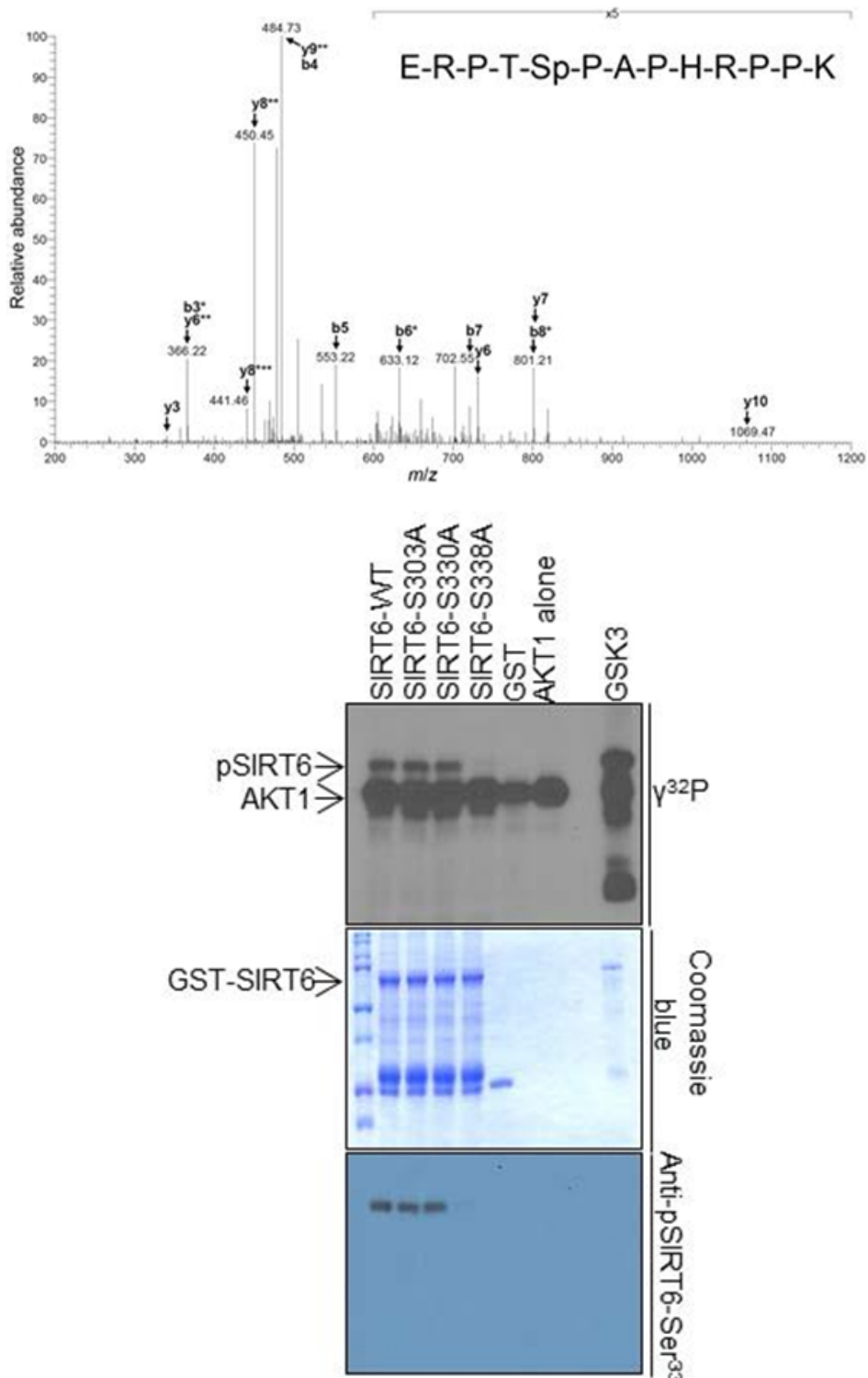

Figure 12: AKT1 phosphorylates SIRT6 on Ser[338].

In vitro kinase assay with recombinant, active AKT1 and either recombinant GST-tagged, full-length WT or mutant SIRT6. pSIRT6: phosphorylated SIRT6; GSK3: control AKT substrate.

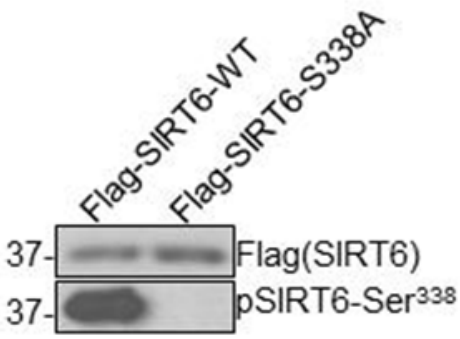

Figure 13A: Immunoblot for phosphorylated SIRT6-Ser338 (pSIRT6-Ser^{338}) in MDA-MB-231 cells that stably express Flag-tagged WT or mutant (S338A) SIRT6, demonstrating specificity of the antibody.

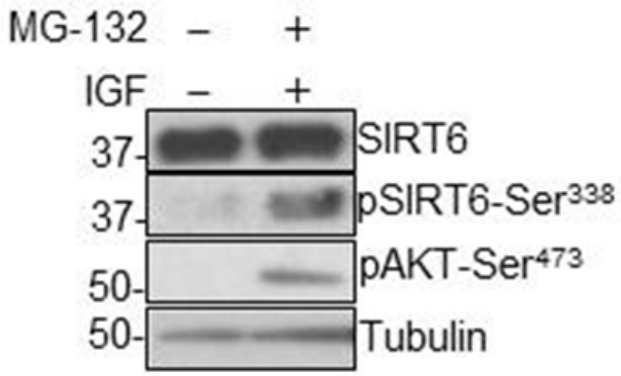

Figure 13B: Western blots for phosphorylated SIRT6 or AKT in lysates from MDA-MB-231 cells serum-starved overnight and then treated with IGF-1 in the presence of MG-132.

Figure 13: SIRT6 is phosphorylated on Ser^{338}.

AKT Phosphorylation Motif: RXRXXS

Human:	330	SPKRERPTSPAP	341
Mouse:	309	VSYKSKPNSPIL	320
Rat:	306	-SYKPKPDSPVP	316
Cow:	334	SPKRERPDSPSP	345
Boar:	334	SPKREQLDSPAP	345

Figure 14: AKT1 phosphorylation site on SIRT6 is conserved.

Sequence alignment of the AKT1 phosphorylation motif of SIRT6 from various species.

Chapter 5

MDM2 is required for AKT1-mediated SIRT6 degradation

MDM2 is required for AKT1-mediated SIRT6 degradation

MDM2 overexpression leads to SIRT6 degradation

MDM2 is the well-characterized oncogenic E3 ligase in the PI3K-AKT pathway and is phosphorylated and activated by AKT [128,129]. Because AKT1 suppresses SIRT6 protein abundance by decreasing its stability, we investigated whether MDM2 is involved in this process.

First, we found that overexpression of wild-type MDM2, but not the MDM2- 9 mutant, which lacks its E3 ligase domain [130], reduced endogenous SIRT6 abundance in HEK293T cells (Figure 15a). In MCF-7 cells, the abundance of SIRT6 increased when MDM2 was knocked down by siRNA (Figure 15b). In addition, when ubiquitin was over expressed concomitantly with MDM2 in HEK293T cells in the presence of MG-132, we observed a polyubiquitination pattern of SIRT6 (Figure 16), suggesting that SIRT6 may be polyubiquitinated for subsequent proteasome degradation.

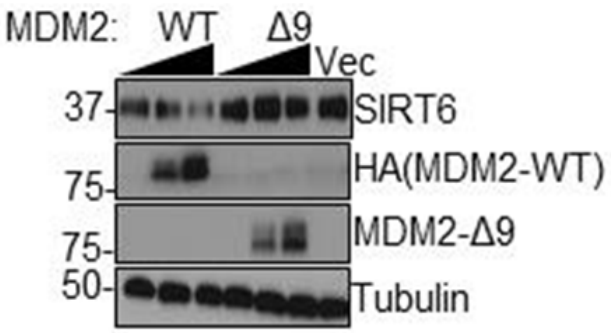

Figure 15 A: Western blotting in lysates from HEK293T cells transfected with 5 or 10 μg of HA-tagged MDM2-WT and MDM2- 9 or a control vector.

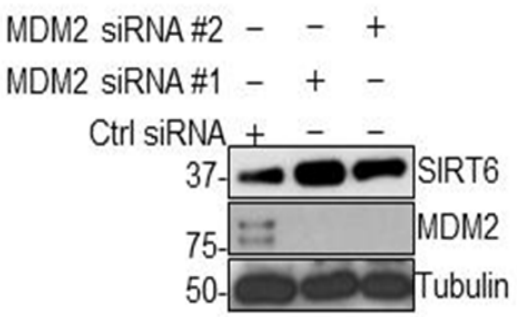

Figure 15B: Western blotting in lysates from MCF-7 cells transfected with one of two siRNAs against MDM2 or a control siRNA for 72 hours.

Figure 15: MDM2 promotes SIRT6 degradation.

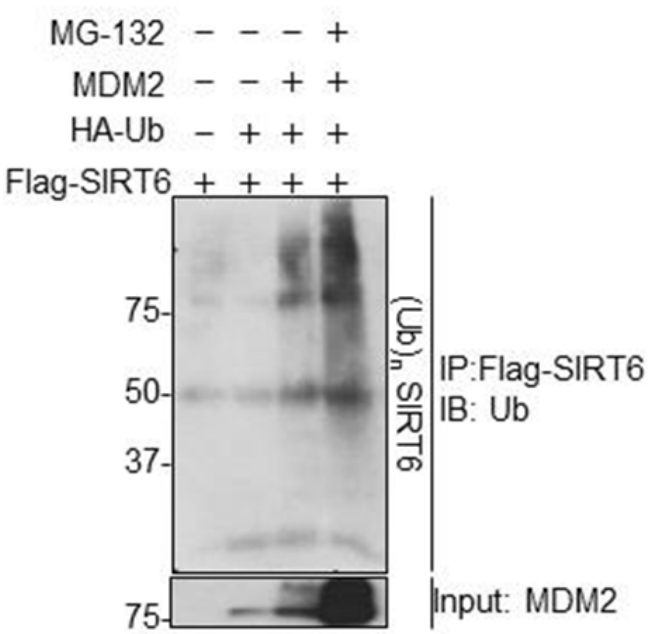

Figure 16: MDM2 induces polyubiquitination of SIRT6.

Immunoprecipitation (IP) for SIRT6 followed by immunoblotting for ubiquitin (Ub) in lysates from HEK293T cells transfected with the indicated plasmids in the presence of MG-132 (10 μM for 7 hours).

MDM2 interacts with SIRT6 and subjects it to proteosome-mediated degradation

Immunoprecipitation showed that MDM2 interacted with endogenous SIRT6 in MCF-7 cells (Figure 17a) and with exogenous Flag-SIRT6 in HEK293T cells (Figure 17b). We then analyzed the half-life of SIRT6 by using the protein synthesis inhibitor cycloheximide. Similar to the observations of SIRT6 abundance in HEK293T cells over expressing a constitutively active AKT1 in the presence of MG-132 (Figure 8b), exogenous SIRT6 abundance decreased by 50% in the presence of MDM2 after 4 hours in the presence of cycloheximide, whereas MG-132 prevented the degradation of SIRT6 even after 8 hours (Figure 18). Furthermore, SIRT6 could no longer be suppressed by IGF stimulation when MDM2 is knocked down by siRNA in MCF-7 cells (Figure 19). These results suggest that MDM2 degrades SIRT6 in a proteasome dependent manner and is required for AKT1-mediated SIRT6 degradation.

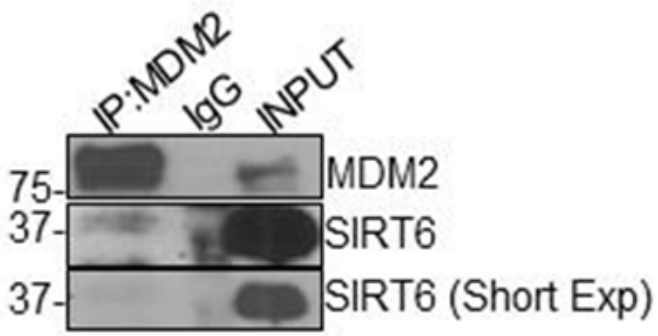

Figure 17 A: Immunoprecipitation for MDM2 followed by immunoblotting for SIRT6 and MDM2 in lysates from MCF-7 cells. Short Exp, shorter exposure time.

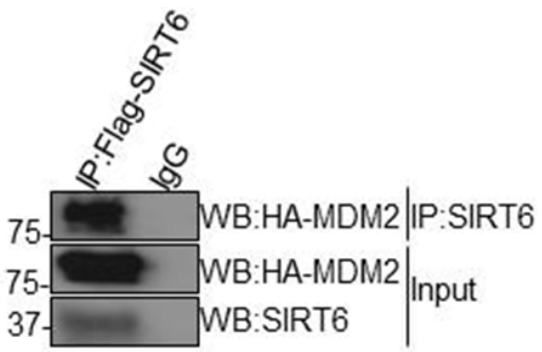

Figure 17B: Immunoprecipitation for Flag followed by immunoblotting (WB) in lysates from HEK293T cells transfected with HA-tagged WT MDM2 (MDM2-WT) and Flag-tagged WT SIRT6 (Flag-SIRT6).

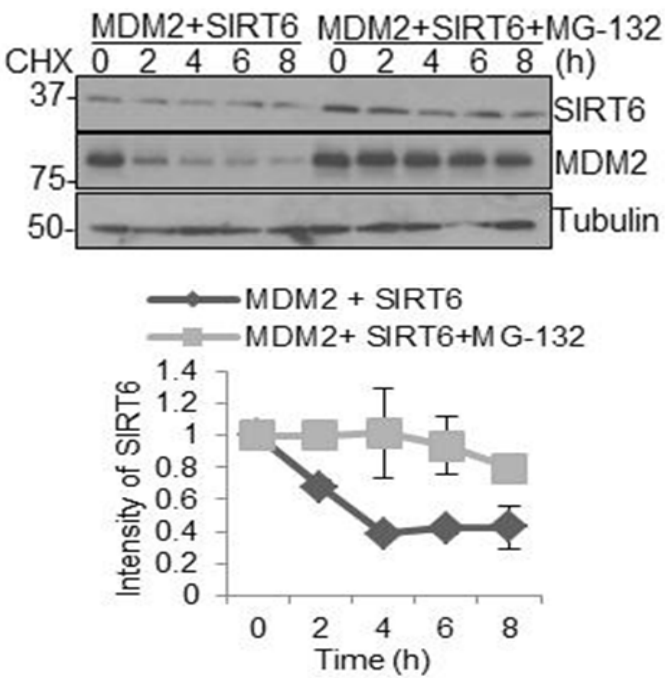

Figure 18: MDM2 degrades SIRT6 in a proteosome dependent manner.

Western blotting in lysates from HEK293T cells transfected with WT MDM2 (MDM2-WT) and Flag-tagged SIRT6 with or without MG-132, in the presence of cycloheximide (CHX) for up to 8 hours.

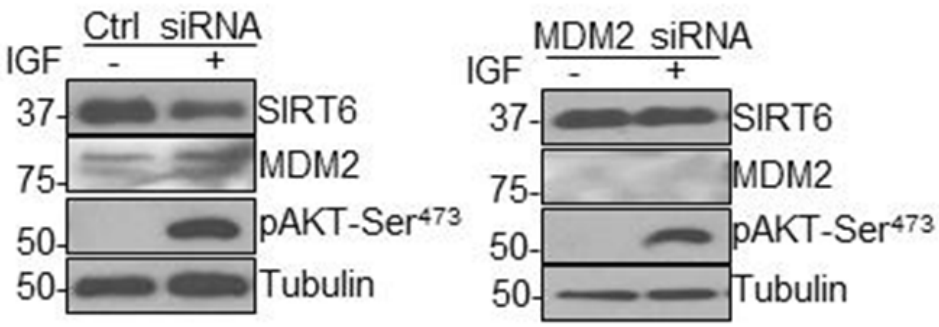

Figure 19: MDM2 is required for AKT mediated SIRT6 degradation.

Western blotting in lysates from MCF-7 cells transfected with an siRNA against MDM2 or a control siRNA for 48 hours, serum-starved for 16 hours, and then cultured with or without IGF (50 ng/ml) for 1 hour.

Chapter 6

Phosphorylation of SIRT6 by AKT1 facilitates MDM2-mediated degradation

Phosphorylation of SIRT6 by AKT1 facilitates MDM2-mediated degradation

Phosphorylation of SIRT6 is a prerequisite for MDM2-mediated degradation

To further show that the phosphorylation of SIRT6 by AKT1 alters its stability, we compared the stability of two SIRT6 mutant proteins: SIRT6-S338A, a nonphosphorylatable mutant, and SIRT6-S338D, a phosphorylation-mimic mutant. Under cycloheximide treatment in MCF-7 cells, the abundance of SIRT6-S338D decreased after 2 hours, whereas SIRT6-S338A abundance remained unsubstantially changed for at least up to 8 hours (Figure 20a). Consistently, the SIRT6-S338D mutant interacted more strongly with MDM2 in MCF-7 cells than did SIRT6-S338A (Figure 20b). These results suggest that AKT1-induced phosphorylation of SIRT6 may recruit MDM2 and ubiquitinate SIRT6 to promote its subsequent degradation. To determine whether this interaction indeed promoted SIRT6 degradation, the SIRT6-S338A or SIRT6-S338D mutant was cotransfected with MDM2 into HEK293T cells. As expected, the abundance of SIRT6-S338D, but not SIRT6-S338A, was decreased in the presence of MDM2 (Figure 20c). Compared with wild-type SIRT6, the SIRT6-S338D mutant was heavily ubiquitinated and the SIRT6-S338A mutant was the least ubiquitinated in the presence of MDM2 and MG-132 in MCF-7 cells (Figure 20d). Together, these data indicate that MDM2 is the E3 ligase that mediates SIRT6 degradation and that the interaction between MDM2 and SIRT6 is dependent on AKT1-mediated SIRT6 phosphorylation on Ser[338].

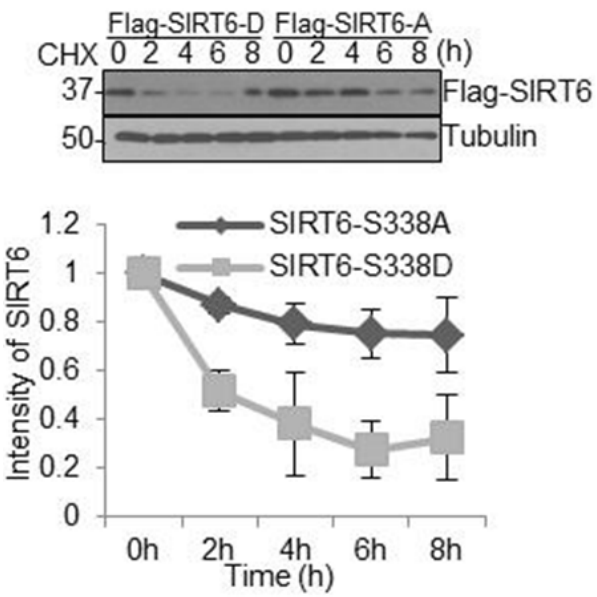

Figure 20A: SIRT6-S338A mutant is stable than the SIRT6-S338D mutant Western blotting in lysates from MCF-7 cells that stably express Flag-tagged SIRT6-S338A (Flag-SIRT6-A) or SIRT6-S338D (Flag-SIRT6-D) in the presence of cycloheximide (CHX) for up to 8 hours.

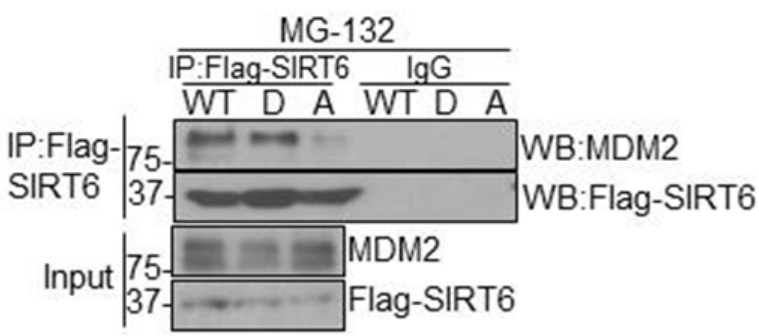

Figure 20B: SIRT6-S338D mutant interacts strongly with MDM2 Immunoprecipitation (IP) with a Flag antibody followed by immunoblotting (WB) in lysates from MCF-7 cells that stably express Flag-tagged, WT SIRT6 (WT), SIRT6-S338A (A), or SIRT6-S338D (D) treated with MG-132 for 7 hours.

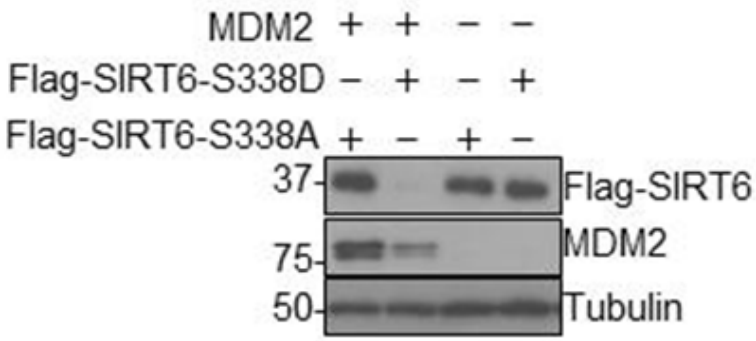

Figure 20C: SIRT6-S338D but not SIRT6-S338A mutant is degraded by MDM2 .

Western blotting in lysates from HEK293T cells transfected with Flag-tagged SIRT6-S338A (Flag-SIRT6-S338A) or Flag-tagged SIRT6-S338D (Flag-SIRT6-S338D) and WT MDM2, harvested 72 hours after transfection.

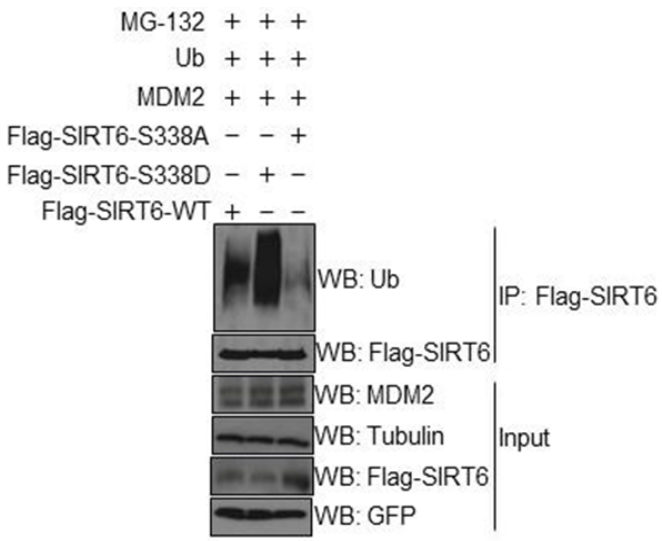

Figure 20D: SIRT6-S338D mutant is highly ubiquitinated Immunoprecipitation with a Flag antibody followed by immunoblotting for ubiquitin in lysates from HEK293T cells transfected with either Flag-tagged WT SIRT6 (Flag-SIRT6-WT) or mutant SIRT6 (S338A or S338D) and WT MDM2 and ubiquitin (Ub) and treated with MG-132 for 7 hours.

Figure 20: Phosphorylation of SIRT6 by AKT1 facilitates MDM2-mediated ubiquitination and degradation.

Figure 20 Phosphorylation of SIRT6 by AKT1 facilitates MDM2-mediated ubiquitination and degradation A. SIRT6-S338A mutant is stable than the SIRT6-S338D mutant Western blotting in lysates from MCF-7 cells that stably express Flag-tagged SIRT6-S338A (Flag-SIRT6-A) or SIRT6-S338D (Flag-SIRT6-D) in the presence of cycloheximide (CHX) for up to 8 hours.

Chapter 7

Nonphosphorylatable SIRT6 inhibits breast cancer tumorigenesis

Nonphosphorylatable SIRT6 inhibits breast cancer tumorigenesis

Nonphosphorylatable SIRT6 inhibits breast cancer tumorigenesis

Because the nonphosphorylatable SIRT6 mutant had enhanced stability and the phosphorylation-mimic mutant had less stability compared to the wild-type SIRT6, we examined the function of SIRT6-WT, SIRT6-S338A, and SIRT6-S338D in cellular proliferation and breast cancer tumorigenesis. Knockdown of endogenous SIRT6 by short hairpin RNA (shRNA) increased the proliferation of MDA-MB-231 cells in culture, as determined by a cell counting assay (Figure 21a), and enhanced the growth of MDA-MB-231 xenografts in the mammary fat pads of nude mice (Figure 21b). We further examined the function of the phosphorylation of SIRT6 at Ser338 in cell proliferation and tumorigenesis by expressing wild-type or either mutant SIRT6 in MDA-MB-231 cells. Expression of the nonphosphorylatable SIRT6-S338A mutant suppressed cell proliferation (Figure 21c) and colony formation on soft agar (Figure 21d) more than the wild-type SIRT6 or the phosphorylation-mimic SIRT6-S338D mutant compared to the vector control.

To further test the tumor-suppressive activity of SIRT6 mutants *in vivo*, we injected MDA-MB-231 cells stably expressing the control vector, wild-type SIRT6, or either mutant SIRT6 into the mammary fat pads of nude mice and monitored tumor development. We found that tumor volume in mice injected with MDA-MB-231 cells stably expressing wild-type SIRT6 was smaller than those injected with cells expressing the control vector. The growth of tumors expressing the SIRT6-S338A mutant was significantly decreased compared with those expressing the control vector or the phosphorylation-mimic SIRT6-S338D mutant (Figure 21e).

To further investigate whether the expression of SIRT6 phospho-mutants affects the endogenous expression of known SIRT6 target genes that are involved in promoting tumorigenesis, we performed a quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of MDA-MB-231 cells expressing vector control, SIRT6-WT, SIRT6-S338A,or SIRT6-S338D. We found that the SIRT6-S338A mutant suppressed the mRNA abundance of a panel of target genes more significantly (AKT1, AKT3, IGF-1R, PDK1, MTOR, and LDHA) than others (GSK3B and PFKM), whereas the SIRT6-S338D mutant had no inhibitory effect on the target genes compared to SIRT6-WT (Figure 22a). SIRT6-deficient mice exhibit increased phosphorylation of AKT compared with controls and subsequently have severe hypoglycemia because of enhanced basal and insulin-stimulated glucose uptake [131]. On the other hand, SIRT6-deficient mouse embryonic fibroblasts (MEFs) showed similar amounts of phosphorylated AKT to wild-type MEFs [132]. Thus, we investigated the phosphorylation of AKT in MDA-MB-231 breast cancer cell line that expressed vector, SIRT6-WT, A-SIRT6, or D-SIRT6. Clones were chosen in such a way that the expression of wild-type and mutant SIRT6 were similar, which would make the phosphorylation of AKT comparable. In our system, although there was a slight decrease in the abundance of phosphorylated AKT in the presence of wild-type SIRT6 as previously reported [131], there was no significant difference between the mutants and the wild-type SIRT6 (Figure 22b), suggesting that the Ser^{338} mutation on SIRT6 might not contribute to SIRT6-mediated suppression of AKT activation.

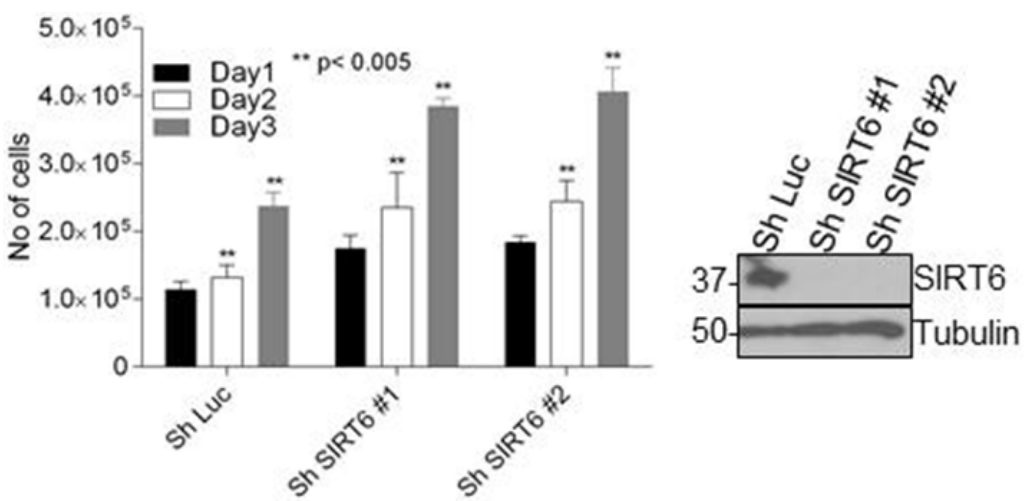

Figure 21A: Proliferation and immunoblot of MDA-MB-231 cells transfected with either shRNA against luciferase or one of two shRNAs against SIRT6. Data are means ± SE from three experiments.

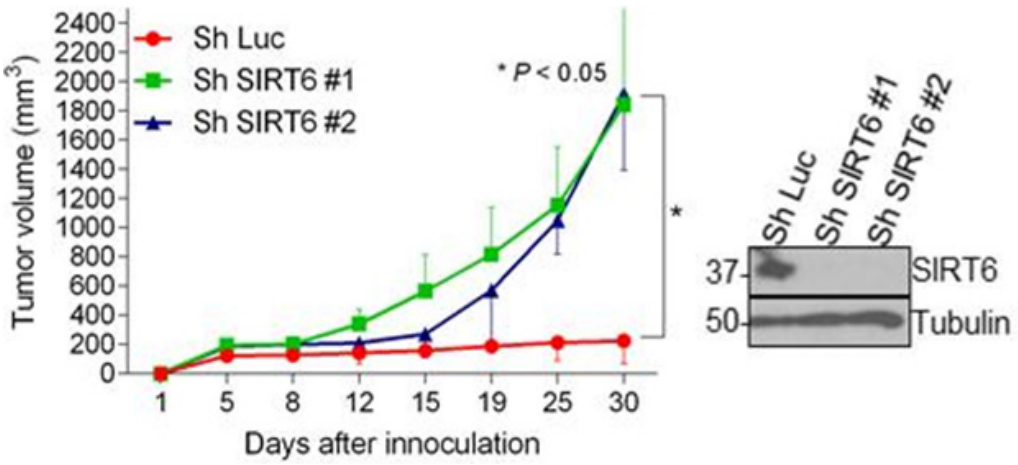

Figure 21B: Growth of mammary fat pad xenografts derived from MDA-MB-231 cells transfected with either luciferase shRNA or one of two SIRT6 shRNAs. Data are means ± SE from five mice per group.

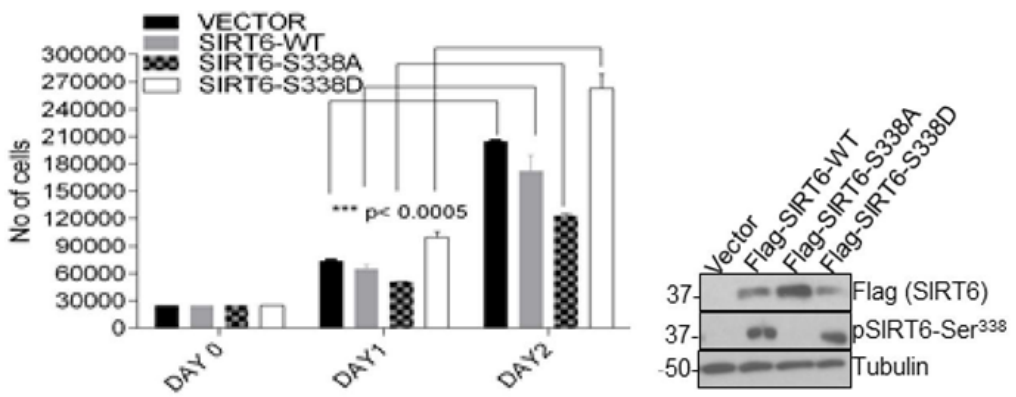

Figure 21C: Proliferation and immunoblot of MDA-MB-231 cells infected with lentiviral vector, WT SIRT6 (SIRT6-WT), SIRT6-S338A, or SIRT6-S338D. Data are means ± SE from three experiments.

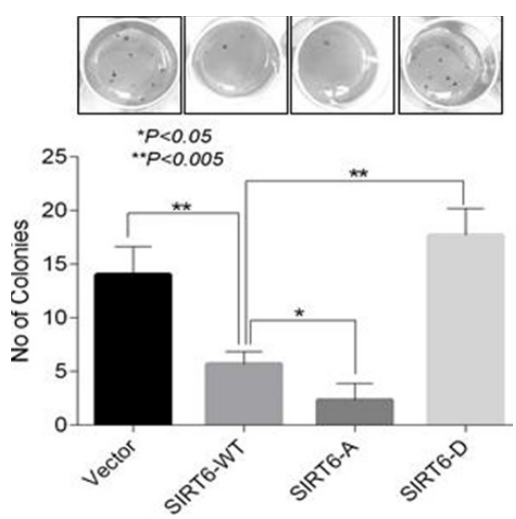

Figure 21D: Soft agar colony formation by MDA-MB-231 cells infected with lentiviral vector, SIRT6-WT, SIRT6-S338A (SIRT6-A), or SIRT6-S338D (SIRT6-D).

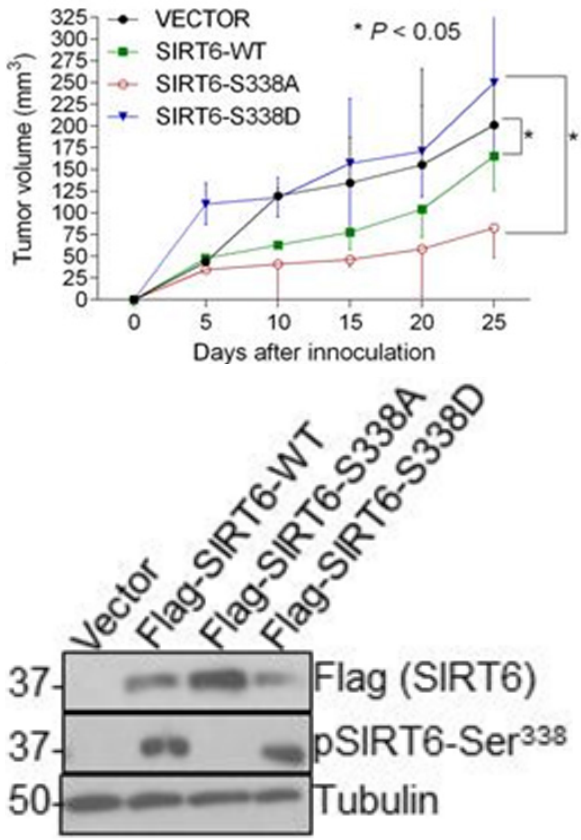

Figure 21E: Tumor growth of orthotopically transplanted MDA-MB-231 cells infected with lentiviral vector, SIRT6-WT, SIRT6-S338A, or SIRT6-S338D. Data are means ± SE from five mice per group.

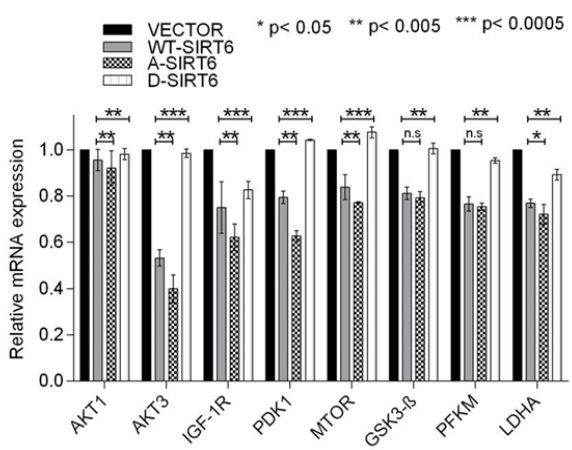

Figure 22A: Quantitative RT-PCR analysis cDNA was isolated from MDA-MB-231 cells infected with lentivirus expressing vector, wild-type (WT) SIRT6, or mutant [S338A (A) or S338D (D)] SIRT6. Data are means ± S.E. from 3 experiments.

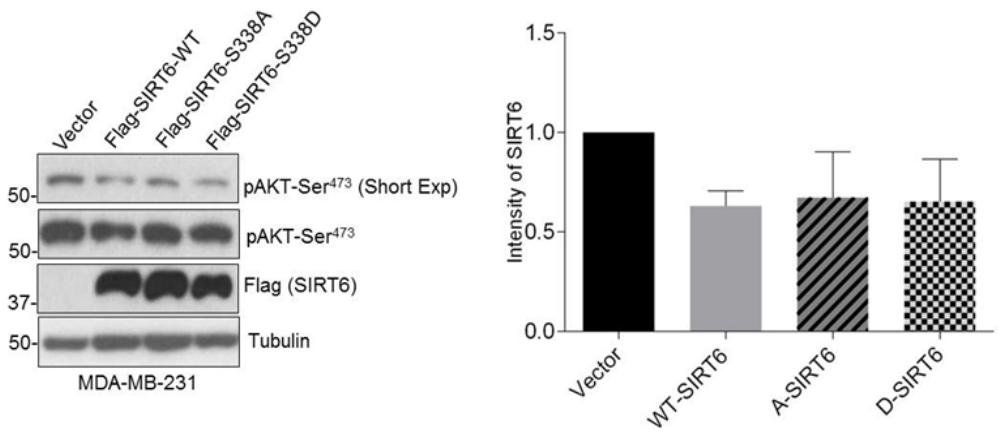

Figure 22B: Western blotting for Flag and phosphorylated AKT in MDA-MB-231 cells infected with lentivirus expressing vector, Flag-tagged wild-type SIRT6 (SIRT6-WT), or Flag-tagged SIRT6 mutant [S338A (Flag-SIRT6-A) or S338D (Flag SIRT6-D)]. Blot is representative; data are means ± S.E. from 3 experiments.

Figure 22: SIRT6 phosphorylation affects expression of endogenous SIRT6 target genes, but not AKT phosphorylation.

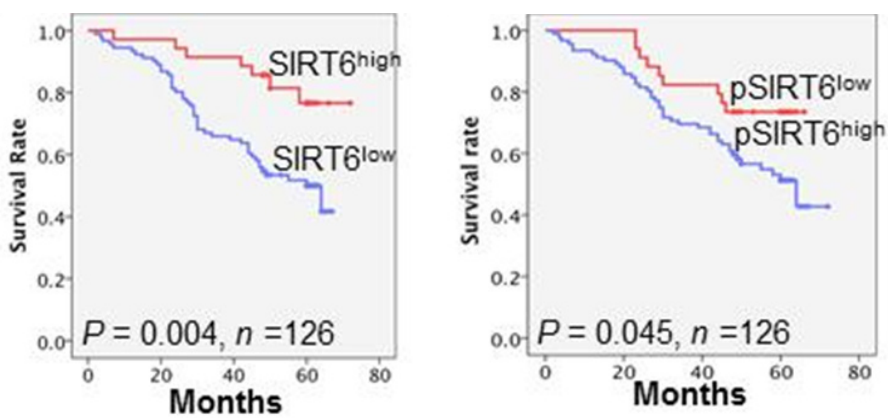

Figure 23A: Survival curves of patients with breast tumors that have high or low abundance of total or phosphorylated SIRT6.

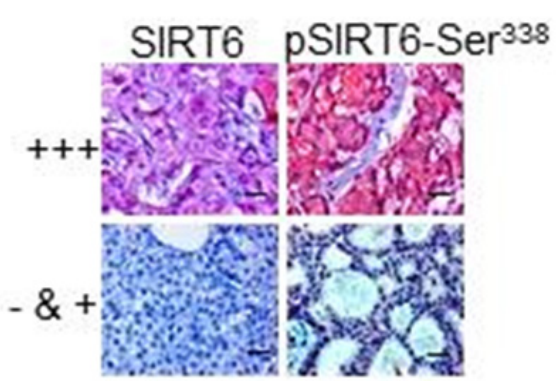

Figure 23B: Immunohistochemistry for SIRT6 and phosphorylated SIRT6 in representative tumor tissues from patients in (F). +++, high expression; − & +, low or no expression. Scale bars, 25 μm.

Figure 23: High SIRT6 levels correlate with better prognosis in breast cancer patients.

High SIRT6 levels correlate with better prognosis in breast cancer patients

To determine the correlation between SIRT6 phosphorylation and breast cancer patient survival or disease progression, Immunohistochemical staining was performed for total and phosphorylated SIRT6 in biopsy tissues from 126 breast cancer patients. Patients whose tumors had high SIRT6 abundance had better overall survival than those whose tumors had low SIRT6 abundance. However, patients whose tumors had high abundance of phosphorylated SIRT6 had poorer overall survival than those whose tumors had low abundance of phosphorylated SIRT6 (Figure 23a & 23b). These results suggest that SIRT6 and its phosphorylation status may have the potential to be predictive of breast cancer patient survival.

Chapter 8

Loss of SIRT6 results in trastuzumab resistance in HER2 over expressing breast cancer cells.

Loss of SIRT6 results in trastuzumab resistance in HER2 over expressing breast cancer cells.

Loss of SIRT6 results in trastuzumab resistance in HER2 over expressing breast cancer cells

Trastuzumab is a standard treatment for patients with HER2-positive breast cancer. However, intrinsic or acquired resistance to this treatment is observed [133,134]. Increased or constitutive activation of AKT appears to be a key factor in trastuzumab resistance [135-138]. Because we found that AKT1 decreased SIRT6 stability through phosphorylation and subsequent proteasome-dependent degradation, we speculated that SIRT6 might also play a role in trastuzumab resistance. Indeed, SIRT6 abundance was lower in two trastuzumab-resistant, HER2-positive breast cancer cell lines (BT474-TtzmR and SKBR3-TtzmR) compared with trastuzumab-sensitive parental lines (BT474-P and SKBR3-P) (Figure 24a). The abundance of SIRT6 increased after the addition of trastuzumab in BT474-P, but not in BT474-TtzmR, cells (Figure 24b). In the BT474-P cells, 24-hour treatment with trastuzumab inhibited the phosphorylation of AKT at Ser^{473}, whereas in the BT474-TtzmR cells, there was residual phosphorylation of AKT even after 48 hours of trastuzumab treatment. This persistent activation of AKT appeared to attenuate the increase in SIRT6 protein abundance seen in the parental cells, suggesting that the induction of SIRT6 contributes to the therapeutic effect of trastuzumab. To further validate the above findings, we knocked down SIRT6 in BT474-P cells and cultured them in trastuzumab for 4 days. Loss of SIRT6 in trastuzumab-sensitive BT474-P cells decreased cell sensitivity to trastuzumab to a similar sensitivity seen in BT474-TtzmR cells as measured by relative metabolic activity in an MTT assay (Figure 24c). Expression of the nonphosphorylatable SIRT6-S338A mutant considerably resensitized BT474-TtzmR cells to trastuzumab compared with expression of either wild-type SIRT6 or the phosphorylation-mimic SIRT6-S338D mutant (Figure 24c). These data suggest a mechanism by which trastuzumab inhibits breast cancer cell proliferation through the induction of SIRT6 and that loss of SIRT6 mediated by AKT1 and MDM2 contributes to trastuzumab resistance.

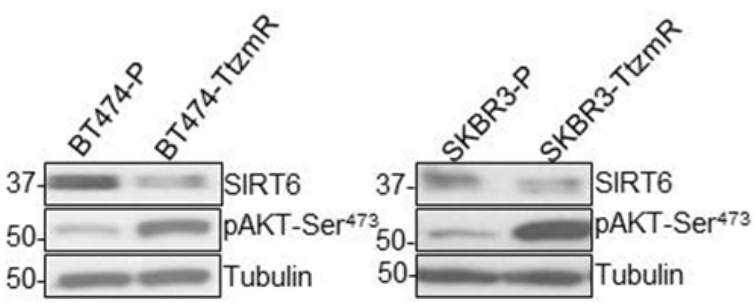

Figure 24A: Western blotting in cell lysates from BT474 parental (BT474-P), BT474 trastuzumab resistant (BT474-TtzmR), SKBR3 parental (SKBR3-P), and SKBR3-trastuzumab– resistant (SKBR3-TtzmR) cells. Blots are representative of three experiments.

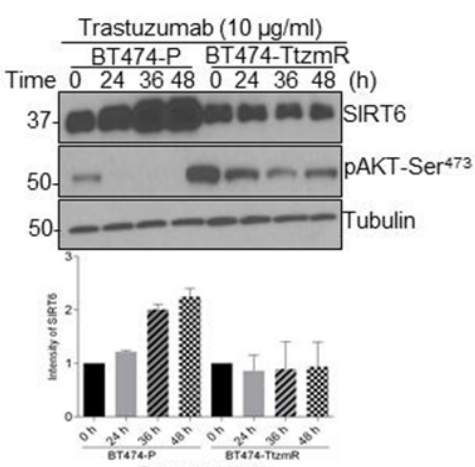

Figure 24B: Western blots for SIRT6 and phosphorylated AKT at Ser^{473}(pAKT-Ser^{473}) in BT474-PandBT474-TtzmRcells treated with trastuzumab for up to 48 hours. Blot is representative, and data in graph are means ± SE abundance of SIRT6 normalized to tubulin from three independent experiments.

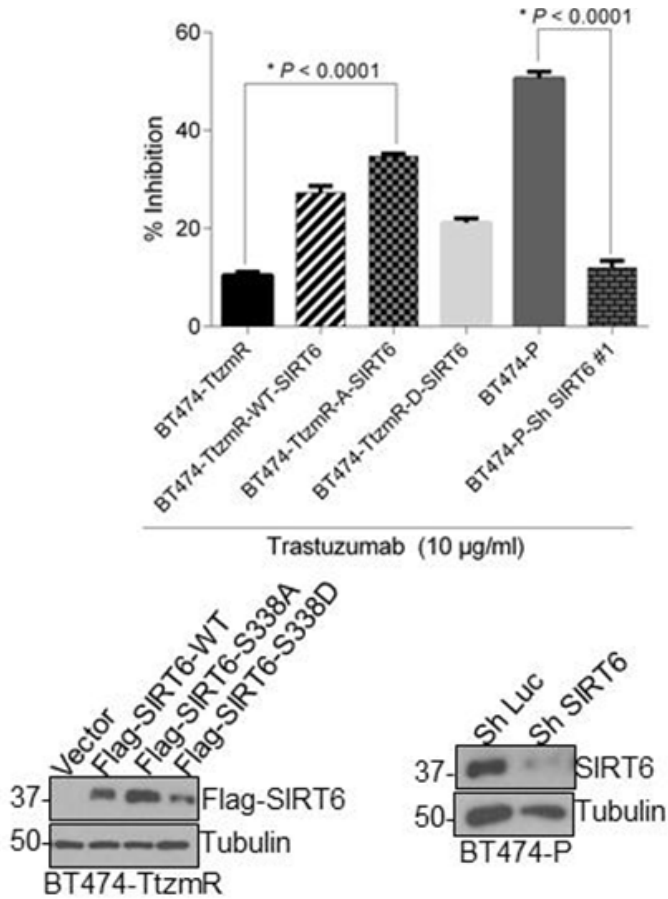

Figure 24C: Cell viability, assessed by relative proliferation by an MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] assay, in BT474-TtzmR cells that stably expressed SIRT6-WT, SIRT6-S338A, or SIRT6-S338D, and in BT474-P cells transfected with luciferase or SIRT6 shRNA, each treated with trastuzumab (10 µg/ml) for 4 days. Blots show representative transfection or knockdown, respectively. Data are means ± SE from three independent experiments. *P < 0.0001, Student's t test.

Figure 24: Loss of SIRT6 results in Trastuzumab resistance**.**

Chapter 9
Summary and Discussion

Summary and Discussion

Summary

Here, we have identified two post translational modifications on SIRT6 that leads to its degradation via the proteosome pathway. The degradation of SIRT6 in turn results in breast cancer tumorigenesis and resistance to trastuzumab in HER2 over expressing breast cancers. In our proposed model, activated AKT1 interacts with SIRT6 and phosphorylates SIRT6 on Ser^{338}. Phosphorylation at this site promotes the interaction between SIRT6 and the E3 ligase MDM2, followed by ubiquitination and degradation of SIRT6 in a proteosome dependent manner. Loss of SIRT6 in breast cancer, leads to enhanced cell proliferation *in vitro* and aggressive breast tumor growth *in vivo* in an orthotropic mouse model, probably through downregulation of genes involved in tumorigenesis. Reconstitution with SIRT6-S338A mutant suppressed breast cancer tumorigenesis effectively than the wild type SIRT6, while the SIRT6-S338D mutant has no tumor suppressive activity at all. Analysis of human breast cancer patient tissues showed that SIRT6 protein expression is indeed down regulated in breast cancer tissues compared to the normal breast tissues and patients with high SIRT6 expression have a better overall survival than patients with low SIRT6. Also, patients with higher levels of phosphorylated SIRT6 have poor survival when compared to those with lower levels. In addition, we also found that SIRT6 protein expression was down regulated in cells that were resistant to trastuzumab when compared to the sensitive parental cells. Knockdown of SIRT6 in the trastuzumab sensitive cells made them resistant to trastuzumab, while reconstituition with the SIRT6-S338A mutant rendered these cells sensitive to trastuzumab to a greater extent that the wild type SIRT6. However, reconstitution with SIRT6-S338D mutant did not resensitize the cells to trastuzumab. Thus this data suggests that both SIRT6 expression and SIRT6 phosphorylation could be used as biomarkers for breast cancer and also for trastuzumab resistance in breast cancer.

Discussion

The histone deacetylase SIRT6 regulates a number of functions including aging, inflammation, glucose metabolism, mitochondrial respiration, DNA damage repair etc. These processes either directly or indirectly contribute to tumorigenesis. Although the role of SIRT6 as a tumor suppressor in a number of cancers has started to unfold, there are no clear thoughts on how SIRT6 might be regulated in these cancers. So, we sought out to identify novel regulations of SIRT6 in breast cancers and how these regulations control breast cancer tumorigenesis.

In this study, we propose a model in which the histone deacetylase SIRT6 is phosphorylated and inhibited by the serine/threonine kinase AKT1. Mechanistically, SIRT6 is phosphorylated by AKT1 and targeted for subsequent degradation by the MDM2-dependent proteosome degradation pathway (Figure 25). We have identified Ser^{338} to be the AKT1 phosphorylation site on SIRT6 and this phosphorylation increased the interaction between MDM2 and SIRT6 and hence its degradation. Thus this work has led to the identification of two novel post translational modifications of SIRT6 that affects its protein stability. Independently, the Ser^{338} site on SIRT6 was identified by another group [127], which shows that this phosphorylation is highly conserved and it modulates interactions of SIRT6.

Prior to our finding that MDM2 is a novel E3 ligase for SIRT6, Ronnebaum et al. had identified C terminus of Hsc70-interacting protein (CHIP) as an E3 ligase that stabilized SIRT6 through non-canonical ubiquitination [139]. But, they had also observed canonical ubiquitination of SIRT6 even in the absence of CHIP. This is interesting, as it is an indication of a possibility for other E3 ligases like MDM2 to ubiquitinate and degrade SIRT6. Moreover, recently, ubiquitin peptidase or deubiquitinase, USP10 was shown to interact with, deubiquitinate, and stabilize SIRT6 in colon cancer cells [140]. Also, USP10 deubiquitinates and stabilizes p53, which is a well-known substrate that is degraded by MDM2-dependent proteosome pathway. This proposes a similar mechanism wherein SIRT6 is destabilized through ubiquitination by MDM2, which could be reversed by deubiquitination by USP10.

To further delineate the significance of SIRT6 phosphorylation with respect to breast cancer tumorigenesis, we mutated the serine 338 residue to alanine (SIRT6-S338A) and to aspartate (SIRT6-S338D) to generate the nonphosphorylatable and the phospho-mimic mutants of SIRT6 respectively. We used various *in vitro* assays and *in vivo* experiments in mice to show that the nonphosphorylatable SIRT6-S338A mutant is resistant to MDM2-mediated degradation, is more stable than wild-type SIRT6, and promotes stronger suppression of cell proliferation *in vitro* and tumor growth *in vivo* in mice. SIRT6 phosphorylation also affected the transcription of SIRT6 target genes that are involved in promoting tumorigenesis, with some genes affected more significantly than the others. The SIRT6-S338A suppressed transcription greater that the WT-SIRT6, while the SIRT6-S338D mutant had no inhibitory effect.

Our results, together with previously published studies [39,141-144], further strengthen the role of SIRT6 as a tumor suppressor in numerous cancers and how loss of SIRT6 is a critical step for the promotion of tumorigenesis.

Increased AKT activation is also associated with the development of trastuzumab resistance in breast tumors overexpressing HER2 [133,134,145]. Various treatment alternatives and combination therapies have been designed to overcome this issue of resistance, including combining trastuzumab with the SRC inhibitor sarcatinib [146] or an AKT inhibitor [147] and, most recently, trastuzumab-DM1 (T-DM1) was developed by Genentech, in which a cytotoxic agent mertansine is linked to the monoclonal antibody against HER2 [148]. Because, we found that AKT signaling can negatively regulate SIRT6, we looked if SIRT6

downregulation could also be associated with resistance to trastuzumab. As expected, SIRT6 protein levels were lower in trastuzumab-resistant cells than in trastuzumab-sensitive cells. When we manipulated SIRT6 abundance in resistance cells, we were able to modulate its sensitivity to trastuzumab. Reconstitution of SIRT6-S338A in the resistant cells rendered the cells sensitive to trastuzumab again to a greater extent compared to the wild type, while the SIRT6-S338D mutant had no effect. Thus these results suggest that by manipulating SIRT6 abundance, we could modulate the sensitivity to trastuzumab and that the loss of SIRT6 might be one of the mechanisms that enable acquired resistance to trastuzumab. This suggests that histochemical analysis of SIRT6 expression in breast cancer patients treated with trastuzumab might be used as a biomarker to determine drug sensitivity in them.

Interestingly, SIRT6 has been shown to activate PARP1 during oxidative stress and thus promotes efficient DNA repair [51]. PARP is a family of proteins consisting of PARP1 and PARP2, and they recruit other DNA repair proteins to the sites of DNA damage [149]. They play a critical role in repair of DNA through Base Excision Repair (BER). This makes PARP effective targets for anti-cancer therapies and there are a number of PARP1 inhibitors that are currently in clinical trials. We and others have shown that SIRT6 acts as a tumor suppressor in various cancers. But the fact that SIRT6 can activate PARP1, implies the possibility of another role for SIRT6 in tumorigenesis. It would be interesting to investigate if SIRT6 also has a role in tumor progression which might be context dependent.

Also, we have seen that the SIRT6 phosphorylation on Ser338 affects its stability and further analysis would have to be done to see if this phosphorylation has any effect on the deacetylase activity or ADP-ribosyl transferase activity of SIRT6.

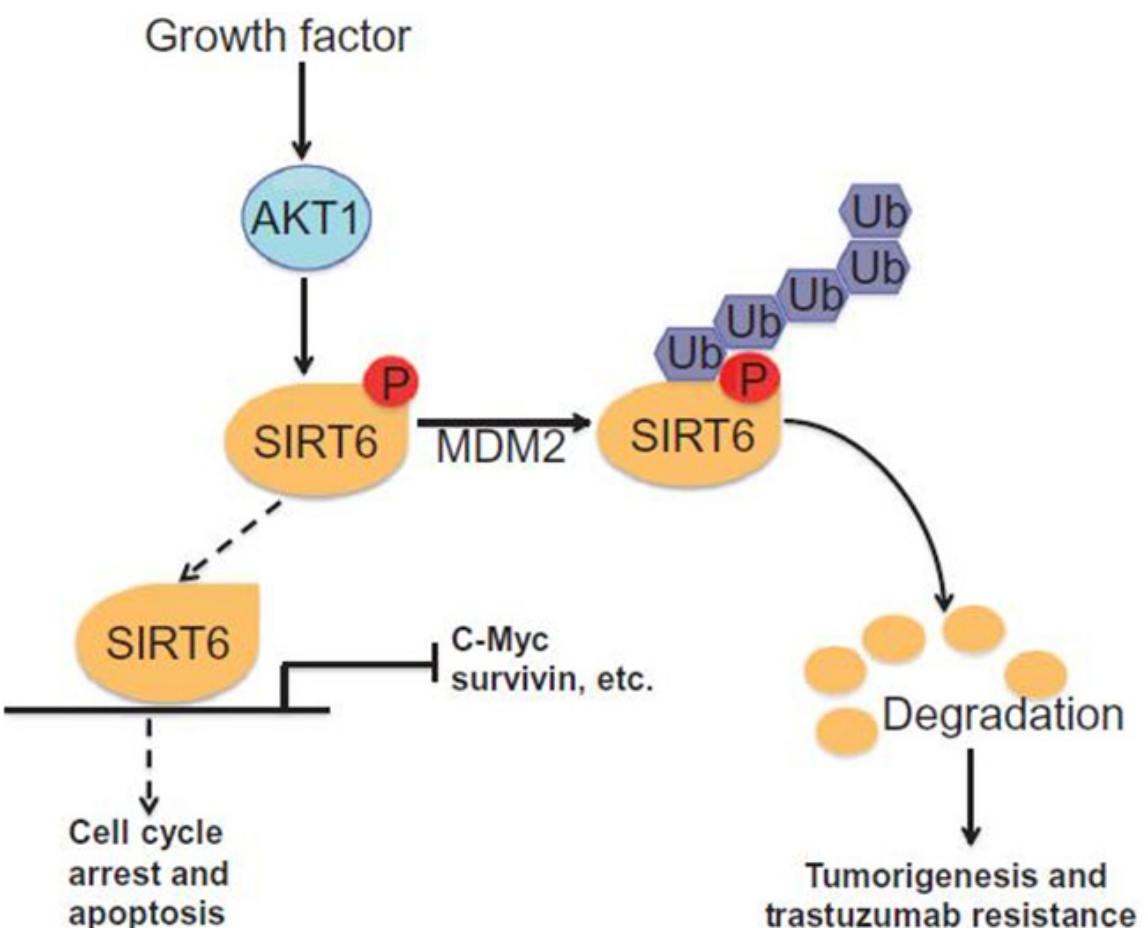

Figure 25: Model of AKT1 dependent SIRT6 degradation.

Future Directions

Future Directions

To study the relation between SIRT6 and trastuzumab resistance

The cell line data showed that SIRT6 protein levels were lower in trastuzumab resistant BT474-TtzmR compared to the sensitive BT474-P cells. Also, reexpression of SIRT6-S338A mutant in the BT474-TtzmR cells overcame the acquired resistance to trastuzumab, more effectively than the SIRT6-WT. We would like to see if these phenomenon exist *in vivo*. To test this, we would perform two independent sets of experiments.

First experiment would be designed to analyze if SIRT6 is lost when the cells become resistant to trastuzumab treatment. Female nude mice would be implanted with 0.72 mg, 60-day release 17β-estradiol pellets before inoculating with the appropriate cell lines. Tumors would be established by injecting BT474-Parental (BT474-P) cells (5×10^6) into the mammary fat pads of mice (20 mice per group). Tumor size would be measured every 3 days. When the tumor volume reaches about 200 mm^3, the mice would be randomly distributed into two groups. The experimental group would be treated with trastuzumab (10 mg/kg) and the control group with vehicle (IgG) intraperitoneally (i.p.), once a week. Two scenarios can be expected from the experimental group- the tumors that are sensitive to trastuzumab would shrink, the tumors that are resistant would continue growing even under treatment and the tumors which acquire resistance would continue to shrink initially, but then starts to grow in the presence of trastuzumab. Tumors would be harvested at these different stages and would be stained for SIRT6 and phospho SIRT6-Ser^{338} expression by immuno histochemistry. These results would indicate if SIRT6 is lost while acquiring resistance to trastuzumab *in vivo* and if the mechanism for loss of SIRT6 is through phosphorylation mediated degradation.

In the second set of experiments, we would like to see if SIRT6 phosphorylation affects the acquired resistance to trastuzumab. BT474-TtzmR cells which express either WT/S33A/S338D SIRT6 would be injected into the mammary fat pads of nude mice, as mentioned above and after the tumors reach around 200 mm^3 in volume, they would be started under trastuzumab treatment and the tumor volumes would then be monitored.

Based on the cell line data, we would expect the BT474-TtzmR that express the SIRT6-S33A mutant to be sensitive to trastuzumab and not acquire resistance.

Analysis of SIRT6 expression in trastuzumab treated patients

Preliminary analysis showed that SIRT6 abundance is reduced in HER2 over expressing breast cancer cells that had acquired resistance to trastuzumab when compared to the parental sensitive cell lines. Also, reconstitution of the nonphosphorylatable SIRT6 mutant in the resistant cells resensitized these cells to trastuzumab again. We would like to further investigate the association between SIRT6 abundance and acquired resistance to trastuzumab in breast cancer patients treated with trastuzumab. Immunohistochemical analysis for SIRT6 and phospho SIRT6-S^{338} in the tissues obtained from breast cancer patients at MD Anderson Cancer Center who were treated with trastuzumab would be performed. Tissue specimens from patients who are sensitive, resistant and who were initially sensitive, but acquired resistance to trastuzumab would be analyzed and compared.

Identification of kinases responsible for phosphorylation of SIRT6 in Ser303 and Ser330

Mass spectrometry analysis had identified two other phosphorylation sites on SIRT6 under growth factor stimulation, Ser^{303} and Ser^{330}. We would like to identify the kinase(s) that are responsible for phosphorylating SIRT6 on these sites. Preliminary analysis of the amino acid sequence of SIRT6 revealed that these two residues lie within a CDK consensus phosphorylation motif. The CDK consensus sequence for the phosphorylation site in the substrate is [S/T*]PX[K/R], where S/T* is the phosphorylated serine or threonine, P is proline, X is any amino acid, K is lysine, and R is arginine(150). We would further confirm which CDK(s) phosphorylates SIRT6 on Ser^{303} and (or) Ser^{330} by performing *in vitro* kinase assay. The phosphorylation site would then be confirmed by mutational analysis of the serine residue(s) to alanine, followed by kinase assay.

After the successful confirmation of the kinase and the phosphorylation site(s), the next step would be the identification of the functional significance of this phosphorylation on SIRT6. As CDKs plays a critical role in cell cycle regulation, we would like to look at the role of SIRT6 in cell cycle regulation, if any and also the role of this phosphorylation with respect to cell cycle.

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