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Regulation of Alteration/Deficiency in Activation 3 (ADA3) by Acetylation and its Role in Cell Cycle Regulation and Oncogenesis

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REGULATION OF ALTERATION/DEFICIENCY IN ACTIVATION 3 (ADA3) BY ACETYLATION AND ITS ROLE IN CELL CYCLE REGULATION AND ONCOGENESIS

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Andrew T. Dudley, Ph.D. Lt. Runqing Lu, Ph.D.

DEDICATION

To my loving parents

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ABSTRACT

The ADA3 (Alteration/Deficiency in Activation 3) protein is a transcriptional adaptor protein that was initially discovered as a component of several HAT (Histone Acetyltransferase) complexes, the enzyme complex responsible for histone acetylation, which is a prerequisite for transcription. Earlier the studies from Dr. Band's laboratory and that of others' have deciphered a crucial role of ADA3 in cell cycle regulation (both through G_1/S and G_2/M phase transitions) and in maintaining the genomic stability.

While our laboratory investigated the mechanism behind the role of ADA3 in G_1/S transition, the same remained unknown for G_2/M phase transition. Based on this prior knowledge about ADA3, I started out my Ph.D. thesis work in Dr. Band's laboratory directed towards examining the role of ADA3 in mitosis. During my doctoral research, I demonstrated that ADA3 governs the recruitment of a key centromeric protein CENP-B on to the centromeres and regulates the chromosome segregation during mitosis.

ADA3 protein has the potential to undergo posttranslational modification, including acetylation, and in the course of my Ph.D. research, I became interested in how these modifications might regulate the function of ADA3. I showed that ADA3 acetylation is regulated by coordinated actions of its associated HATs, GCN5, PCAF and p300, and a new partner I discovered, the deacetylase SIRT1. We used mass-spectrometry and site-directed mutagenesis to identify major sites of ADA3 acetylated by GCN5 and p300 and found that acetylation defective mutants were capable of interacting with HATs and other components of HAT complexes but deficient in their ability to restore ADA3-dependent global or locus-specific histone acetylation marks and cell proliferation in *Ada3* deleted MEFs.

A parallel focus of my studies was to define the role of ADA3 in HER2+ breast cancers, which basically emanates from a clinical study from our laboratory that revealed that ADA3 is overexpressed/mislocalized in these types of aggressive tumors. By using cell culture models I

have established a link between ADA3 and HER2 signaling pathways. In these cell lines, I found that ADA3 is a downstream target of HER2 and discovered a novel phospho-AKT-phospho-p300-Ac-ADA3 signaling pathway. Importantly, *ADA3* knockdown in these cells recapitulates the cell cycle inhibitory effects of a tyrosine kinase inhibitor lapatinib such as accumulation of CDK inhibitor p27 and reduced mitotic index. Taken together these results highlight the importance of ADA3 as a marker for treatment efficacy and a promising therapeutic target. Given the key importance of ADA3-containing HAT complexes in the regulation of various biological processes, including cell cycle, my thesis work provides an insight for the regulation of the function of these complexes through dynamic ADA3 acetylation.

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ABBREVIATIONS

ADA3	Alteration/Deficiency in Activation 3
ATP	Adenosine triphosphate
BSA	Bovine serum albumin fraction V
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
DAPI	4'-6-Diamidino-2-phenylindole
DTT	Dithiothreitol
EBNA-6	EBV-encoded nuclear antigen 6
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent proteins
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
Flox(ed)	Flanked by two loxP sites
GCN5	General Control Nonderepressible 5
GST	Glutathione *S*-transferase
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid
HER2	Human Epidermal growth factor Receptor 2
hMEC	Human mammary epithelial cell
HPV	Human papilloma virus

IB	Immunoblotting
IF	Immunofluorescence
IP	Immunoprecipitation
KAT	Lysine Acetyltransferase
KCl	Potassium chloride
KD	Knockdown
KO	Knockout
MEF	Mouse embryonic fibroblast
$MgCl_2$	Magnesium chloride
MW	Molecular weight
NaCl	Sodium chloride
NAM	Nicotinamide
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
NP-40	Nonidet P-40
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	Progesterone receptor
qRT-PCR	Quantitative real-time PCR
Rb	Retinoblastoma
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline with 0.1 % Tween-20
TF	Transcription factor
TNFα	Tumor Necrosis Factor-α

Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol with hydrochloric acid
TSA	Trichostatin A
WB	Western blotting

CHAPTER 1

INTRODUCTION

ADA3, as a component of SAGA complex

In eukaryotes histone acetylation is one of the major epigenetic modifications by which gene expression is regulated (1, 2) . Acetylation of histones is mediated by histone acetyl transferases (HATs) that exist as multi-subunit complex and one of the well-studied HAT complexes is known as Spt-Ada-Gcn5-Acetyltransferase (SAGA) complex that was originally identified in yeast (3) . The SAGA complex is highly conserved in eukaryotes, however the number of SAGA like complexes have increased during evolution and mammals have two SAGA like complex; SAGA itself (contains Ada2b) and ATAC complex (contains Ada2a), and both these complexes can be further diversified on the basis of their catalytic unit i.e. Gcn5 or the highly related HAT PCAF (4) . In general, the 1.8 MDa SAGA complex is composed of 18 to 20 subunits that can be divided into four separate groups of proteins (Table 1.1 and Figure 1.1) (5, 6) . The first group consists of Ada (alteration/deficiency in activation) proteins that form the acetyl transferase module of the complex. Second group consists of Spt (Suppressor of Ty) proteins that interact with TATA binding protein (TBP). Third group of proteins in the complex includes TAF proteins (TATA binding protein associated factors) that interact with basal transcription machinery and the last group is comprised of the protein that forms the de-ubiquitin (DUB) module of the complex (5, 7) .

Initial studies in yeast have established the partial structural organization of SAGA complex and phenotypic defects observed upon specific subunit deletion demonstrated that Ada1, Spt7 and Spt20 are required for structural integrity of the complex (8) . On the other hand Ada2/Ada3/Gcn5 are required for nucleosome acetylation and have no major impact on structural integrity of the complex (8) . Based on this a model was proposed for the structural organization of SAGA complex in which Ada2, Ada3 and Gcn5 were predicted to be peripheral whereas Ada1/Spt7/Spt20 were assumed to be localized in the center of complex (8) . To a large extent this model was consistent in subsequent studies (9-11) However defining direct interacting

Table 1.1: Eukaryotic GCN5/PCAF Complexes

ADA	SAGA-type			ATAC-type	
(y)ADA	(y)SAGA	(d)SAGA	(h)STAGA	(d)ATAC	(h)ATAC
0.7 MDa	1.8 MDa	1.8 MDa	1.8 MDa	1.3 MDa	1.8 MDa
Gcn5	Gcn5	GCN5	GCN5 or PCAF	GCN5	**GCN5 or PCAF**
Ada2	Ada2	ADA2-B	ADA2-B	ADA2-A	**ADA2-A**
Ada3	Ada3	ADA3	ADA3	ADA3	**ADA3**
Sgf29	Sgf29	CG30390	STAF36	CG30390	**STAF36**
-	Ada1	ADA1	STAF42	ATAC1	**ZZZ3**
-	Spt3	SPT3	SPT3	ATAC2	**CSRP2BP**
-	Spt7	CG6506	STAF65γ	D12	**YEATS2**
-	(Spt8)				**(UBAP2L)**
-	Spt20		**FAM48A/p38IP**	CG10238	**MBIP**
-	TAF5	WDA	TAF5L	WDS	**WDR5**
-	TAF6		TAF6L		**MAP3K7**
-	TAF9	TAF9	TAF9	CG32343	
-	TAF10	TAF10	TAF10	NC2β	**NC2β**
-	TAF12	TAF12	TAF12	CHRAC14	**POLE3**
-	Tra1	TRA1	TRRAP		**POLE4**
-	Sgf73		ATXN7		
-	(Sgf11)		ATXN7L3		
-	(Ubp8)		(USP22)		&
-	(Sus1)	E(y)2	ENY2		Others?
	(Rtg2)				
Ahc1			(SAP130)		
Ahc2	Chd1				
		-	**(HCF-1)**	HCF	**(HCF-1)**

Table 1.1: Eukaryotic GCN5/PCAF complexes. The identified protein components of the distinct types of complexes currently known in yeast (y), Drosophila (d) and humans (h), and the total estimated size of the complexes are indicated. Subunits indicated in parentheses are variably or less stably associated (e.g. salt concentration-dependent) in the respective complexes. Shaded blocks are groups of homologs specific of each type of complex. The GCN5-ADA3-STAF36/Sgf29 module is conserved in all complexes. HCF-1 is an abundant nuclear cofactor and promiscuous adaptor for several different co-activators; it was not detectable by LC-MS/MS in highly purified STAGA or ATAC, and thus may only be weakly associated (adapted from Wang et al., 2008).

partners of an individual protein in the complex has remained a challenge and subtle differences in subunit organization within the complex always existed from one study to another. The SAGA complex acts as a transcriptional co-activator complex that mediates transcriptional activation by at least four different ways. First, it can directly recruit the basal transcription machinery to the gene promoters and SAGA subunits such as Spt3 and Spt8 have been reported to facilitate the recruitment of TBP and formation of pre initiation complex at target genes (12) . Second mode of activation comes from intrinsic histone acetyl transferase activity of Gcn5 (global and promoter specific) that helps opening of chromatin and making it accessible to transcription factors (7) . Third mode of transcriptional activation by SAGA complex is attributed to another enzymatic activity i.e. deubiquitinase. Ubiquitin Specific Protease 22 (USP22) catalyzes the deubiquitinase activity of SAGA complex and mediates transcriptional activation primarily by deubiquitination of histone H2B; nonetheless, deubiquitination of histone H2A by USP22 is also reported (13-15) . The fourth mode by which SAGA complex regulates transcriptional activation involves posttranslational modification of non-histone proteins most of them belong to transcription factor. For example Gcn5 have been shown to acetylate p53 (16) and c-MYC (17) to positively regulate their activity. Similarly USP22 has been shown to regulate the ubiquitination of number of transcription regulators (18) .

The structural and functional analysis of SAGA complex reveals two important features. First, each subunit in the complex has a distinct role and secondly certain subunits assemble to form a module in the complex that performs a specific function (Figure 1.1). Early studies done yeast indicate that both HAT and DUB enzymatic module of the complex can perform their function independently however a recent study demonstrates that deletion of Gcn5 decreases the ubiquitin protease activity of USP22 thus suggesting a cross talk between HAT and DUB module of the complex (19) explicitly suggesting that in order for SAGA to perform its complete function the overall integrity of the complex is required.

Figure 1.1: Modular Organization of SAGA Complex

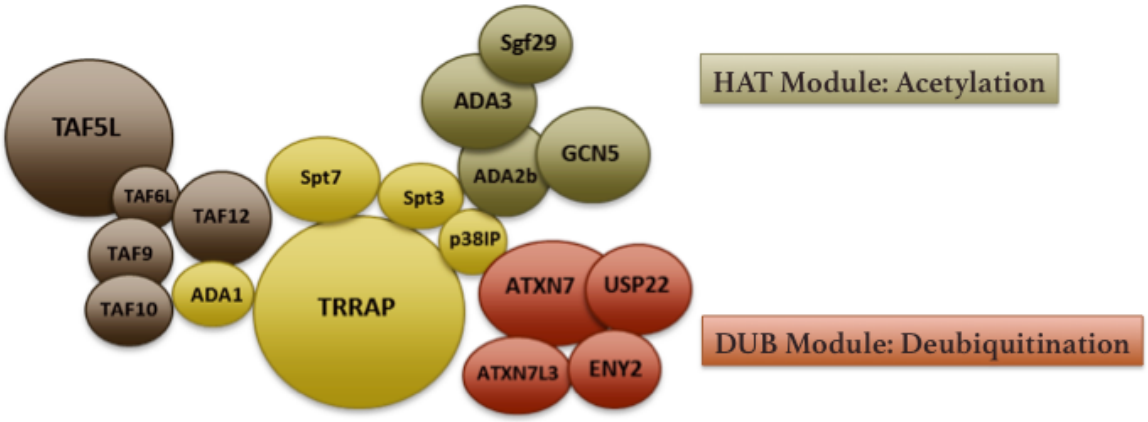

Figure 1.1: Organization of SAGA Complex. Based on various studies the image depicts the modular organization of SAGA complex. Please note that this is not an accurate model.

The alteration/deficiency in activation-3 (*ada3*) gene was discovered in yeast based on the mutation that confers resistance to GAL4-VP16 toxicity (20) and later on the protein was found to be an essential component of SAGA/SAGA like complexes (3, 21) . In yeast SAGA complex, Ada3 forms a trimeric sub-complex with Ada2 and Gcn5 where Ada2 acts as a bridging molecule between Ada3 and Gcn5 (3, 22) . Both Ada2 and Ada3 significantly enhance the ability of Gcn5 to acetylate nucleosomal histones and this trimeric sub-complex form the catalytic core of histone acetyl transferase module of the SAGA complex (23) . Consistent with yeast data a similar trimeric sub-complex also exists in humans that facilitate the GCN5 HAT activity (24) . Although as a component of SAGA complex, the primary function of Ada3 is in histone acetylation, work from our laboratory and that of others have reported several other functions of Ada3. Our laboratory has identified ADA3 as a novel human papilloma virus E6 oncoprotein binding protein (25) . Ada3 has also been reported to regulate the function of a non-histone protein p53, by mediating its acetylation (26, 27) and recent findings from our laboratory show that ADA3 is a novel cell cycle regulator (28) and is crucial for maintaining the genomic stability (29) .

Many of SAGA complex components have distinct domains that play certain functional role and as a whole complex the activity of SAGA appears to be autoregulated where one subunit regulates the function of other. For example, as described earlier, both Ada2 and Ada3 positively regulate the HAT activity of Gcn5. In the context of HAT activity of SAGA complex, Gcn5 and Ada2 have been studied extensively but how Ada3 regulate the SAGA HAT activity is not clear yet. Gcn5, the catalytic component of HAT module regulate the SAGA HAT activity is two manners. First, it possesses the intrinsic lysine acetyl transferase activity that catalyzes the transfer of acetyl group on histones (and non- histone proteins also) and second level of regulation of HAT activity is through bromodomain. The bromodomain is a protein module that recognizes the acetylated lysine residue. Thus binding of Gcn5 bromodomain to acetylated targets facilitates to localize and increase the retention of SAGA complex to its targets and thereby promoting the lysine acetyl transferase function in cooperative manner (30, 31) . The second

component of HAT module Ada2 has two distinct domains namely SANT (**S**wi3, **A**da2, **N**-Cor, and **T**FIIB) and SWIRM (**Swi**3, **R**sc8, and **M**oira). Both these domains are also found in many other chromatin modifying proteins (32, 33) . In particular, the SANT domain of Ada2 is required for SAGA complex interaction with histone H3 tails and is indispensible for Gcn5 HAT activity towards nucleosomes (34) . Although not known mechanistically, the Ada2 SWIRM domain also plays an important role in SAGA mediated nucleosome acetylation (24) . Similarly, the DUB module catalytic component, USP22 contains an N-terminal Zn-finger domain in addition to C-terminal peptidase domain. The ZnF domain of USP22 facilitates protein-protein interactions (35) and other subunits of DUB module ATXN7L3 and ENY2 are required for USP22 assembly into SAGA complex and thus regulating the DUB activity of USP22 (36) .

On the other hand third regulatory subunit of SAGA HAT module Ada3 does not possess any defined domain. Truncation mutation analysis of Ada3 has provided only a larger view of the protein according to which C terminus half of the protein is required for the interaction with Ada2 and N terminus half is required for interaction with transcriptional activators (22, 24, 26, 37) and how Ada3 regulates the HAT activity of SAGA complex remains unclear.

ADA3 as a p300 Interacting Protein

Although ADA3 was initially identified and classically known as an integral component of SAGA complex, it also interacts with KAT p300 (26, 38) . Unlike GNAT and MYST family KATs, p300 does not have canonical KAT domain thus called as orphan class KAT (2) . The large structure and presence of three cysteine-histidine rich domains in p300 facilitate its interaction with other proteins and to date more than 400 cellular targets have been identified as p300 interacting partners (39) . Wang et al. first discovered that ADA3, in addition to HATs GCN5 and PCAF, also interacts with p300. Furthermore, domain mapping of ADA3 revealed that C-terminus of ADA3 interacts with p300 (26) . Notably, the SAGA complex KAT GCN5 also interacts with C-terminus of ADA3 through ADA2 suggesting that C-terminus of ADA3 is critical for its interaction with KATs (24) . Later on using a combination of glycerol gradient sedimentation and co-immunoprecipitation analysis, our laboratory also identified ADA3 as a p300 interacting protein (38) . ADA3 performs at least two distinct functions by interacting with p300. First, it helps recruit p300 to the promoter region to facilitate transcription and acts as a co-activator of co-activator p300. For example ADA3 mediates the recruitment of p300 to the transcription factor Estrogen Receptor (ER) targets in estrogen dependent manner and the recruitment of ADA3 at ER responsive genes was found to be concomitant with RNA polymerase II (37, 38) . The second function of ADA3 is correlated with its classical role i.e. the facilitator of acetylation. ADA3 enhances p300 KAT activity towards both histone and non-histone substrates. We have previously demonstrated that ADA3 not only increases the acetylation of histones in solution but also upon *Ada3* deletion histone acetylation at various lysine residues is abrogated (28) . The acetylation of p53, a known substrate of p300, has been reported to be dependent of ADA3 (26, 27, 40) . p53 is acetylated by p300 upon DNA damage and acetylation of p53 stabilizes the protein and thus ADA3 plays an important role in stabilization of p53 (26, 27) . So, how does ADA3 act as facilitator of p300 dependent acetylation? One interesting observation from our study was the significant reduction in p300 levels upon *Ada3* deletion (28) . Notably

this reduction in p300 was not at transcript levels, suggesting that ADA3 plays a crucial role in determining the stability of p300 protein that probably is attributed to its KAT activity.

The Non-KAT Interactome of ADA3

In addition to various KATs and KAT complex components, a myriad of interacting partners of ADA3 have been identified so far. The human ADA3 was initially identified as HPV16 E6 interacting protein in the screening of a mammary epithelial cell yeast two-hybrid library (25) . Our laboratory found that high risk but not low risk E6 binds to ADA3 (25) . ADA3 does not have any recognizable protein domain however, the amino acid sequence analysis reveals that it contains five putative LxxLL motifs where L is leucine and x stands for any amino acid. The LxxLL motif is a generic feature of nuclear hormone receptor co-activators that facilitates its binding with nuclear hormone receptors. Many groups including ours have shown the direct association of ADA3 with nuclear hormone receptors such as RXR and ER (37, 41, 42) . The other well-characterized non-KAT binding partner of ADA3 is p53 and this interaction is governed by DNA damage and N-terminal phosphorylation of p53 (25-27) . Studies directed towards understanding the molecular mechanisms of gene expression by transcriptional activators have further discovered novel ADA3 interacting partners and one such example of ADA3 associated protein is ankyrin repeats-containing co-factors (ANCO). ANCO1 functions as a tumor suppressor protein by inhibiting ER mediated transactivation and also by augmenting the p53 transcriptional activity. ADA3 directly interacts with C-terminal co-activator domain of ANCO1 and related family member ANCO2 (43, 44) . Albeit the interaction of ANCO2 with ADA3 was direct, the same was weaker as compared to ANCO1 and interacting domain was not mapped (43) . ADA3 has also been shown to interact with β-catenin and armadillo domains present in the C terminus half of β-catenin facilitate its interaction with ADA3 (45) . A study also found ADA3 interaction with IL-1α N-terminal peptide (IL-1 NTP), the matured and processed form of IL-1α (46) . Identification of AATF (apoptosis-antagonizing transcription factor), and regulatory subunits of the PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) [PPP1R7 (PP1 regulatory subunit 7) and PPP2R5D (PP2A 56 kDa regulatory subunit δ isoform) respectively] as

interacting partners of ADA3 have further extended the list of non-KAT interactome of ADA3 (47) .

Physiological and Pathological Roles of ADA3

ADA3 is primarily involved in the regulation of transcription by recruiting various HATs and transcription factors to the promoter region. This function of ADA3 led biologists to place it under the category of co-activators. As described in preceding sections, ADA3 also enhances the acetylation non-histone substrates. In addition to these two major functions, ADA3 regulates wide spectrum of physiological and pathological processes including cell cycle, oncogenesis, DNA damage response, cellular senescence, and apoptosis. Before I discuss about the role of ADA3 in these cellular processes, it is noteworthy to mention that some of in the these functions have been studied *vis-à-vis* protein complexes that ADA3 is part of. An independent function of ADA3 is a debatable topic and still remains to be explored; nonetheless, ADA3 plays a very important role in maintaining cellular homeostasis.

Ada3 null mice were embryonically lethal at day E3.5 (note that $Ada3^{+/-}$ mice are haplosufficient) and thus ADA3 was though to play a vital role of ADA3 in embryogenesis and cell proliferation (28) . Further studies have deciphered a role of ADA3 in cell cycle (both interphase and mitosis) progression. ADA3 regulates c-MYC-SKP2-p27 pathway and conditional deletion of *Ada3* has been shown to prolong the half-life of CDK inhibitor p27, thus causing G_1 arrest (28) . An obvious consequence of G_1 arrest is delay in cell cycle progression through G_2/M phase. Indeed deletion/knockdown of *Ada3* displays various mitotic, karyokinesis and cytokinesis defects such as lobulation/fragmentation in nuclei, formation of anaphase bridges, accumulation of lagging chromosome, increased number of centrosomes and increased midbody size (28, 48) .

Uncontrolled cell cycle is the hallmark of cancer and it is not surprising that ADA3, as a cell cycle regulator, has been found to be associated with cancer. ADA3 regulates *c-MYC* transcription that is overexpressed in many types of cancer (28, 49) . ADA3 also promotes the proliferation of ER positive breast cancer cells under the hormonal response (38) . Furthermore, a study in large cohort of breast cancer patients has reported that expression/localization of ADA3 closely related with clinical outcomes. In particular, ADA3 nuclear localization was found to be

co-related with ER positive breast cancer patients, the breast cancer with good prognosis whereas predominant cytoplasmic localization of ADA3 serves as a marker for ERBB2/EGFR positive breast cancer patients that more aggressive and bear poorer clinical outcomes (50) .

The effect of *Ada3* deletion in cells is manifold and one such effect is genomic instability. *Ada3* deleted cells manifest various chromosomal aberrations spontaneously, which is further exacerbated upon DNA damage. At molecular level, lack of ADA3 is associated with various DNA damage response markers such as increase in pATM, p53BP1, pRAD51 and γ-H2AX (29) . The role of ADA3 in DNA damage response is indeed well studied and reveals ADA3 as a positive regulator of p53, the guardian of genome. ADA3 not only interacts with p53 upon DNA damage caused by ionizing radiations, but also stabilizes it by enhancing its acetylation and as a consequence transcriptional activity of p53 is increased towards CDK inhibitor p21 (26, 27) . The evidence that ADA3 functions as positive regulator of p53 and the discovery of human ADA3 as E6 binding protein have further provided a link of ADA3 in E6 mediated oncogenesis. E6 targets ADA3 for proteasomal degradation that accounts for the abrogation of p53 mediated transactivation and its tumor suppressor function induced by DNA damage. Given the fact p53 also tagged for degradation by E6, the observation that an E6 mutant incompetent to induce p53 degradation still causes the inactivation of p53; certainly provides a bypass mechanism for E6 mediated oncogenesis (25) . These findings were further substantiated and ADA3 degradation by E6 demonstrated inhibition of RXR mediated transactivation and abolition of p14ARF-p53 mediated senescence (41, 51) . More recently, in a gene knockdown-screening *ADA3* has emerged as a regulator of granzyme-B (GrzB) mediated mitochondrial dependent apoptosis. GrzB is a protease involved in Bid processing and in this study authors demonstrated that ADA3 promotes apoptosis by regulating the expression of phosphofurin acidic-cluster sorting-protein 2 (PACS2) that assists in GrzB mediated Bid cleavage (52) .

Regulation of ADA3 by Post-translational Modifications

Given that ADA3 plays an important role in the regulation of many physiological processes, one important question arises how ADA3 itself is regulated? Based on the studies mentioned in preceding sections, ADA3 levels generally do not change in response to any alteration in physiological conditions. For instance, ADA3 remains fairly constant during cell cycle progression or DNA damage response. Post-translational modifications of proteins are one common way by which their function is tightly regulated. Along the same line there has been some indirect evidences that ADA3 also post-transnationally modified that might regulate ADA3 levels and consequentially its function. For example, viral oncoprotein E6 directed proteasomal degradation of ADA3 suggests that the protein is likely to be ubiquitinated (25) . This observation was further substantiated and moreover ADA3 was also found to be modified by sumolyation. (25, 53)

One of the main and well-defined functions of ADA3 is to mediate histone acetylation. Serendipitously, while studying the effect of ADA3 on nucleosome/free-histone acetylation by GCN5 and p300 ADA3 itself was found to be a substrate of these HATs, which was another inkling for posttranslational modification of ADA3 (24, 28) . Mass spectrometry is widely used technique in proteomics and recent advancements in this area have allowed researchers to accumulate information about post-translational modifications of number of proteins. In fact high-throughput mass spectrometry have revealed some phosphorylated and acetylated ADA3 peptides (54, 55) . In addition to this many bioinformatics tools have also predicted the amino acid residues in ADA3 that have the potential for modification. A detailed list of such modifications can be viewed on http://www.phosphosite.org. Although substantial amount of evidence are there that ADA3 is post-transnationally modified, detailed studies are still warranted to explore molecular cues for these modifications and their functional relevance.

Based on this prior knowledge about ADA3 described above, I have addressed three broader questions in my thesis, which are addressed in detail as freestanding manuscripts in subsequent chapters. The three questions are as follows:

1. How ADA3 regulates mitosis and maintains the genomic stability?
2. How ADA3 is regulated by acetylation and is it a functionally relevant modification?
3. Given the critical role of ADA3 in cell cycle progression, is it connected with cancer?

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CHAPTER 2

ALTERATION/DEFICIENCY IN ACTIVATION 3 (ADA3) PROTEIN, A CELL CYCLE REGULATOR, ASSOCIATES WITH CENTROMERE THROUGH CENP-B AND REGULATES CHROMOSOME SEGREGATION

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INTRODUCTION

The centromere is a chromatin region that is essential for driving chromosome segregation in cell division, and is responsible for accurate inheritance of eukaryotic chromosomes during this process (1-3). It serves as the site of kinetochore assembly to which microtubule attachment occurs (4). The centromere-kinetochore is a complex network of proteins that work in concert for the faithful segregation of chromosomes (5,6). A major class of this network of proteins is, centromere proteins (CENPs)[3] that includes CENP-A, -B, -C, -E, -F, -H, -I and others (2,7).

In CENP group of proteins, CENP-B is highly conserved in several mammalian species (8). CENP-B specifically binds to a 17-bp sequence, known as CENP-B box through its amino-terminal region and dimerizes through its carboxyl-terminal region (9,10). The CENP-B box is conserved in centromeric human alpha-satellite and mouse minor satellite region (11). CENP-B is required for *de novo* assembly of centromere and kinetochore nucleation (12,13). Yeast CENP-B homolog acts as a site-specific nucleation factor for the formation of centromeric heterochromatin by heterochromatin-specific modifications of histone tails (14). The centromere function mainly entails CENP-A, -B and -C, in which CENP-B plays a crucial role by recruiting CENP-A and stabilizing CENP-C at centromeres (15-17).

Recent studies from our laboratory and that of others' have shown a critical role of Alteration/deficiency in activation 3 (ADA3) in cell cycle regulation (18,19). ADA3 is an essential component of several transcriptional adaptor and HAT (histone acetyl transferase) complexes conserved among eukaryotes (20). HATs and histone deacetylases (HDACs) are required to maintain steady-state levels of acetylation (21-24). A number of HAT enzymes, including general control non-repressed 5 (GCN5), p300, p300/CBP associated factor (PCAF), and CREB-binding protein (CBP) have been demonstrated as part of large complexes, such as Spt/Ada/Gcn5 acetyltransferase (SAGA), TBP-free TAF (TFTC), and Ada2.2A-containing (ATAC) complexes in human (21-24).

Our laboratory previously reported that germ line deletion of *Ada3* in mouse is embryonic lethal, and lack of ADA3 in mouse embryonic fibroblasts (MEFs) results in severe proliferation defect, dramatic changes in global histone acetylation, delay in G1 to S phase transition, mitotic defects, and delay in G2/M to G1 transition (18). Furthermore, we have shown a novel role for ADA3 in maintaining DNA repair process and genomic stability by controlling DNA repair checkpoints (25). Consistently, we observed ADA3 is over-expressed/mis-localized in breast cancers, and its over-expression predicts poor survival and poor prognosis in breast cancer patients, underscoring the critical function of ADA3 in physiology and pathology (26).

To better understand how ADA3 is involved in multiple biological processes, we recently performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) and found that ADA3 was significantly associated with human centromere regions across most chromosomes (unpublished) Interestingly, in yeast, GCN5 has been shown to play an important role in mitosis, by binding to centromeres (27). Given that GCN5 and ADA3 form an integral part of various HAT complexes and based on a clear role of ADA3 protein in mitosis, we explored if ADA3 associates with centromeres. In this study, using a series of PCR primers corresponding to the centromere region of human X chromosome, we demonstrate that ADA3 specifically binds to high order repeat (HOR) region of centromere which is the site of kinetochore attachment.

Given the known role of CENPs in centromere regulation, we examined if ADA3 associates with centromeric proteins such as CENP-A and CENP-B. We observed ADA3 is associated with CENP-B and this interaction of ADA3 with CENP-B was noticed throughout all phases of the cell cycle. Significantly, centromere binding of CENP-B was decreased with knockdown of *ADA3*. More importantly, in contrast to wild type ADA3, an ADA3 mutant that lacks the binding ability with CENP-B failed to rescue cell proliferation defects caused by the deletion of endogenous *Ada3*. Finally, we demonstrate that ablation of *Ada3* leads to defective chromosomal segregation with increase in anaphase bridges and lagging chromosomes. Taken together, these results provide a novel connection for the role of ADA3 in mitosis.

MATERIALS AND METHODS

Constructs

To generate N-terminally GST-tagged FLAG-ADA3 bacterial expression vector, full length FLAG-ADA3 was PCR amplified from pMSCV puro FLAG-ADA3 construct (18). Subsequently, BglII-SalI digested FLAG-ADA3 PCR amplicon was cloned into BamHI-SalI sites of pGEX6P-1 vector (GE Healthcare). Similarly, GST-tagged ADA3 C-terminal deletion (1-369, 1-214 and 1-110) and N-terminal deletion (111-432) bacterial expression vectors were constructed by cloning the respective BglII-SalI digested PCR amplicons into BamHI-SalI sites of pGEX6P-1 vector. Generation of retroviral pMSCV puro FLAG-ADA3 construct has been previously described (18). Retroviral construct for FLAG-ADA3 (111-432) was generated by cloning BglII-SalI digested FLAG-ADA3 (111-432) PCR amplicon into BglII-XhoI site of the pMSCV puro vector (Clontech).

Cell Culture, Transfections and Viral Infections

76NTERT cells were cultured in DFCI media as described before (28). *Ada3*$^{FL/FL}$ MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. *Ada3*$^{FL/FL}$ MEFs stably expressing full length FLAG-ADA3 or FLAG-ADA3 (111-432) were generated as previously described (18). Adenoviruses expressing EGFP-Cre or EGFP alone were purchased from University of Iowa (Gene transfer vector core). Cre mediated deletion of *Ada3* was performed as described previously (18). For *ADA3* knockdown experiments in 76NTERT, cells were transfected with 50 nM of control (sc-37007, Santa Cruz Biotechnology) or *ADA3* siRNA (sc-78466, Santa Cruz Biotechnology), using the DharmaFECT 1 Transfection Reagent (T-2001-03, Dharmacon).

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed using ChIP-IT Express kit from Active Motif according to manufacturer's protocol with slight modifications in fixation and sonication conditions. 76NTERT cells were washed twice with 1X PBS and fixed in EGS [ethylene glycol bis (succinimidyl succinate)] and formaldehyde at room temperature. In particular, cells were incubated in 1.5 mM EGS in 1X PBS on shaking platform for 15 min. To this formaldehyde (1% working concentration) was added for another 15 min. Fixation reaction was then stopped by 1X glycine at room temperature for 5 min. Chromatin from 76NTERT cells was isolated and sonicated for 12 minutes to obtain a fragment size of 200-1000 bp. Remaining steps for ChIP were followed exactly as per manufacturer's protocol. Antibodies used for ChIP assay were: anti-ADA3 monoclonal antibody (18); anti-CENP-A (ab13939, Abcam) and anti-CENP-B antibody (07-735, EMD Millipore). PCR amplification was performed using primers as described in Table 2.1.

Table 2.1 - PCR primer sets used for ChIP-PCR ±

Genomic Site	Description	Position (hg19)
A: γ-ALR jxn	F: 5' agcccgaggaaaatactggtgagg 3'	chX: 58319092—58319315
	R: 5' gctgtctttctagtttttgtcgtgggttat 3'	
B: Xp mono sat	F: 5' tgcagagggatatttgtaagcat 3'	chX: 58514700—58514948
	R: 5' tgcttctgtctaattttcgtgtg 3'	
C: Xp mono-HOR jxn	F: 5' aacgctgcgctatcaaagggaaagt 3'	chX: 58560457—58560769
	R: 5' ggacatgtggagcgctttgtgc 3'	
D: HOR satellite	F: 5' aaagggtgtttcgaacctga 3'	chX: 61718416—61718622
	R: 5' tgaacatgccttttgatgga 3'	
D': HOR satellite	F: 5' ataatttcccataactaaacaca 3'	chX: 58605896—58606430
	R: 5' tgtgaagataaaggaaaaggctt 3'	
E: Xq mono-HOR jxn	F: 5' gacctcaaagcactctaaatacac 3'	chX: 61725908—61726403
	R: 5' cttcacataaaaactagacagacag 3'	
F: Xq mono sat	F: 5' aaaattgaggtttcaaaactgct 3'	chX: 61745729—61745968
	R: 5' ttccttttcatagcgcactt 3'	
G: Xq sat jxn	F: 5' cctgctgaatcaaaacaatggt 3'	chX: 62045588—62045976
	R: 5' caaagaaggctgggtgagaag 3'	
Universal alpha satellite	F: 5' cattctcagaaacttctttgtg 3'	N/A

± PCR primers sequences were aligned with human genome (hg19). Sequences of PCR primer sets of γ-ALR jxn, Xp mono-HOR jxn, HOR satellite (D'), Xq mono-HOR jxn, and Xq sat jxn were from Mravinac *et. al.* (32)

Immunofluorescence

For immunofluorescence, 76NTERT cells were grown to 50% confluence on glass cover slips in 12-well plates. After knocking down *ADA3* in 76NTERT cells by siRNA, the cover slips were fixed in 4% paraformaldehyde for 20 minutes. Staining was performed as described earlier (25). The primary antibodies used were FITC labeled human anti-centromere antibody (ACA) (15-235-F, Antibodies Incorporated), anti-CENP-B antibody (ab25734, Abcam or 07-735, EMD Millipore) and anti-ADA3 antibody (18). Secondary antibodies used were Alexa fluor 488, Alexa fluor 594 and Alexa fluor 647 from Life Technologies. Nuclei were counterstained with DAPI. The cover slips were then placed on slides using the mounting medium. Fluorescent images were captured using LSM 510 META Confocal fluorescence microscope (Zeiss).

Duolink in situ Proximity Ligation Assay

Anti-mouse PLA probe plus, anti-rabbit PLA probe minus, and detection kit Red 563 were purchased from OLink Bioscience. 4% Formaldehyde fixed cells were blocked with PBS containing 10% goat serum and 0.001% Triton X-100 for 1 hr, and incubated with primary antibodies for ADA3 and CENP-B (07-735, EMD Millipore or ab25734, Abcam) or p53 (sc-6243, Santa Cruz Biotechnology) for overnight at 4^{o}C. PLA probes were diluted 1:8 in blocking solution. Detection of the PLA signals was carried out with LSM 510 META Confocal fluorescence microscope (Zeiss).

In vitro Binding Assays

GST-FLAG-ADA3 full length or various GST-ADA3 truncated mutants were purified from bacterial lysates based on the protocol by Frangioni and Neel (29). 1µg of GST, GST-FLAG ADA3 full length or GST-ADA3 deletion mutants non-covalently bound to glutathione beads were incubated with 300 ng of purified 6xHis tagged CENP-B (ab73636, Abcam) in NETN

buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM Na_4VO3, 1 mM NaF, and protease inhibitor mixture) for 2 hours at 4^oC and washed 5 times with NETN buffer. The bound proteins were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted using anti-CENP-B antibody (07-735, EMD Millipore or ab25734, Abcam).

CellTiter-Glo® Luminescent Cell Viability Assay

The assay was performed three times independently. 2 x 10^5 *Ada3*$^{FL/FL/Vector}$, *Ada3*$^{FL/FL/FLAG\text{-}ADA3\ (Full\ Length)}$ or *Ada3*$^{FL/FL/FLAG\text{-}ADA3\ (111\text{-}432)}$ MEFs were plated in p100 dishes. After overnight attachment, cells were infected either with Adeno-EGFP or Adeno-EGFP-Cre as previously described (18). 24 h after infection (day 1), each plate was divided in six well plates and 96 well plates (one plate for each day). In particular, 1 x 10^4 cells were plated in one well of six well plate (for western blotting) while 150 cells per well were plated in six replicates of 96 well plate (for luminescence). Cells were cultured with change of media every alternate day. At day 1, 3, 5, 7 and 9 cell viability was measured by CellTiter-Glo® luminescent cell viability assay (Promega) following manufacturer's protocol. To confirm the deletion of endogenous *Ada3* and ectopic expression of ADA3 full length and (111-432), cells were also harvested for western blotting at aforementioned days and immunoblotted with indicated antibodies.

Colony Formation Assay

Cells were infected with either Adeno-EGFP or Adeno-EGFP-Cre as described above. 24 h after infection (day 1), 10,000 cells per well were plated in six well plate and cultured till day 9 with change of media every alternate day. At day 9, cells were fixed and stained with crystal violet solution (0.25% crystal violet in 25% methanol) and imaged as described previously (18)

Cell Fractionation and Immunoblotting

72 hours after infecting *Ada3*$^{FL/FL}$ MEFs with control or Cre adenovirus, cells were trypsinized, collected and washed once with PBS. Cell fractionation was performed according to previously published protocols with modifications (30,31). A fraction of the harvested cells was used to make whole cell extracts. The remaining cell pellet was suspended in lysis buffer (10 mM HEPES pH 7.4, 10 mM KCl, 0.05% NP-40, 0.1 mM Na_4VO_3, 1 mM NaF, 10 mM Nicotinamide, 2 μM Trichostatin A and protease inhibitor mixture), incubated on ice for 30 min, vortexed twice at high speed followed by centrifugation at 14000 rpm for 10 min at 4^{o}C. The supernatant obtained was kept as cytoplasmic fraction and the pellet containing nuclei was washed once with lysis buffer. Nuclei were then resuspended in low salt buffer (10 mM Tris-HCl pH 7.4, 0.2 mM $MgCl_2$, 1% Triton X-100, 0.1 mM Na_4VO_3, 1 mM NaF, 10 mM Nicotinamide, 2 μM Trichostatin A and protease inhibitor mixture) and incubated on ice for 15 min followed by centrifugation at 14000 rpm for 10 min at 4^{o}C. The supernatant was stored as the nucleoplasmic fraction and the pellet was resuspended in 0.2N HCl and incubated on ice for 20 min. The soluble fraction was neutralized with 1M Tris-HCl pH 8 and used as the chromatin fraction. The cell fractions were quantitated using the BCA protein assay reagent (Pierce). The proteins were resolved by SDS/PAGE and transferred onto PVDF membrane. Immunoblotting was performed with primary antibodies against ADA3 [(mouse monoclonal antibody (18) or rabbit polyclonal antibody (HPA042250, Sigma)], CENP-B (ab25734, Abcam), FLAG (A8592, Sigma), HSC70 (sc-7298, Santa Cruz Biotechnology) GAPDH (MAB374, EMD Millipore) and Histone H3 (06-755, EMD Millipore).

Chromosome Mis-segregation Analyses

For analyzing chromosome mis-segregations, *Ada3*$^{FL/FL}$ MEFs were infected with control or Cre adenovirus. 24 hours after infection, cells were trypsinized and plated on 18-mm coverslips in 12 well plates. The following day (48 hours after infection), cells were synchronized in S phase by souble thymidine block (18 hour first block with 2 mM thymidine, then release for 9 hours in

complete medium and then 2nd block with 2 mM thymidine for another 18 hours). After the second block, the cells were released in complete medium for 6 hours (approximately the time for cells to go into mitosis) followed by fixing cells in 4% PFA and mounting the coverslips in DAPI containing mounting medium (Vectashield). The images were captured at 63x using LSM 510 META Confocal fluorescence microscope (Zeiss).

Statistical Analyses

The cell viability assay was performed three times independently. For each independent experiment, the luminescence from six replicates were recorded and averaged. The standard errors were calculated using luminescence from three independent experiments and *p* values were computed by Student's *t* test (two tailed, unpaired) using Microsoft Excel 2010 and p value $\leq$ 0.05 was considered as statistically significant. For the effect of ADA3 depletion on CENP-B and ACA co-localization a total of 120 cells were counted in control or *ADA3* siRNA without bias and cells with > 5 CENP-B and ACA co-localization foci were considered positive for co-localization. For chromosomal abnormalities, at least 50 anaphase chromosomes were counted in control or *Ada3* deleted cells without bias and examined for segregation defects. The Chi square test was performed using SAS 9.3 (SAS Institute, Cary, NC) and p value ≤ 0.05 was considered as statistically significant.

RESULTS

ADA3 Associates with HOR Region of X Chromosome Centromere

As mentioned above, we observed association of ADA3 with centromeric regions of most human chromosomes by performing ChIP-seq (unpublished)[4]. Since the centromere is an essential chromosomal domain that is required for chromosome segregation, and ensures the faithful inheritance of the chromosome during cell division (1-4), we assessed ADA3 interaction with centromere. We first confirmed the binding of ADA3 with centromere by performing ADA3 ChIP-PCR using a series of PCR primers on human X chromosome centromere region in an immortal human mammary epithelial cell line, 76NTERT (32) (Table 2.1). To test the specificity of the ChIP primers, as well as the binding of ADA3 to centromeres, we performed ChIP using anti-CENP-A or CENP-B antibodies as both CENP-A and CENP-B are known to bind to high order repeats (HOR) in the centromeres (33). As expected, we observed binding of CENP-A and CENP-B only to HOR regions of the centromere as previously reported. Interestingly, ADA3 also associated with the alpha-satellite region in higher order repeats (HOR), which is the site of kinetochore assembly (Figure 2.1A and 2.1B) (34). The universal primer set which recognizes the alpha-satellite region on human genome was also amplified in ADA3 ChIP-PCR (Figure 2.1A and 2.1B); suggesting ADA3 may maintain genomic stability by regulating chromosome separation through association with centromere.

FIGURE 2.1. Association of ADA3 with HOR region of human X chromosome centromeric alpha satellite region by ChIP-PCR

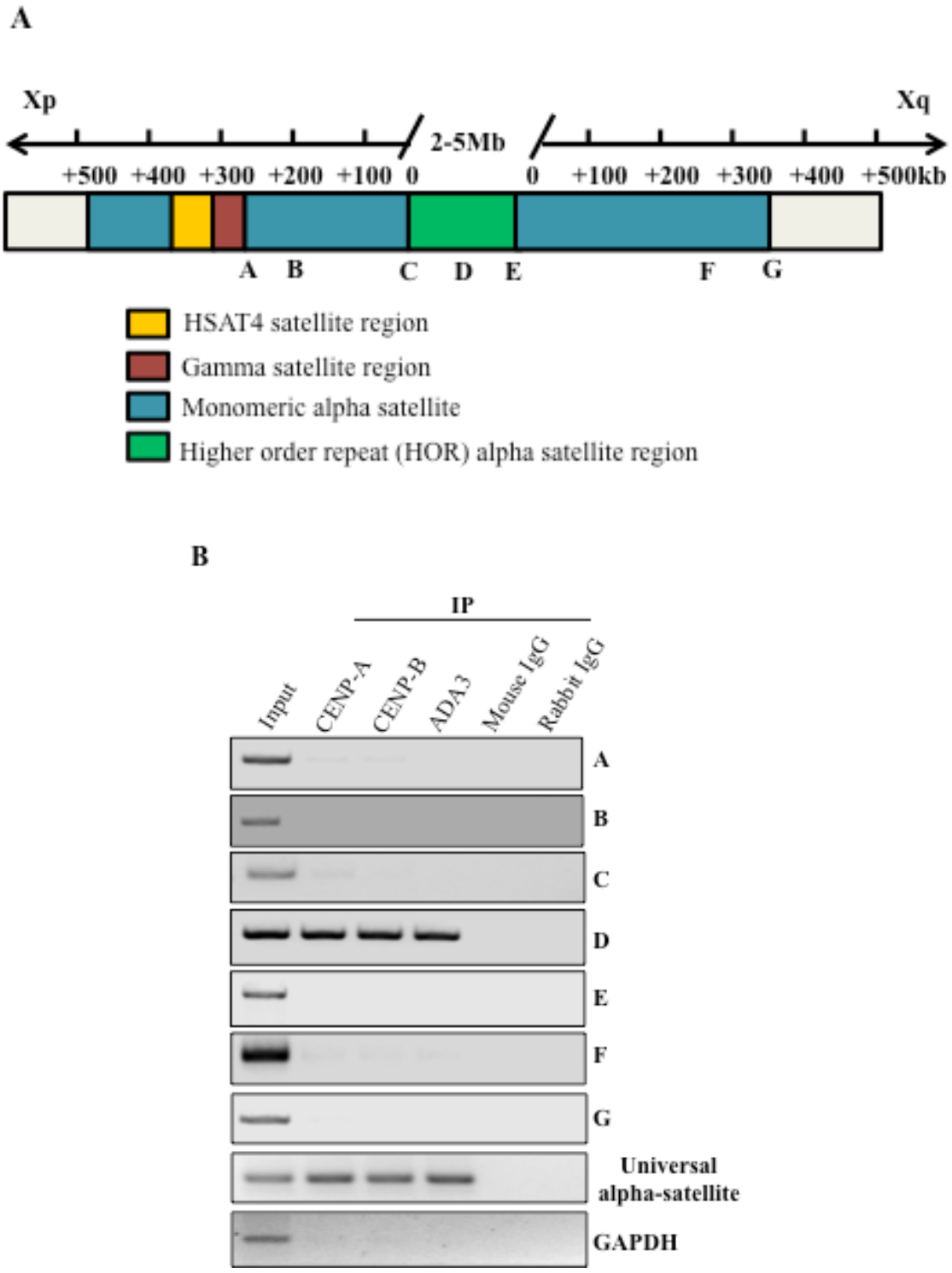


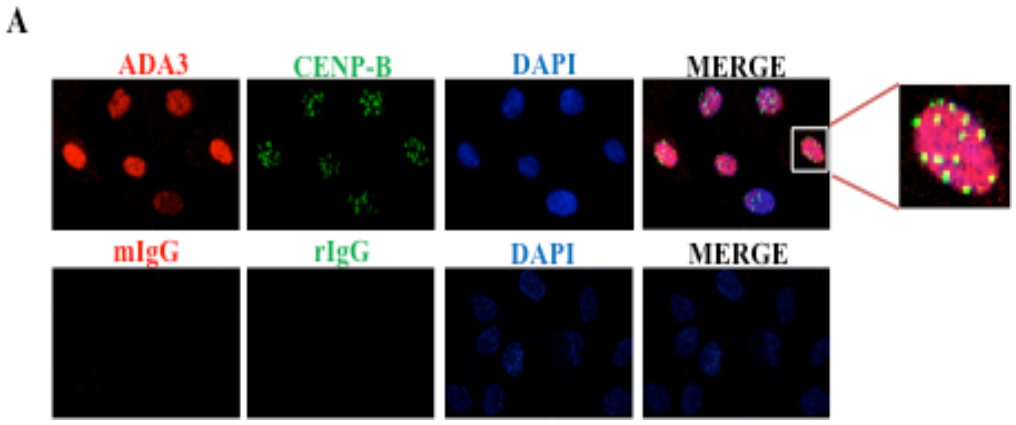
FIGURE 2.1. Association of ADA3 with HOR region of human X chromosome centromeric alpha satellite region by ChIP-PCR. *A,* Structure of the centromere of human X chromosome along with putative CENP-A, CENP-B and ADA3 binding region shown in black color on the centromere of X chromosome as obtained from *B*. *Note: The diagram is not drawn to scale. Xp: short arm; Xq: long arm. B,* ADA3 associates with HOR region of human X chromosome centromere: ADA3, CENP-A or CENP-B protein was immunoprecipitated from cross-linked chromatin-protein complex prepared from 76NTERT cells. Associated chromatin was then eluted and amplified by PCR using primers against centromere regions depicted in *A* (Also see Table 2.1). Mouse and rabbit IgGs were used as negative controls for immunoprecipitation. Universal alpha satellite primers were used as positive control whereas GAPDH primers were used as negative control in PCR.

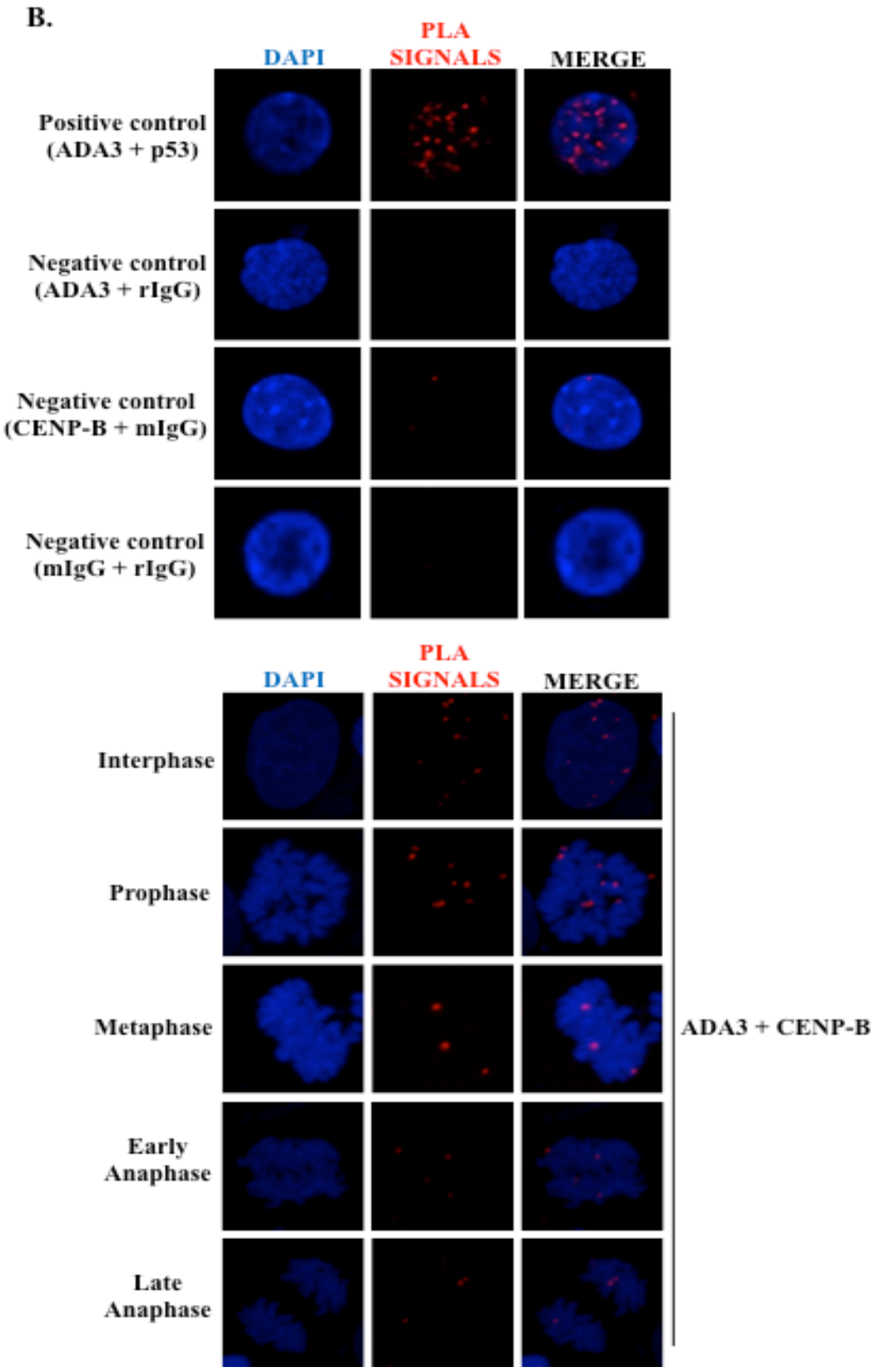
ADA3 Associates with CENP-B Protein at the Centromere

Several studies have identified a number of protein components that associate with centromere including CENPs (2). Given the fact that ADA3 is a transcriptional co-activator and itself does not contain DNA binding domain, we hypothesized that some other centromeric protein may mediate ADA3 interaction with centromere. Among the well-known centromere binding proteins, CENP-B is highly conserved in several mammalian species, specifically binds to a 17-bp sequence (CENP-B box) which is conserved in centromeric human alpha-satellite region and associates with centromeric heterochromatin (10,11). Since ADA3 associated with the HOR region in alpha-satellite region on centromere where CENP-B also binds to, we examined whether ADA3 co-localizes with centromere marker CENP-B. Co-immunofluorescence assay using anti-ADA3 and anti-CENP-B antibodies clearly demonstrated CENP-B and ADA3 are co-localized in the nucleus (Figure 2.2A). The specificity of ADA3 antibody used in this assay has been extensively determined in our previous publications (18,25,26). To further confirm ADA3 association with centromeres we analyzed ADA3 interaction with CENP-B by DuoLink *in situ* proximity ligation assay (PLA). In addition to CENP-B, we assessed whether ADA3 also interacts with another centromeric protein, CENP-A. To determine the specificity of interactions in PLA, we used p53 protein, known to directly interact with ADA3 (35,36), and rabbit or mouse IgG as negative controls (Figure 2.2B). In this assay, primary antibodies raised in different species are used against two interacting proteins and when species specific secondary antibodies linked with complementary DNA probes come in close proximity (30-40 nm) the linked DNA can be amplified and visualized with a fluorescent probe as distinct foci. ADA3 and CENP-A / CENP-B were immunostained with anti-mouse and anti-rabbit secondary antibodies, respectively, that were linked to complementary oligonucleotides. PLA exhibited only CENP-B but not CENP-A (data not shown) interaction with ADA3, particularly in interphase of cell cycle (Figure 2.2B). Out of total cells quantified in various cell cycle phases, approximately 80% of cells

showed more than three ADA3-CENP-B interaction foci (Figure 2.2C) and the interaction signals persisted when cells entered into prophase, but the signals were reduced when cells entered into metaphase and anaphase (Figure 2.2B). Taken together, these results demonstrate that ADA3 associates with CENP-B during interphase and prophase, but its interaction with CENP-B is reduced in metaphase and anaphase, suggesting an important role of ADA3 in early phase of mitosis.

FIGURE 2.2: ADA3 associates with CENP-B protein at the centromeres




B.
DAPI
PLA SIGNALS
MERGE
Positive control (ADA3 + p53)
Negative control (ADA3 + rIgG)
Negative control (CENP-B + mIgG)
Negative control (mIgG + rIgG)
DAPI
PLA SIGNALS
MERGE
Interphase
Prophase
Metaphase
Early Anaphase
Late Anaphase
ADA3 + CENP-B

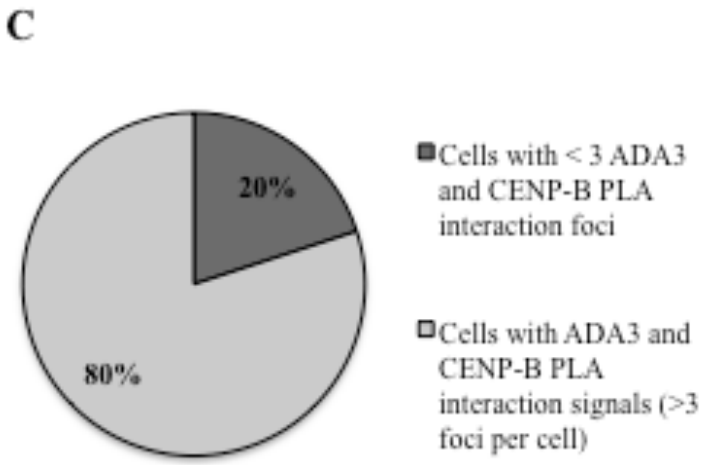


FIGURE 2. ADA3 associates with CENP-B protein at the centromeres. *A,* Co-localization of ADA3 and CENP-B in 76NTERT cells. Cells were cultured on coverslips, fixed with 1% formaldehyde and co-immunostained for CENP-B and ADA3, followed by fluorescence microscopy using appropriate filters (Red: ADA3, Green: CENP-B, and Blue: DAPI) Mouse and Rabbit IgG (mIgG or rIgG) served as negative controls. *B,* Interaction of ADA3 with CENP-B during cell cycle using proximity ligation assay (PLA). 76NTERT cells were treated for 16.5 h with 100 ng/ml of Nocodazole, released in culture medium and harvested at different time points. Fixed cells were incubated with antibodies against ADA3 and CENP-B followed by DuoLink *in situ* PLA and fluorescence microscopy using appropriate filters (Blue: DAPI; Red: PLA signals). Interaction of ADA3 with p53 is shown as positive control whereas mouse or rabbit IgG served as negative controls for PLA. *C,* Quantification of cells with ADA3-CENP-B PLA interaction signals from B. More than 100 cells in different cell cycle phases were quantified for PLA interaction foci present in the nucleus. Cells with greater than 3 PLA interaction signals were considered positive for ADA3-CENP-B interaction.

ADA3 Directly Interacts with CENP-B through its N-terminus

Next, to assess if ADA3 and CENP-B directly interact, we performed GST pull down assay using purified GST-tagged human FLAG-ADA3 and 6xHis-CENP-B protein. As shown in Figure 2.3A, CENP-B is detected in the GST pull down lysates after incubation of GST-FLAG-tagged ADA3 with CENP-B protein, demonstrating ADA3 directly interacts with CENP-B *in vitro*. Next, to map the region of ADA3 essential for binding to CENP-B, we generated a series of GST-ADA3 constructs; (1-369), (1-214), (1-110); in which a coding region from C-terminus was sequentially removed along with the one, (111-432); in which codons that code for the first 110 amino acids from N-terminus were deleted (Figure 2.3B). Recombinant proteins from these constructs were used as baits and purified CENP-B as prey in our GST pull down assays. Immunoblotting with anti CENP-B antibody showed that all ADA3 fragments were able to efficiently pull down CENP-B except 111-432 fragment of ADA3, suggesting that N-terminus of ADA3 is critical for its interaction with CENP-B (Figure 2.3C).

Figure 2.3: ADA3 Directly Interacts with CENP-B through its N-terminus

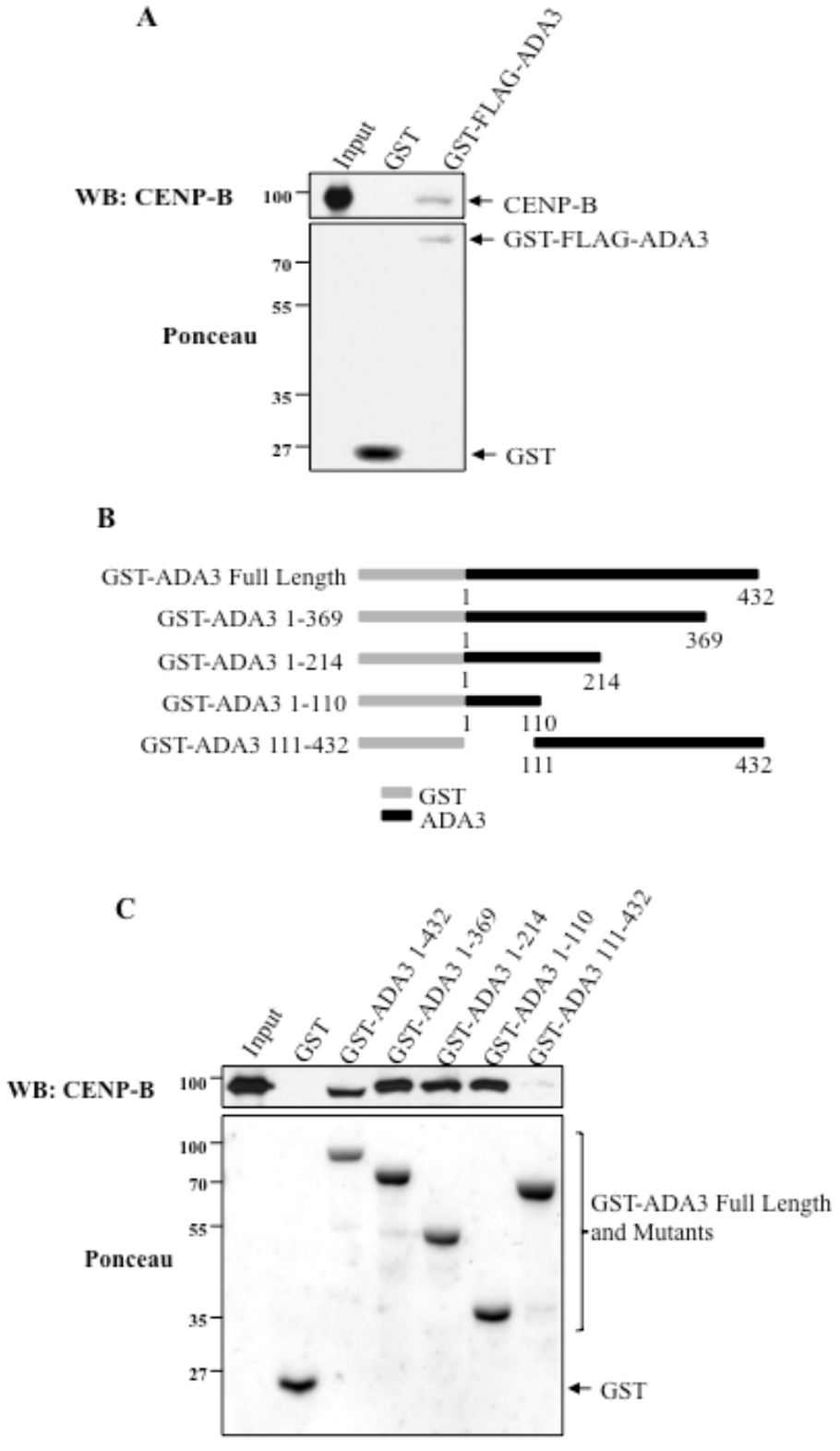
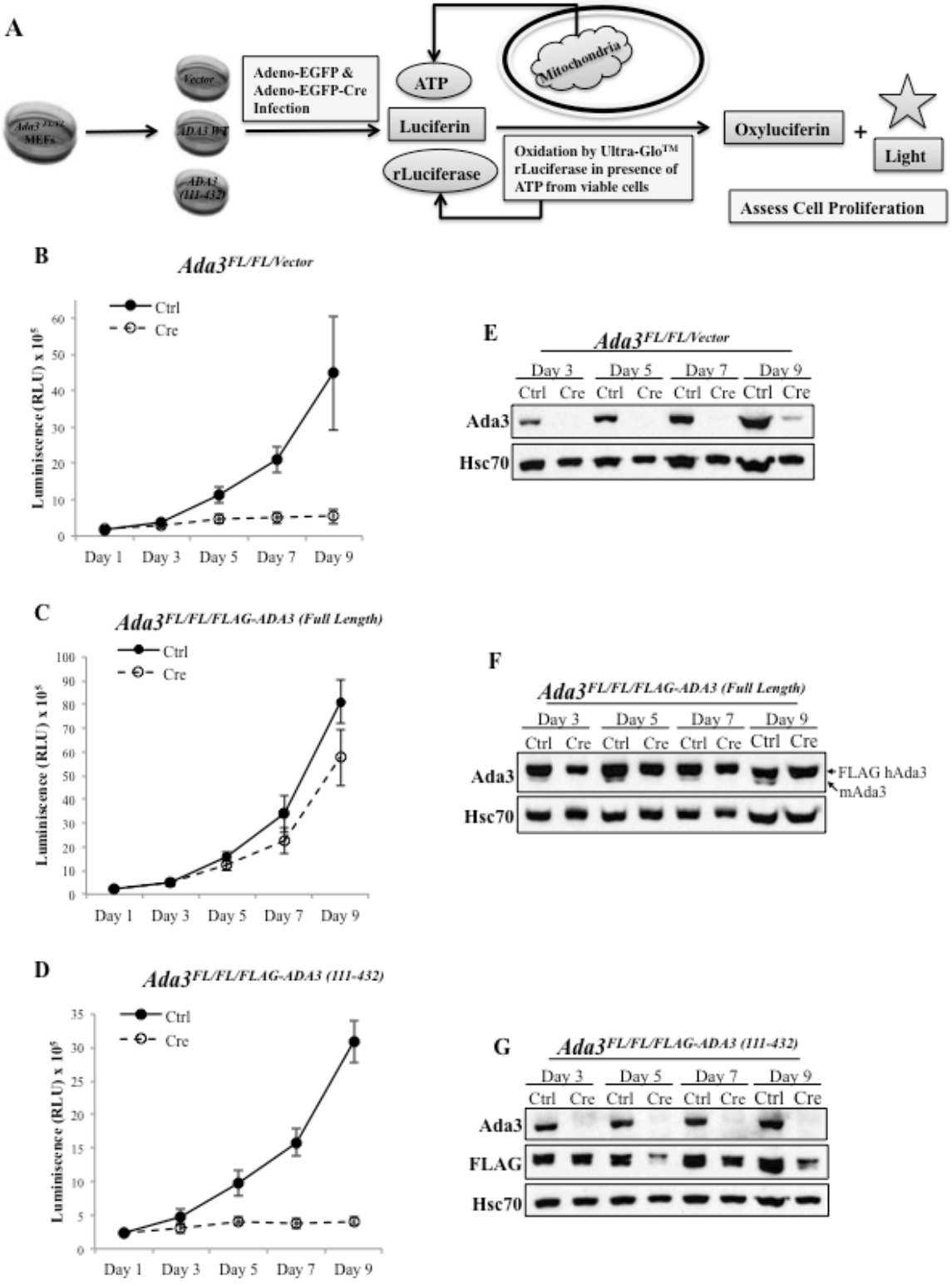


FIGURE 2.3. ADA3 directly interacts with CENP-B through its N-terminus. *A, In vitro* binding assays were performed to determine if ADA3 directly interacts with CENP-B. 1μg of GST or GST-FLAG ADA3 bound to glutathione beads was incubated with 300 ng of purified CENP-B protein. After washes, the beads were loaded onto SDS-PAGE, transferred to PVDF membrane and immunoblotted using anti-CENP-B antibody. Input is 100%. *B,* Schematic representation of GST-ADA3 constructs used to determine the region in ADA3 required for its interaction with CENP-B. *C, In vitro* GST pull down assays were performed as in *A* using GST-ADA3 constructs shown in *B*. Input is 10%.

CENP-B Binding Defective ADA3 Mutant Fails to Rescue Cell Proliferation Arrest Caused by the Deletion of Endogenous *Ada3*

We have previously demonstrated that conditional deletion of endogenous *Ada3* from $Ada3^{FL/FL}$ MEFs causes cell proliferation arrest (18). However, $Ada3^{FL/FL/ADA3}$ MEFs in which human *ADA3* is ectopically expressed rescues the cell proliferation defects. Therefore, we tested the ability of 111-432 fragment of ADA3, which lacks the binding ability with CENP-B, to rescue cell cycle arrest. For this purpose, FLAG tagged ADA3 full length or 111-432 fragment were stably expressed in $Ada3^{FL/FL}$ MEFs, followed by the deletion of endogenous *Ada3* by adenovirus expressing Cre recombinase and cell proliferation was assessed at regular intervals up to 9 days by CellTiter-Glo® luminescent cell viability assay that determines the number of viable cells in culture based on quantitation of ATP (Figure 2.4A). Deletion of endogenous *Ada3* and expression of ectopically expressed *ADA3* was confirmed by immunoblotting (Figure 2.4E, 2.4F & 4G). Consistent with our previous report (18), we observed that in contrast to vector alone, wild type full length *ADA3* was able to restore the cell proliferation arrest caused by the deletion of endogenous *Ada3* (Figure 2.4B & 2.4C). Interestingly, we observed that the CENP-B binding defective 111-432 mutant failed to rescue the cell cycle defect (Figure 2.4D). To further confirm that 111-432 ADA3 mutant is defective in cell proliferation rescue, a colony formation assay was also performed at day 9. Similar results were obtained in this assay (Figure 4H & 4I) indicating CENP-B and ADA3 interaction may be required for ADA3's ability to regulate cell proliferation.

FIGURE 2.4: CENP-B binding defective ADA3 mutant fails to rescue cell proliferation arrest caused by the deletion of endogenous *Ada3.*



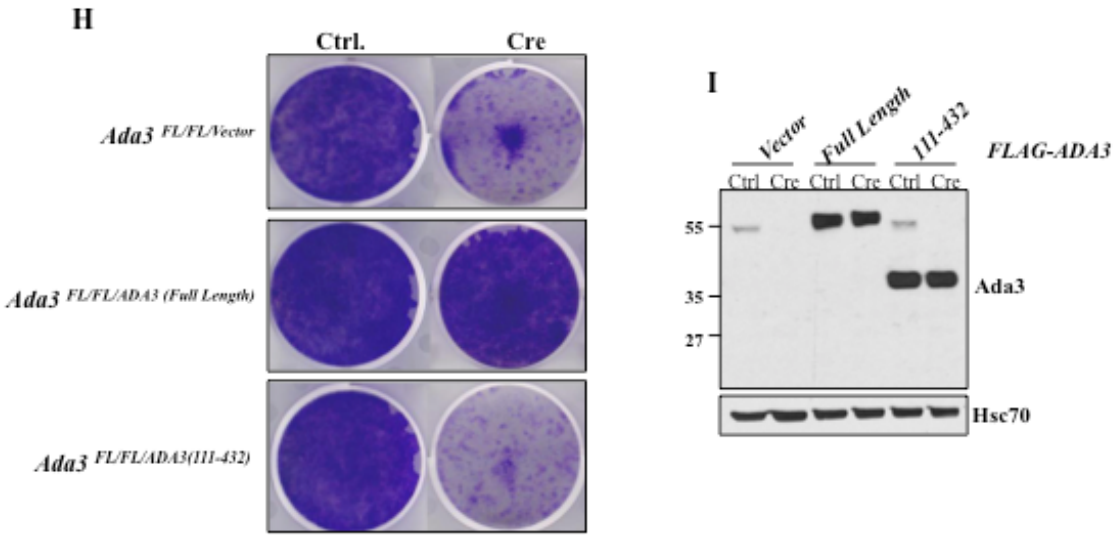
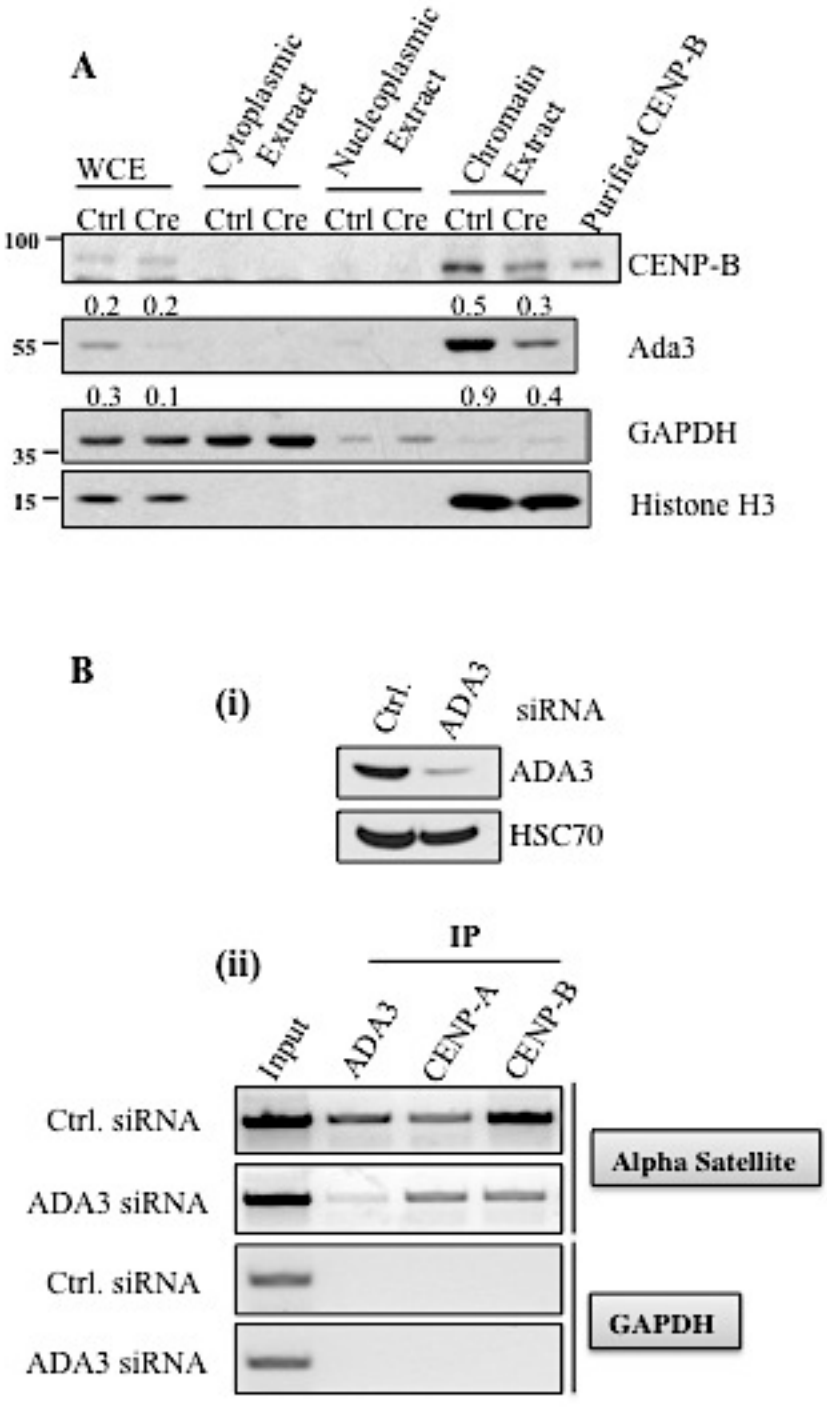


FIGURE 2.4. CENP-B binding defective ADA3 mutant fails to rescue cell proliferation arrest caused by the deletion of endogenous *Ada3*. *A,* Strategy used to perform ADA3 rescue cell proliferation assays. *B-D,* Cell viabilities of *(B)* $Ada3^{FL/FL/Vector}$, *(C)* $Ada3^{FL/FL/FLAG\text{-}ADA3(Full\ Length)}$, and *(D)* $Ada3^{FL/FL/FLAG\text{-}ADA3\ (111\text{-}432)}$ MEFs after control adenovirus (*Ctrl*) or Cre adenovirus (*Cre*) infection obtained using CellTiter-GLO luminescent cell viability assay as described under "Experimental Procedures". Data shown here are mean ± S.E. from three independent experiments performed in six replicates and *p* values were computed using Student's *t* test. *E-G*, ADA3 protein levels at different time points after Cre adenovirus infection of indicated cell lines. Note that full length or mutant ADA3 reconstituted control cells express both mouse ADA3 and human FLAG-ADA3 proteins, whereas only human FLAG-ADA3 is seen in Cre adenovirus-infected cells. *H*, Colony formation assay. Crystal violet staining of the indicated cells infected with control virus or Cre adenovirus grown for 9 days is shown. *I*, Western blotting of lysates from *H* showing exogenous and endogenous ADA3.

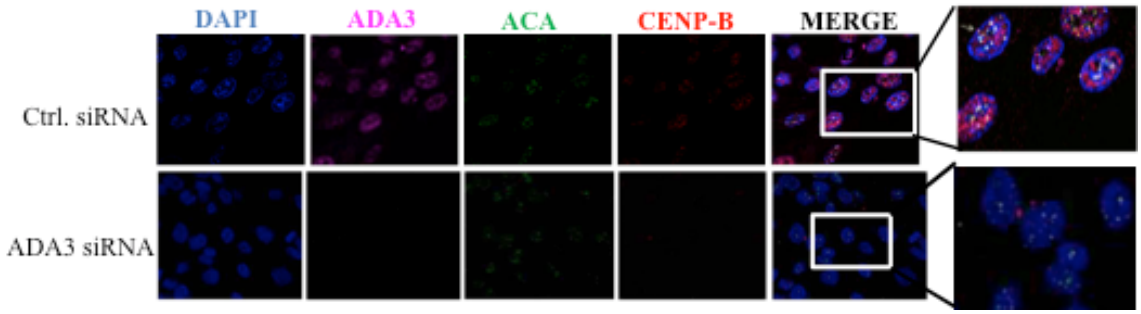
Depletion of ADA3 Abrogates CENP-B Recruitment to Centromeres

Given the critical roles of CENPs in centromere regulation, we examined the role of ADA3 on CENP-B association to centromeres. For this purpose, we deleted *Ada3* from *Ada3*$^{FL/FL}$ MEFs by Cre-mediated deletion, fractionated cell compartments and assessed the levels of CENP-B in whole cells, cytoplasmic, nucleoplasmic or chromatin fractions. As seen in Figure 2.5A, CENP-B protein was only observed in whole cell extract (WCE) and chromatin fraction. Notably, the levels of CENP-B were not altered in the whole cell extracts, consistent with our recent report where mRNA levels of CENP-B do not change upon deletion of *Ada3* in microarray (18); however a significant decrease in CENP-B levels were seen in chromatin fraction (Figure 2.5A). To confirm the effect of ADA3 on CENP-B binding ability to centromere, we performed ChIP assay using PCR primers specific to HOR region on the centromere of human X chromosome (primer D' in Table 2.1), and observed that binding of CENP-B to the centromere of X chromosome is dramatically decreased upon *ADA3* knockdown. Notably, 80% knockdown of *ADA3* led to 63% reduction in CENP-B recruitment to X-chromosome HOR region (Figure 2.5B). However, no significant change in the recruitment of CENP-A onto HOR region upon *ADA3* knockdown was noticed. In a different strategy, we knocked down *ADA3* in 76NTERT cells using siRNA and then examined binding of CENP-B to centromeres by co-immunofluorescence using anti-CENP-B and anti-centromere antibodies (ACA). As seen in Figure 2.5C & 2.5D, we observed a significant reduction (Chi square test p value < 0.0001) in co-localization of CENP-B with ACA after knockdown of *ADA3*. Taken together, our results demonstrate ADA3 is required for association of CENP-B to centromeres.

FIGURE 2.5. Depletion of ADA3 abrogates CENP-B recruitment to centromeres



C


DAPI
ADA3
ACA
CENP-B
MERGE
Ctrl. siRNA
ADA3 siRNA

D

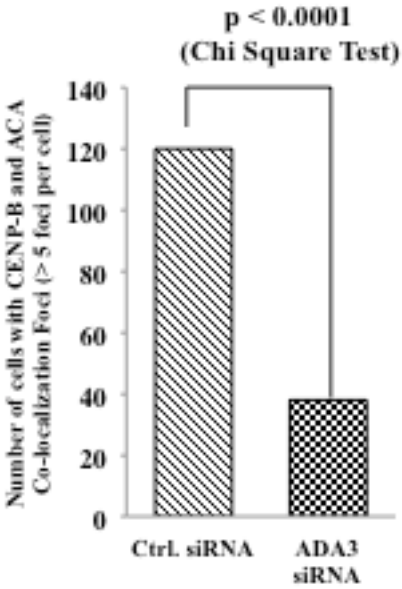

p < 0.0001
(Chi Square Test)
Number of cells with CENP-B and ACA Co-localization Foci (> 5 foci per cell)
140
120
100
80
60
40
20
0
Ctrl. siRNA
ADA3 siRNA

FIGURE 2.5. Depletion of ADA3 abrogates CENP-B recruitment to centromeres. *A, Ada3*$^{FL/FL}$ MEFs were infected with Control (Ctrl) or Cre adenovirus. 72 hrs after infection, cells were trypsinized and harvested. Cell compartments were fractionated as described under "Experimental Procedures". The fractions were run on SDS-PAGE, transferred to PVDF membrane and immunoblotted with indicated antibodies. The values indicate intensities measured using ImageJ. The intensities were normalized against GAPDH (for WCE and cytoplasmic extract) or Histone H3 (for chromatin extract). *WCE,* Whole cell extract. *B,* 76NTERT cells were transfected with control or *ADA3* siRNA. *i*, Western blot showing ADA3 depletion by *ADA3* siRNA. *ii*, ADA3, CENP-A or CENP-B protein was immunoprecipitated from cross-linked chromatin-protein complex and ChIP-PCR was performed using PCR primer D' corresponding to the higher order repeat alpha satellite region on the X chromosome (Table 2.1). GAPDH was used as a negative control. The table shows the band intensities estimated by ImageJ software, normalized against inputs of Ctrl and *ADA3* siRNA independently. *C,* Representative immunofluorescence images of the co-localization of CENP-B and ACA performed in 76NTERT cells after transfecting with either control siRNA or siRNA against *ADA3*. Cells were fixed on 18-mm coverslips with 4% paraformaldehyde after 48 hours of transfection. Indicated antibodies were used for immunofluorescence. ACA staining was used to identify centromeres. *D,* Quantification of cells exhibiting CENP-B and ACA co-localization from *C*. Control or *ADA3* siRNA infected cells co-stained with ACA and CENP-B were quantified based on ACA-CENP-B co-localization foci. Cells with more than 5 CENP-B-ACA interaction foci were considered positive (n=120).

Deletion of Ada3 Causes Defects in Chromosome Segregation

Previous studies have demonstrated CENP-B protein is important in ensuring faithful chromosome segregation during mitosis and thus assuring highest fidelity of centromere function (16,17) as deletion of CENP-B causes significant elevation in chromosome mis-segregation (17). As *ADA3* knockdown/deletion in cells causes significant reduction in CENP-B recruitment onto centromeres, we speculated that deletion of *Ada3* might cause chromosome mis-segregation in cells as seen upon depletion of CENP-B. To test this, we deleted *Ada3* from $Ada3^{FL/FL}$ MEFs and measured defects in chromosome segregation. To enrich cells in mitosis, we synchronized control and Cre infected cells by double thymidine block in S phase and then fixed cells after release with complete medium for 6 hours (Figure 2.6A). Analyses of DAPI stained anaphase chromosomes in both control and Cre infected cells by Confocal microscopy revealed significantly higher percentage of anaphase bridges and lagging chromosomes in *Ada3* deleted cells compared to control cells (Chi square test p value < 0.0001) (Figure 2.6B & 2.6C). These results reveal a novel role of ADA3 in the process of chromosome segregation through its binding to centromeres. Thus, ADA3 is required for maintaining the fidelity of chromosome segregation in mitosis (Figure 2.7).

FIGURE 2.6. Deletion of *Ada3* causes defects in chromosome segregation

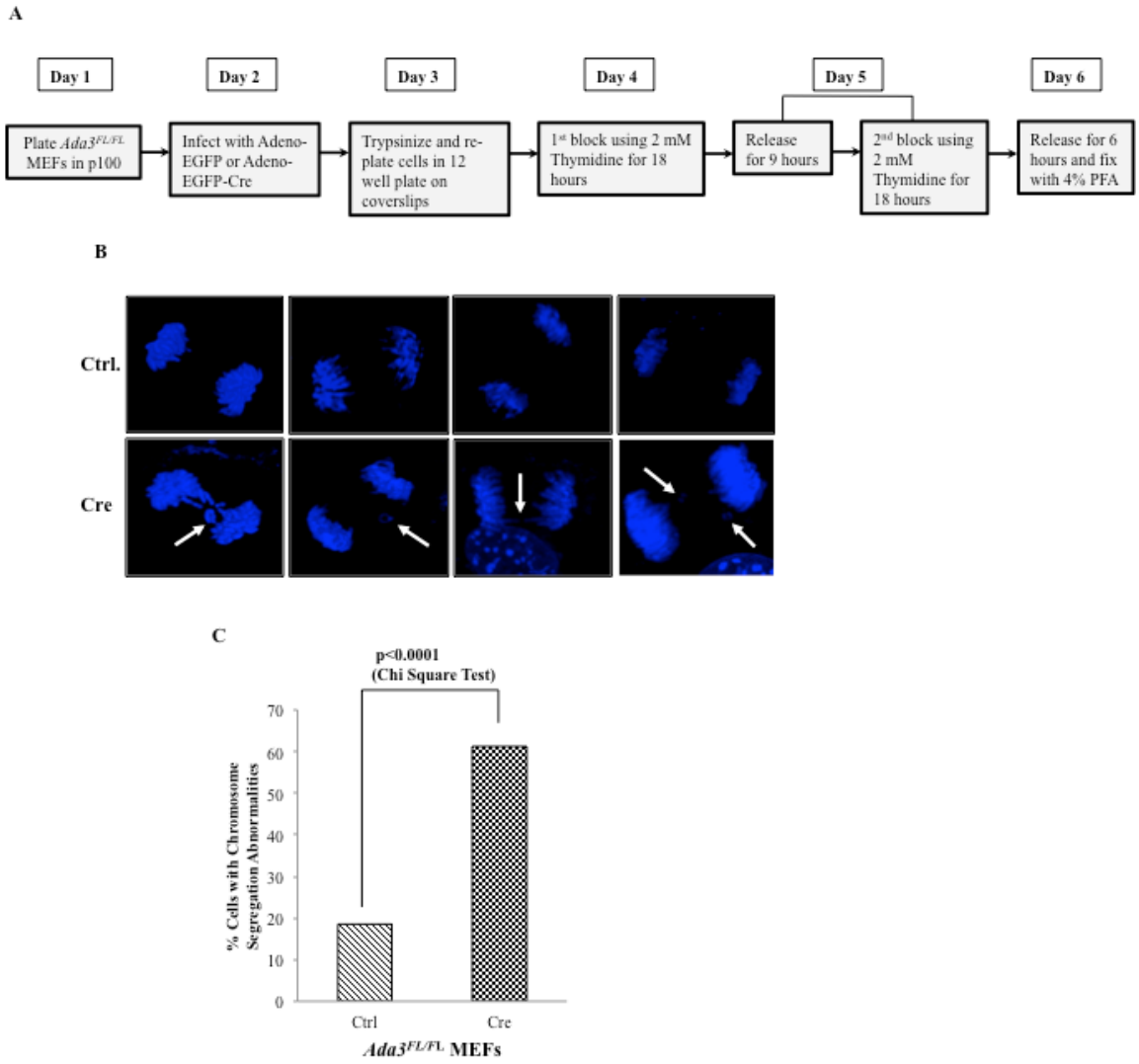


FIGURE 2.6. Deletion of *Ada3* causes defects in chromosome segregation. *A,* Strategy used to assess chromosome mis-segregation in *Ada3* deleted cells. *B,* Representative confocal images of DAPI stained anaphase chromosomes from control or Cre infected $Ada3^{FL/FL}$ MEFs. Note that *Ada3* deleted cells show various chromosomal segregation abnormalities, such as lagging chromosomes and anaphase bridges (indicated by white arrows). *C,* Quantification of chromosomal segregation defects in control or Cre infected $Ada3^{FL/FL}$ MEFs from *B*. *Note: At least 50 anaphase cells each from Ctrl and Cre infected* $Ada3^{FL/FL}$ *MEFs were analyzed for quantification.*

DISCUSSION

Human centromeres are multi-megabase regions of highly ordered arrays of alpha satellite DNA that are separated from chromosome arms by unordered alpha satellite monomers and other repetitive sequences. The centromere is an essential chromosomal domain that is required for chromosome segregation, and ensures the faithful inheritance of the chromosome during cell division (1-4).

Our recent study where conditional deletion of *Ada3* from $Ada3^{FL/FL}$ MEFs using adenovirus Cre system demonstrated a critical role of ADA3 in cell cycle progression with defect in mitosis and another study where knockdown of *Ada3* in NIH3T3 cells demonstrated role of ATAC (ADA3 associated complex) in mitosis provided the rationale to examine mechanism of ADA3 regulation of mitosis (18,19). We performed ChIP-seq followed by ChIP assay using anti-ADA3 antibody and demonstrated that ADA3 associates with high order repeat region in alpha satellite of X chromosome centromere, which is the site of kinetochore assembly. Our results together with a recent study that showed direct binding of GCN5 to centromere (27) suggests a role of ATAC/SAGA complex in mitosis through regulation of centromere function.

Several CENPs, including CENP-B, are known to bind to centromere and regulate centromere function and thus chromosome segregation (33,37). Amongst centromeric proteins, CENP-B is the only protein which has DNA sequence specific binding ability that is conferred by a 17-bp sequence, known as the CENP-B box, in alpha satellite region of the human and minor satellite region of the mouse centromere (8,38) suggesting a role of CENP-B in centromere identity. While studies of human artificial chromosome formation have revealed an essential role of CENP-B in *de novo* centromerization (12,13), the less severe mitotic defects in *Cenpb* knockout mice (39-41) and lack of CENP-B boxes in Y chromosome and neocentromeres (42,43) have led to the idea that CENP-B might be dispensable in centromere function. However, the two recent *ne plus ultra* studies (16,17) by Cleveland and colleagues have provided several lines of evidences that uncover the role of CENP-B in centromere function. They observed an increased

rate of chromosome mis-segregation in *Cenpb* null MEFs and CENP-B devoid Y and neocentromeres, a new concept according to which CENP-B ensures the highest fidelity of chromosome segregation.

Our results using PLA and GST pull down assays showed direct interaction of ADA3 with CENP-B. Further, to determine the region of ADA3 required for its interaction with CENP-B, we generated various GST-tagged truncated mutants of ADA3 and demonstrated that the N-terminal 110 amino acids of ADA3 are essential for its interaction with CENP-B. Accordingly, previous studies from our laboratory and that of others' have demonstrated that N-terminal half of ADA3 facilitates its interaction with transcription factors such as p53, AATF, estrogen receptor, ANCO-1 and other non-HAT-complex components; whereas C-terminus of ADA3 is essential for its incorporation into HAT complexes through its direct interaction with ADA2 subunit and is also required for its association with p300 HAT (36,44-49). Thus, our finding that N-terminus of ADA3 directly interacts with CENP-B defines a new interactor of ADA3 N-terminus. More importantly, we showed that the ADA3 truncated mutant that does not interact with CENP-B failed to rescue the cell proliferation defects caused upon deletion of endogenous *Ada3* in $Ada3^{FL/FL}$ MEFs. These findings imply that ADA3-CENP-B interaction is important for cell proliferation.

Furthermore, sub-cellular protein fractionation, ChIP assay and direct immunofluorescence demonstrated the role of ADA3 in CENP-B recruitment to centromeres. These results along with published findings implicate the role of ADA3 in regulation of centromere. Nevertheless, how ADA3 controls the localization of CENP-B on chromatin still remains to be answered. We speculated that ADA3 might regulate the localization of CENP-B by mediating its acetylation by various KATs; however, in our assays neither p300 nor GCN5 was able to acetylate CENP-B protein (data not shown). One possibility is that direct interaction of ADA3 with CENP-B might enhance its DNA binding ability without the acetylation of CENP-B as we have demonstrated in the case of estrogen receptor (46,50). The second possibility is that ADA3 might regulate the

DNA binding ability of CENP-B indirectly. A recent report suggests that α-N-trimethylation of CENP-B protein enhances its binding ability to CENP-B box (51) and the role of ADA3 in this context is subject of future studies that might reveal a novel function of ADA3.

Additionally, based on recent studies that show a vital role of CENP-B in maintaining the fidelity of chromosome segregation, we examined if ADA3 could regulate the chromosome segregation process through its interaction with CENP-B/centromere. Analyses of DAPI stained anaphase chromosomes in *Ada3* deleted cells revealed a dramatic increase in the chromosome mis-segregation events compared to control cells suggesting an essential role of ADA3 in maintaining the faithful segregation of chromosomes during mitosis (Figure 2.7). These findings also provide a rationale for our earlier study that demonstrated an important role of ADA3 in maintaining genomic stability (25). In this study, we showed that deletion of *Ada3* caused an increase in chromosomal aberrations such as chromosome breaks, fragments, deletions and translocations. As defect in the process of chromosome segregation is an important element leading to chromosomal aberrations in cells (3), the genomic instability observed in *Ada3* deleted cells could be attributed to the critical role of ADA3 in regulating the process of chromosome segregation through its centromere binding ability.

Although we demonstrated ADA3 association with centromere via CENP-B in the present study, given the role of ADA3 as a mediator protein of global acetylation of histones, it is possible that ADA3 also regulates histone acetylation at centromeric region. Indeed H3K9 and histone H4 acetylation have been shown to regulate kinetochore assembly (52,53). However, in our ChIP experiments we were unable to detect any significant changes in the levels of H3K9 acetylation or H3K9 trimethylation at X-chromosome HOR region upon *ADA3* knockdown (data not shown). However, we cannot rule out the possibility that *ADA3* knockdown may change histone acetylation at centromeres of other chromosomes. Besides regulating the acetylation of histone proteins, ADA3 also promotes the acetylation of non-histone proteins (35) and we cannot rule out the possibility that ADA3 might play a role in acetylation of other centromeric proteins.

This study together with our previous studies where we demonstrated halt in cell cycle and genomic instability upon deletion of *Ada3* (18,25) clearly demonstrate an important role for ADA3 in the regulation of mitosis. Given that ADA3 level/localization alter in breast cancers and predict poor prognosis and poor survival in patients (26), this study underscores the important role of ADA3 in abnormal proliferation that leads to oncogenesis.

FIGURE 2.7. Model displaying the role of ADA3 in chromosome segregation.

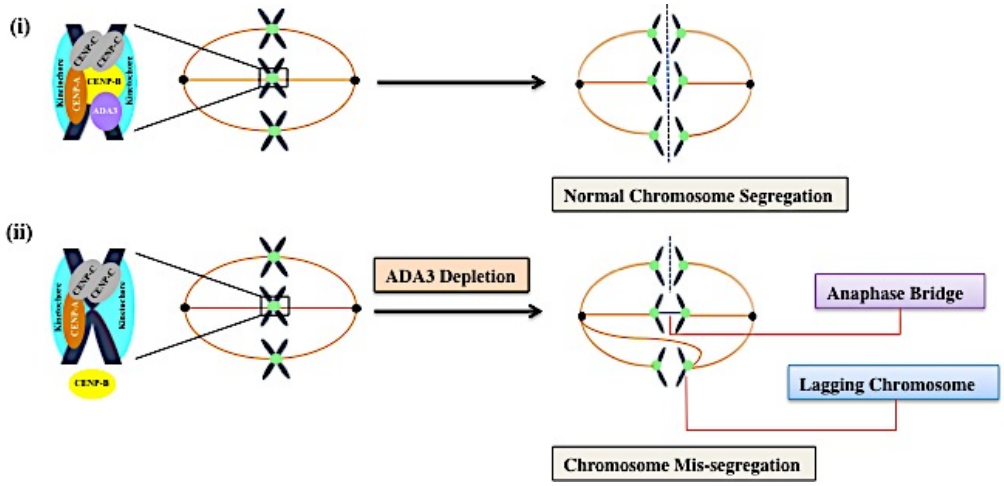


FIGURE 2.7. Model displaying the role of ADA3 in chromosome segregation. *i.* ADA3 associates with centromere by directly binding to CENP-B that interacts with centromeric proteins CENP-A and CENP-C to form functional centromere, which is required for proper chromosome segregation. *ii,* In conditions where ADA3 protein is depleted the recruitment of CENP-B at centromere is diminished leading to chromosomal segregation abnormalities viz. anaphase bridges and lagging chromosomes (*ii*). *Note: For the simplicity of the model, the association of ADA3 and centromeric proteins with centromere is enlarged at only one chromosome and not shown for each chromosome.*

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CHAPTER 3

ACETYLATION OF MAMMALIAN ADA3 IS REQUIRED FOR ITS FUNCTIONAL ROLES IN HISTONE ACETYLATION AND CELL PROLIFERATION

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Introduction

Alteration/deficiency in activation 3 (ADA3) protein is a conserved component of key chromatin-modifying complexes that contain either GCN5 or PCAF histone acetyl transferases (HATs), such as SAGA (Spt/Ada/Gcn5) in yeast, ATAC (ADA2a containing complex), STAGA (SPT3/TAFII31/GCN5 Acetyltransferase) and TFTC (TATA binding protein free-TAF containing complex) in metazoans (1-7). Within these complexes, ADA3 associates with GCN5 and ADA2 to form the HAT module. ADA3 has also been shown to associate with p300, the most well defined HAT of mammalian system (8, 9). ADA3 is essential for the HAT activity of p300 and GCN5 containing HAT complexes towards histones (10-14) as well as of non-histone proteins such as p53 and β-catenin (15, 16).

Although strongly implicated in the regulation of HAT activity of ADA3-containing complexes, additional functions for ADA3 have been reported. For example, we identified ADA3 as a novel human papilloma virus E6 oncoprotein-binding protein (17), and additional studies revealed that ADA3 binds to nuclear hormone receptors, such as estrogen receptor and retinoid acid receptor, and enhances their transcriptional activation function (8, 18-21). Recent studies have identified an essential role of ADA3 in normal cell cycle progression and maintenance of genomic stability (5, 13, 22, 23).

Whether ADA3's role in these processes is merely a passive structural one or is actively regulated is unknown. Post-translational modification represents one potential mechanism to regulate ADA3 function and in fact yeast ADA3 was found to be modified by acetylation (24). Consistent with this idea we observed that human ADA3 is also acetylated *in vitro* by its interacting HAT p300 (13). Here, we present evidence that, in addition to p300, mammalian ADA3 is acetylated by GCN5 and PCAF, and that ADA3 acetylation is balanced by deacetylation by histone deacetylase (HDAC) SIRT1. Mass spectrometry analyses identified seven p300 and one GCN5 acetylation sites on ADA3. ADA3 acetylation is cell cycle- phase dependent, and acetylation-defective mutants of ADA3 fail to restore global histone acetylation patterns or H3K9

acetylation at the *c-myc* enhancer, and failed to rescue cell cycle progression block caused by endogenous *Ada3* deletion, demonstrating that acetylation plays an important role in ADA3 function in histone modification and cell cycle progression. Taken together, our findings demonstrate that acetylation of ADA3 by its associated HATs is essential for its key role in histone acetylation and cell cycle progression.

Materials & Methods

Plasmids, siRNA and Transient Transfections

Construction of FLAG-ADA3 has been described previously (13). Various FLAG-ADA3 point mutants were generated using Invitrogen GeneArt site directed mutagenesis kit and then verified by DNA sequencing. Primers for site directed mutagenesis were designed using the GeneArt Primer design tool on the manufacturer's website (http://www.thermofisher.com/order/oligoDesigner//). Primer sequences are available upon request. His-GCN5L2 HAT domain was a gift from Cheryl Arrowsmith (Addgene plasmid # 25482). p300 wild-type, p300ΔHAT mutant, FLAG-HDACs, FLAG-SIRT1, -2 and -3 were generous gifts from Dr. Kishor Bhakat. FLAG-SIRT4, -5, -6 and -7 were purchased from Addgene (plasmid # 13815, 13816, 13817, 13818, respectively). FLAG-SIRT1-H363Y was generated using Invitrogen GeneArt site directed mutagenesis system kit and verified by DNA sequencing. For transient transfection experiments the indicated plasmids were transfected using X-tremeGene HP transfection reagent (Roche # 06366236001) according to manufacturer's protocol. Control and *SIRT1* (sc-40986) siRNA were purchased from Santa Cruz Biotechnology. For co-transfection of FLAG-ADA3 and control or *SIRT1* siRNA, 3 µg FLAG-ADA3 and 20 nM siRNA was co-transfected using X-tremeGENE siRNA Transfection Reagent (04476093001) following manufacturer's protocol.

Cell Culture, Viral Infections and Cell Cycle Analysis

76NTERT cells were cultured in DFCI media as described before (25). A549, HEK293T and *Ada3*$^{FL/FL}$ MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. *Ada3*$^{FL/FL}$ MEFs stably expressing wild-type FLAG-ADA3 or acetylation defective mutants were generated as previously described (13). Adenoviruses expressing EGFP-Cre or EGFP alone were purchased from University of Iowa (Gene transfer vector core). Cre

mediated deletion of *Ada3* was performed as described previously (13). Cell cycle analysis by FACS in 76NTERT cells was performed as described previously (26).

Antibodies

FLAG-HRP (A8592), β-actin (A2228), and α-tubulin (T-5168) antibodies were purchased from Sigma. Anti-acetyl-H3K56 (04-1135), anti-acetyl-H3K9 (07-352), Histone H3 (06-755) and GAPDH (MAB374, EMD Millipore) were purchased from Millipore. ADA2a (ab-57489) and ADA2b (ab-57953) were purchased from Abcam, p300 (sc-584 and sc-585), PCAF (sc-13124), TRRAP (sc-5405), HSC-70 (sc-7298) and PARP (sc-8007) antibodies were from Santa Cruz Biotechnology, anti-acetyl lysine (9681), anti-acetyl lysine-HRP (6952), GCN5 (3305), SIRT1 (9475) and HA-HRP (2999) were from Cell Signaling. Generation of mouse monoclonal anti-ADA3 was described previously (Mohibi et al., 2012). ADA3 antibody was labeled with HRP using lightening-link HRP kit from Novus Biologicals (701-0030) following manufacturer's protocol.

Reagents

Trichostatin A (TSA, T8552), Nicotinamide (NAM, N0636), β-Nicotinamide adenine dinucleotide sodium salt (NAD^+, N0632), Acetyl co-enzyme A sodium salt (A2056) were purchased from Sigma. EX-527 was purchased from Selleckchem (S1541). Recombinant p300 HAT domain was purchased from Active Motif, (31205).

Immunoprecipitation and Immunoblotting

For immunoprecipitation, cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM Na_4VO_3, 1 mM NaF, and protease inhibitor mixture, 2 μM TSA and 10 mM NAM for acetylation experiments) and whole cell extracts were subjected to

anti-FLAG M2 affinity gel (Sigma) overnight at 4^{o}C and beads were then washed five times at 5000 rpm for 1 minute with lysis buffer. Unless otherwise indicated, the immunoprecipitated FLAG tag proteins were eluted with 0.25 µg/µl 3X FLAG peptide (Sigma) into lysis buffer. The elutes were subjected to SDS-PAGE and analyzed by immunoblotting, as indicated. For FLAG-ADA3 and p300 co-immunoprecipitation, HEK-293T cells were cross-linked by Dithiobis (succinimidyl propionate) (DSP; Thermo Scientific 22585) before immunoprecipitation. In brief, cells were incubated with 1.5 mM DSP in 1x PBS for 15 min at room temperature followed by quenching with excess Tris, pH 7.4. Cells were lysed in RIPA buffer and, FLAG immunoprecipitation remained the same as mentioned above except washing the beads with RIPA buffer. For endogenous ADA3 immunoprecipitation, equal amount of lysates were incubated with 5 µg anti-ADA3 mouse monoclonal antibody overnight at 4^{o}C followed by incubation with protein A/G-agarose (sc-2003, Santa Cruz Biotechnology) for 2 h. Beads were washed with lysis buffer 5 times as mentioned above and eluted in 2x SDS sample buffer. Elutes were then subjected to SDS-PAGE analysis and immunoblotted with indicated antibodies.

Immunofluorescence

Ada3$^{FL/FL}$ MEFs stably expressing wild-type FLAG-ADA3 or acetylation defective mutants were infected with Adeno-EGFP or Adeno-EGFP-Cre in P-100 dishes, as described above. One day after infection 2000 cells were re-plated on glass cover slips in 12-well plates and four days later cells were fixed in 4% paraformaldehyde for 20 minutes. Staining was performed with anti-ADA3 antibody. Secondary antibody used was Alexa fluor 594 from Life Technologies. Nuclei were counterstained with DAPI. The cover slips were then placed on slides using the mounting medium. Fluorescent images were captured using LSM 510 META Confocal fluorescence microscope (Zeiss).

***In vitro* acetylation and deacetylation assays**

Purified GST-ADA3 or various GST-ADA3 mutants were acetylated by recombinant HATs GCN5 or p300. Briefly, 1 μg GST-ADA3 WT or mutants were incubated with 25 ng p300 HAT domain or 50 ng GCN5 HAT domain in HAT buffer (50 mM Tris HCl pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1mM DTT, 2 μM TSA, 50 μM Acetyl co-enzyme A sodium salt and 1 mM PMSF) at 30°C for 30 minutes. The reaction was stopped by adding 3x SDS sample buffer and products were subjected to SDS-PAGE analysis and immunoblotted with the indicated antibodies. For deacetylation reaction, the products of GST-ADA3 WT acetylation reactions (acetylated with p300 or GCN5) were then incubated with 20 ng BSA equivalent FLAG SIRT1 wild-type (WT) or H363Y (FLAG-SIRT1 WT or H363Y were ectopically expressed and immunoprecipitated from HEK293T cells. The immunoprecipitates were eluted with 3X FLAG peptide and a fraction of it was analyzed on SDS-PAGE by CBB staining along with various amounts of BSA) in HDAC buffer (50 mM Tris HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NAD^+, 200 nM TSA) at 37°C for 1 hour. Reaction was then stopped by 6x SDS sample buffer and analyzed by immunoblotting with pan anti-acetyl lysine antibody.

GST-pull down assays

GST-pull down assay was performed as described previously (23). Briefly 1 μg of bacterially purified GST or GST-ADA3 proteins bound to beads were used as bait and incubated with 300 ng of baculovirally purified SIRT1 protein purchased from R&D Systems (Catalog#7714-DA). Following incubation for 2 hours at 4^oC, the beads were washed 5 times with lysis buffer and samples subjected to SDS-PAGE followed by immunoblotting with anti-SIRT1 antibody.

Identification of acetylation sites on ADA3 by mass spectrometry

For identification of acetylation sites *in vivo,* 76NTERT cells stably overexpressing FLAG-ADA3 or 293T cells transiently transfected with FLAG-ADA3, were treated with 1 μM Trichostatin A (TSA) and 5 mM Nicotinamide (NAM) for 10 hours. Following this procedure, whole cell lysates were immunoprecipitated with anti-FLAG conjugated agarose beads. Immunoprecipitates were then eluted with 3X FLAG peptide, ran on SDS-PAGE and stained with Coomassie stain. Band corresponding to FLAG-ADA3 was cut from the gel (about 1.5 μg) and then subjected to either chymotrypsin or trypsin digestion. The samples were cleaned up using Millipore μC18 ZipTip, resuspended in 0.1% Formic acid and injected through Eksigent cHiPLC column onto 5600 TripleTOF. Database searching was performed using PEAKS studio 6 software and peptides identified with 95% confidence level. For identification of lysines specifically acetylated by either GCN5 or p300, 1.5 μg of bacterially purified GST-ADA3 was incubated with 50 ng of GCN5 catalytic domain or with 25 ng of p300 catalytic domain (aa 965-1810) for 30 min at 30^{o}C. The samples were eluted on SDS-PAGE, stained with Coomassie stain and subjected to Mass spectrometric analysis, as indicated above.

Examining ADA3 acetylation during cell cycle

For examining ADA3 acetylation during cell cycle, 76NTERT cells were synchronized in G1 phase by growth factor deprivation and released into cell cycle by stimulating with growth factor containing medium as described previously (26). Following stimulation, cells were harvested at various time points for FACS analysis by PI staining and lysates were prepared in the IP lysis buffer (described above). 450 μg lysates were used to perform endogenous ADA3 IP using anti-ADA3 monoclonal antibody followed by immunoblotting with anti-acetyl lysine-HRP antibody and anti-ADA3-HRP antibody. 10 μg lysates (~2%) were loaded as input to determine the total levels of ADA3 during cell cycle.

Rescue proliferation and rescue colony formation assays

Rescue proliferation assays for various acetylation defective mutants were performed as previously described (23). Briefly, *Ada3* $^{FL/FL}$MEFs stably overexpressing empty vector, *ADA3 WT*, *K418R*, *7KR* or *8KR* were plated in triplicates in 96 well plates (150 cells/well) 24 h after infection with Adenovirus EGFP or EGFP-Cre. The cell proliferation was measured at various days after plating using CellTiter® Glo assay (Promega) according to manufacturer's protocol. For calculating percentage rescue for each cell line, two levels of normalization were used. First, CellTiter® Glo values for each day for Cre infected cells were divided to their corresponding control infected cell values. Subsequently, the data obtained was normalized to Day 1 for each cell line. Rescue colony formation assays were performed as previously described (13) and colony numbers were counted followed by calculation of percentage rescue. Percentage rescues from both CellTiter® Glo assay or colony formation assay represent mean ± S.D. from three independent experiments and *p* values were computed using Student's *t* test.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed using ChIP-IT Express kit from Active Motif according to manufacturer's protocol with slight modifications in fixation and sonication conditions. Stably overexpressing empty vector, *ADA3 WT*, *K418R*, *7KR* or *8KR Ada3* $^{FL/FL}$MEFs were infected with adenovirus expressing GFP-Cre in order to delete endogenous *Ada3*. 48 h after infection cells were switched to 0.1% serum containing media for 72 h followed by stimulation with 10% serum containing media for 40 min. Cells were then fixed in EGS [ethylene glycol bis (succinimidyl succinate), Thermo Scientific] and formaldehyde at room temperature. In particular, cells were incubated in 1.5 mM EGS in 1X PBS on shaking platform for 15 min. To this formaldehyde (1%

working concentration) was added for another 15 min. Fixation reaction was then stopped by 1X glycine at room temperature for 5 min. Chromatin was isolated and sonicated for 12 minutes in Biotuptor^{TM} UCD-200 (Diagenode) attached with NESLAB RTE7 water bath circulator (Thermo Scientific). Remaining steps for ChIP were followed exactly as per manufacturer's protocol. Eluted chromatin from immunoprecipitation and input was then ethanol precipitated and used as templates in RT-PCR reactions preformed in four replicates. ChIP RT-PCR data was analyzed using percentage input method and normalized against vector. In brief, the percentage input was calculated as 100 x 2^ (Adjusted Input-Ct of IP) where adjusted input = Ct of input-$\log_2$(dilution factor).

Figure 3.1: ADA3 is subject to acetylation and deacetylation across different cell types

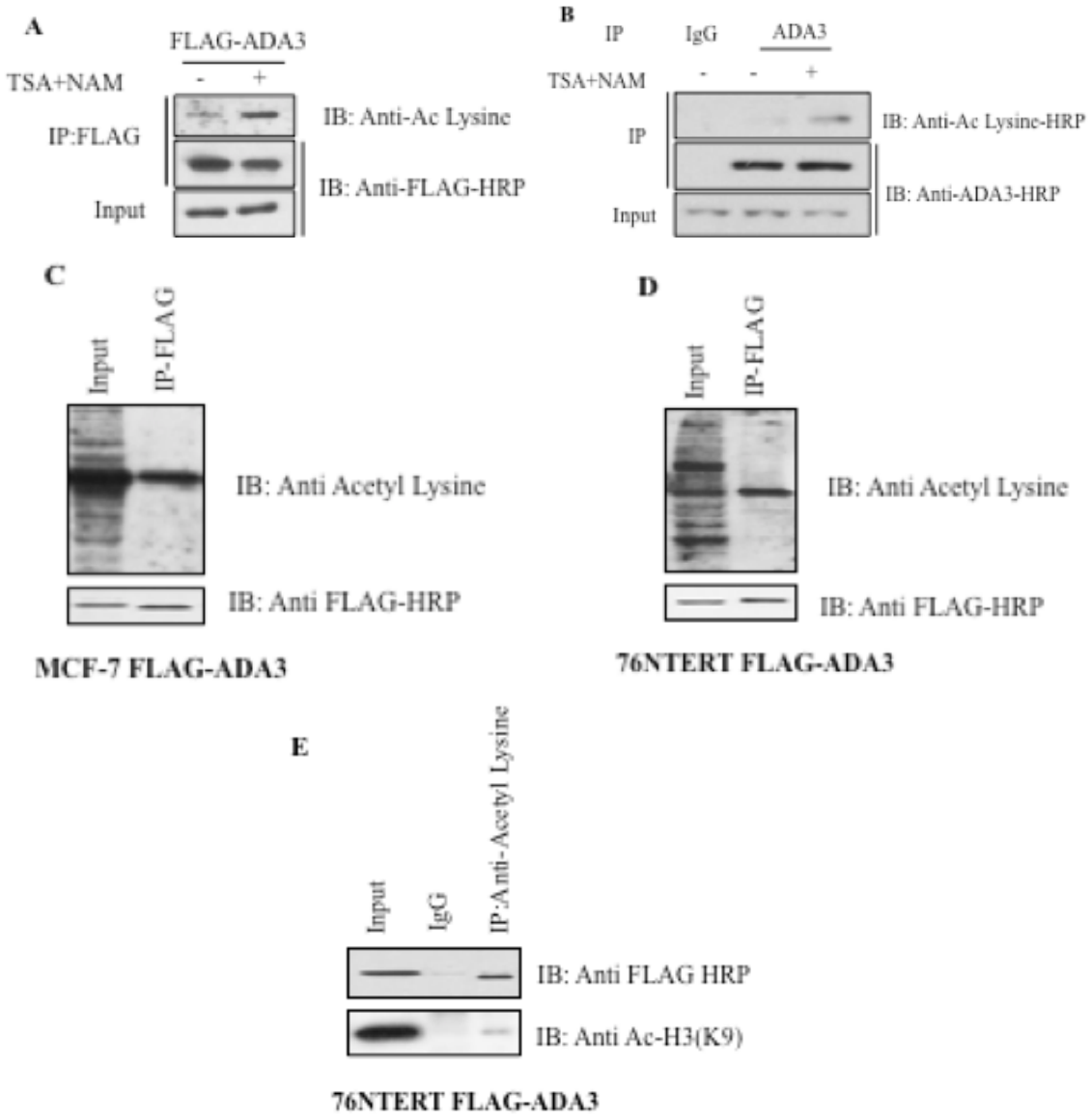
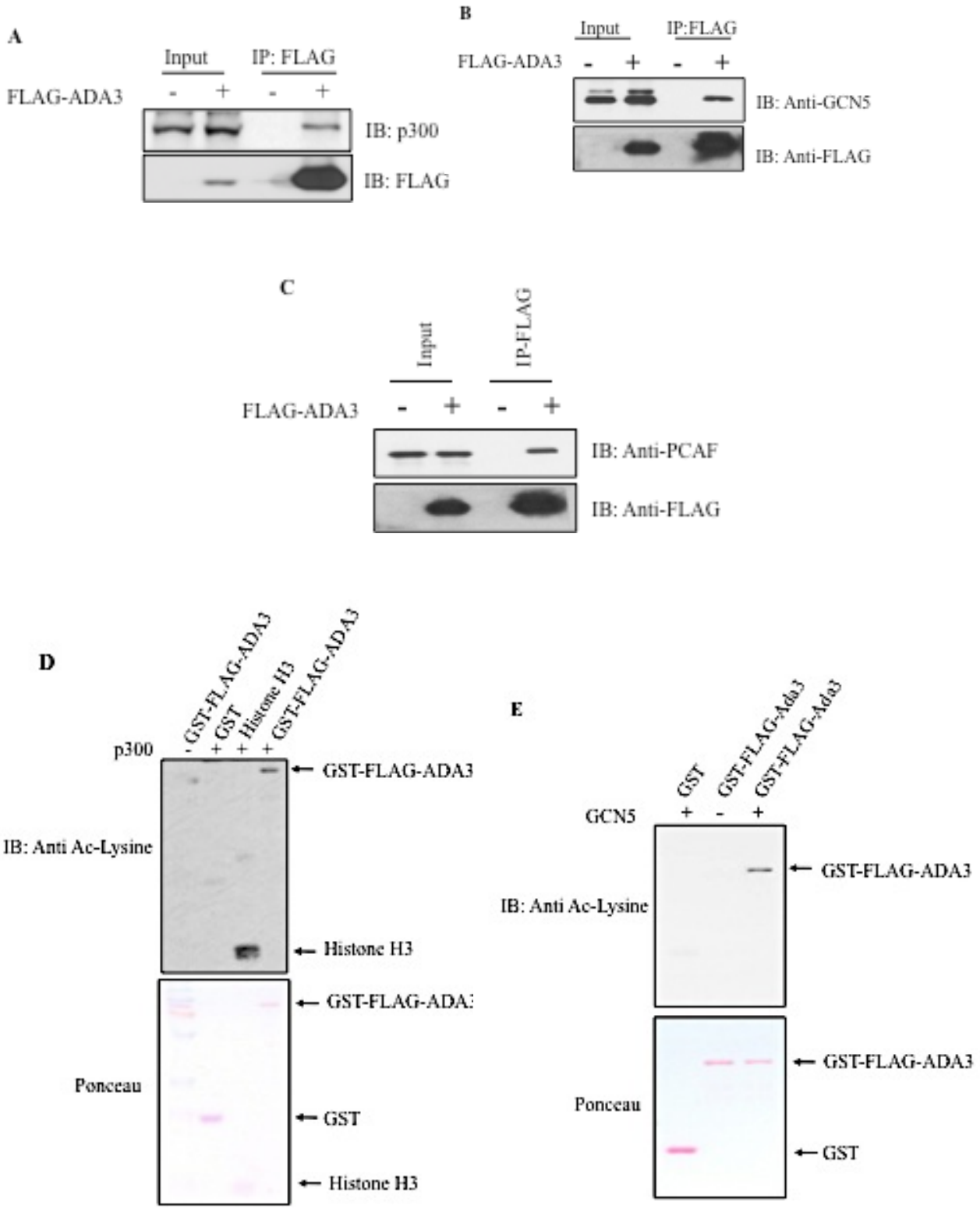


Figure 3.1 - ADA3 is subject to acetylation and deacetylation across different cell types (A) 293T cells was transfected with FLAG-ADA3 and 30 h post transfection, as indicated, cells were treated with 1 μM Trichostatin A (TSA) and 5 mM Nicotinamide (NAM) or vehicle, for additional 10 h. Whole cell lysate were immunoprecipitated with anti-M2 FLAG agarose beads. Immunoprecipitates were then eluted with 3X FLAG peptide and analyzed on SDS-PAGE using anti-acetyl lysine antibody. Same blot was stripped and probed with HRP labeled FLAG antibody. **(B)** A549 cells were treated either with TSA (1 μM) and NAM (5 mM) for 10 h or vehicle. Whole cell extracts were then immunoprecipitated with normal mouse IgG or anti-ADA3 antibodies and immunoblotted with indicated antibodies **. (C, D & E)** MCF7 (C) and 76NTERT (D & E) cells stably overexpressing FLAG-ADA3 were treated with TSA+NAM and whole cell lysate were immunoprecipitated with M2 agarose FLAG beads (C & D) or pan acetyl lysine antibodies (D) followed by immunoblotting with indicated antibodies.

ADA3 acetylation is mediated by its interacting HATs GCN5, PCAF and p300

Since ADA3 is in a complex with HATs GCN5, PCAF or p300 (8-10), we reasoned that these HATs mediate ADA3 acetylation. We first validated the association of ADA3 with these HATs by probing anti-FLAG immunoprecipitates from FLAG-ADA3 transfected HEK-293T cells for p300 (Figure 3.2A), GCN5 (Figure 3.2B) or PCAF (Figure 3.2C). These experiments showed efficient co-immunoprecipitation of endogenous p300, GCN5 or PCAF with FLAG-ADA3. Next, we used *in vitro* lysine acetyl transferase assays to assess the acetylation of bacterially purified ADA3 by the catalytic domains of recombinant p300 or GCN5. As shown in Figure 3.2D and E, both p300 and GCN5 were able to efficiently acetylate ADA3 *in vitro*. We corroborated these findings by co-expressing FLAG-ADA3 with wild-type p300 or GCN5, or their respective HAT activity-deficient mutants (p300ΔHAT or GCN5ΔHAT). Consistent with the *in vitro* analyses, we observed that acetylation of FLAG-ADA3 was increased with co-expression of wild-type p300 or GCN5, but not when their HAT activity-deficient mutant were co-expressed (Figure 3.2F and G). Expression of PCAF also led to increased ADA3 acetylation in HEK-293T cells (Figure 3.2H). Taken together, these results demonstrate that ADA3-interacting lysine acetyl transferases GCN5, PCAF or p300 can acetylate ADA3.

Figure3.2: ADA3 interacts with and acetylated by GCN5, PCAF and p300



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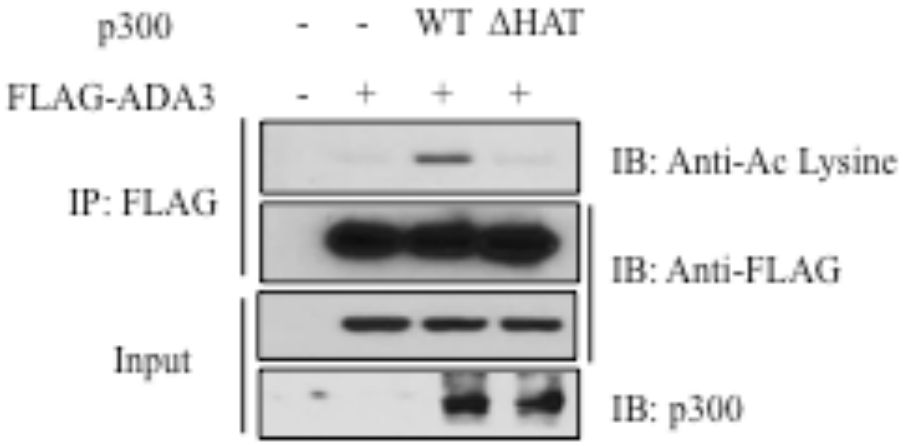
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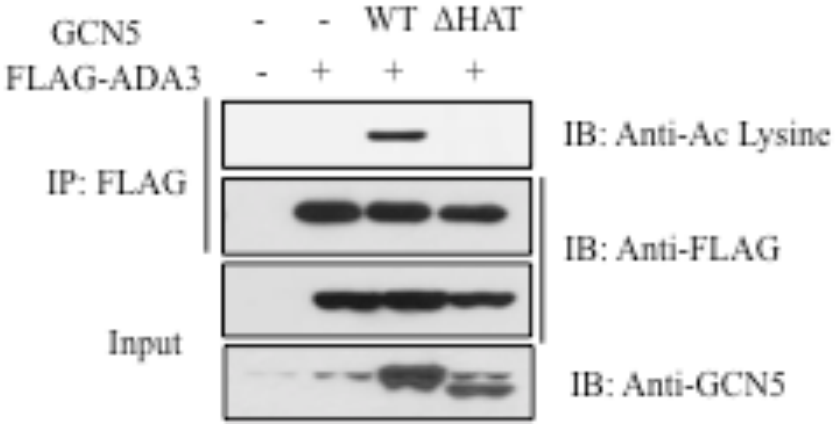
FLAG-ADA3 - + + +
IP: FLAG
IB: Anti-Ac Lysine
IB: Anti-FLAG
Input
IB: p300

G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FLAG-ADA3 - + + +
IP: FLAG
IB: Anti-Ac Lysine
IB: Anti-FLAG
Input
IB: Anti-GCN5

H

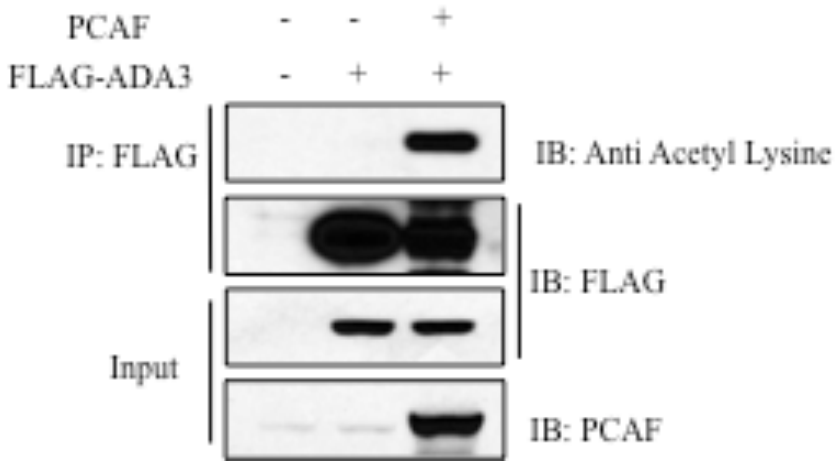

PCAF - - +
FLAG-ADA3 - + +
IP: FLAG
IB: Anti Acetyl Lysine
IB: FLAG
Input
IB: PCAF

Figure 3.2: ADA3 interacts with and acetylated by GCN5, PCAF and p300: (A, B & C) Whole cell lysate from vector or FLAG-ADA3 transfected HEK293T cells were subjected to immunoprecipitation with M2 FLAG agarose beads and interaction with p300 and GCN5 and PCAF was assessed by immunoblotting with anti-p300 (C), anti-GCN5 (D), or anti-PCAF (C) antibodies **(D & E)** *In vitro* HAT assay using GST or GST-ADA3 as substrates and catalytic domains of p300 (D) GCN5 (E) as enzymes. The assay was followed by immunoblotting with anti-acetyl lysine antibody. **(F, G & H)** HEK293T cells were transfected with FLAG-ADA3 along with empty vector, p300 WT or p300 ΔHAT mutant (F) GCN5 WT or GCN5 ΔHAT mutant (G) or PCAF (H). 48 h after transfection whole cell lysates were subjected to immunoprecipitation by M2 agarose beads and immunoblotted with indicated antibodies.

Acetylation of ADA3 is counteracted by class III HDACs (SIRTs)

The marked increase in the levels of ADA3 acetylation upon global inhibition of deacetylation supported the likelihood that ADA3 is dynamically regulated by deacetylation. Histone deacetylases (HDACs), enzymes that remove the acetyl group from lysine, have been categorized into three classes: Class I, consisting of HDACs 1, 2 and 3; Class II, consisting of HDACs 4, 5 and 6; and Class III, consisting of sirtuins (SIRTs) 1 to 7 (30). Trichostatin A (TSA) has been widely used as an inhibitor of class I and II HDACs activities (28) whereas Nicotinamide (NAM) has been demonstrated to be a broad inhibitor of SIRTs (29). In order to determine the class of HDACs responsible for ADA3 deacetylation, we ectopically expressed FLAG-ADA3 in HEK-293T cells and treated the cells with either TSA or NAM. We observed that ADA3 acetylation was markedly enhanced in the presence of NAM, whereas TSA had little or no effect on ADA3 acetylation (Figure 3.3A & B), suggesting that one or more SIRTs are likely to function in ADA3 deacetylation.

Figure 3.3 - Acetylation of ADA3 is counteracted by class III HDACs

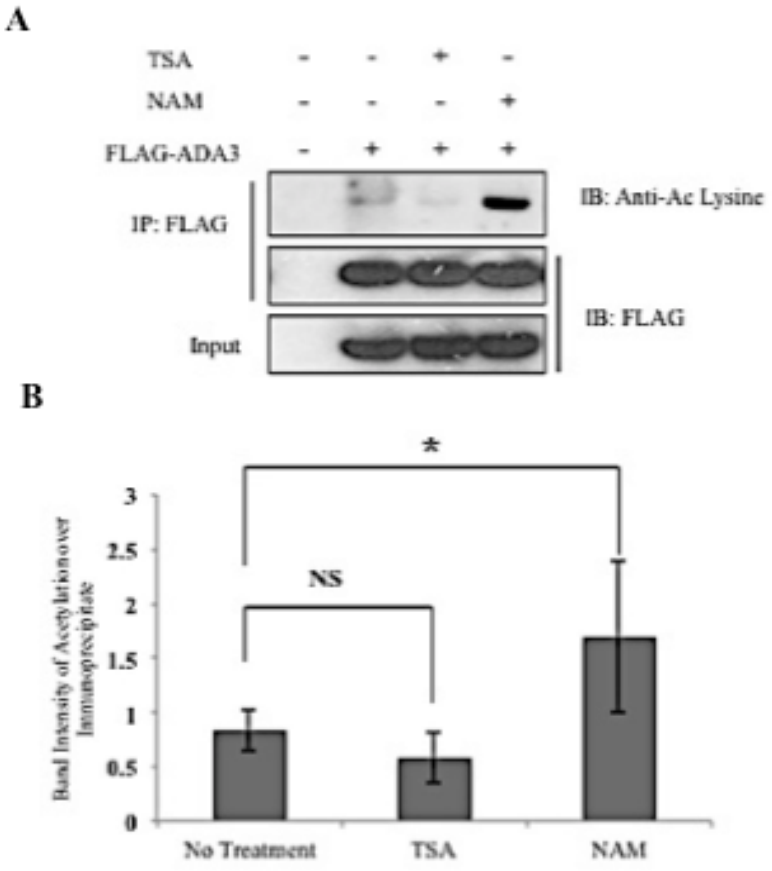


Figure 3.3 - Acetylation of ADA3 is counteracted by class III HDACs (SIRTs). (A) HEK-293T cells were transfected either with FLAG-ADA3 or empty vector. 30 h after transfection cells were treated either with vehicle or 1 μM HDAC class I and II inhibitor Trichostatin A (TSA) or 5 mM HDAC class III inhibitor Nicotinamide (NAM) for 10 h. Whole cell lysates were then subjected to immunoprecipitation by M2 FLAG agarose beads and immunoblotted with either anti acetyl lysine or FLAG-HRP antibodies. **(B)** The band intensities of acetylated FLAG-ADA3 over immunoprecipitated FLAG-ADA3 were quantified using imageJ software and averaged from four independent experiments. The statistical significance between different groups was computed using Student's *t* test. *NS, Not Significant *, $p \leq 0.05$*

ADA3 exclusively interacts with SIRT1 among class I, -II and -III deacetylases

Based on increased ADA3 acetylation specifically with a SIRT inhibitor, we further explored if one or more of SIRTs are involved in deacetylation of ADA3. We first examined the interaction of endogenous ADA3 with ectopically expressed FLAG-tagged HDACs or SIRTs in HEK-239T cells. Co-immunoprecipitation analyses demonstrated that, out of 13 HDACs (HDAC1 through 6 and SIRT1 through 7) tested, only SIRT1 specifically interacted with ADA3 (Figure 3.4A). A reciprocal co-immunoprecipitation further verified the strong association of ectopically expressed FLAG-ADA3 with endogenous SIRT1 (Figure 3.4B). More importantly, immunoprecipitation with anti-SIRT1 antibody followed by western blotting with anti-ADA3 antibody confirmed the association of endogenous ADA3 and SIRT1 (Figure 3.4C).

Next, we assessed whether the interaction of ADA3 with SIRT1 is direct using purified recombinant GST-ADA3 and recombinant SIRT1 proteins. GST-ADA3, but not GST alone, was indeed able to bind to recombinant SIRT1, indicating a direct interaction (Figure 3.4D). Next, we tested if the catalytic activity of SIRT1 is required for its interaction with ADA3, by expressing either wild-type or catalytically inactive (H363Y) SIRT1 in HEK-293T cells. Notably, while wild-type SIRT1 was able co-immunoprecipitate ADA3, the interaction with SIRT1 (H363Y) mutant was markedly lower (Figure 3.4E), suggesting that SIRT1 activity is facilitating its physical interaction with ADA3.

Figure 3.4: ADA3 exclusively interacts with SIRT1 among class I, -II and -III deacetylases

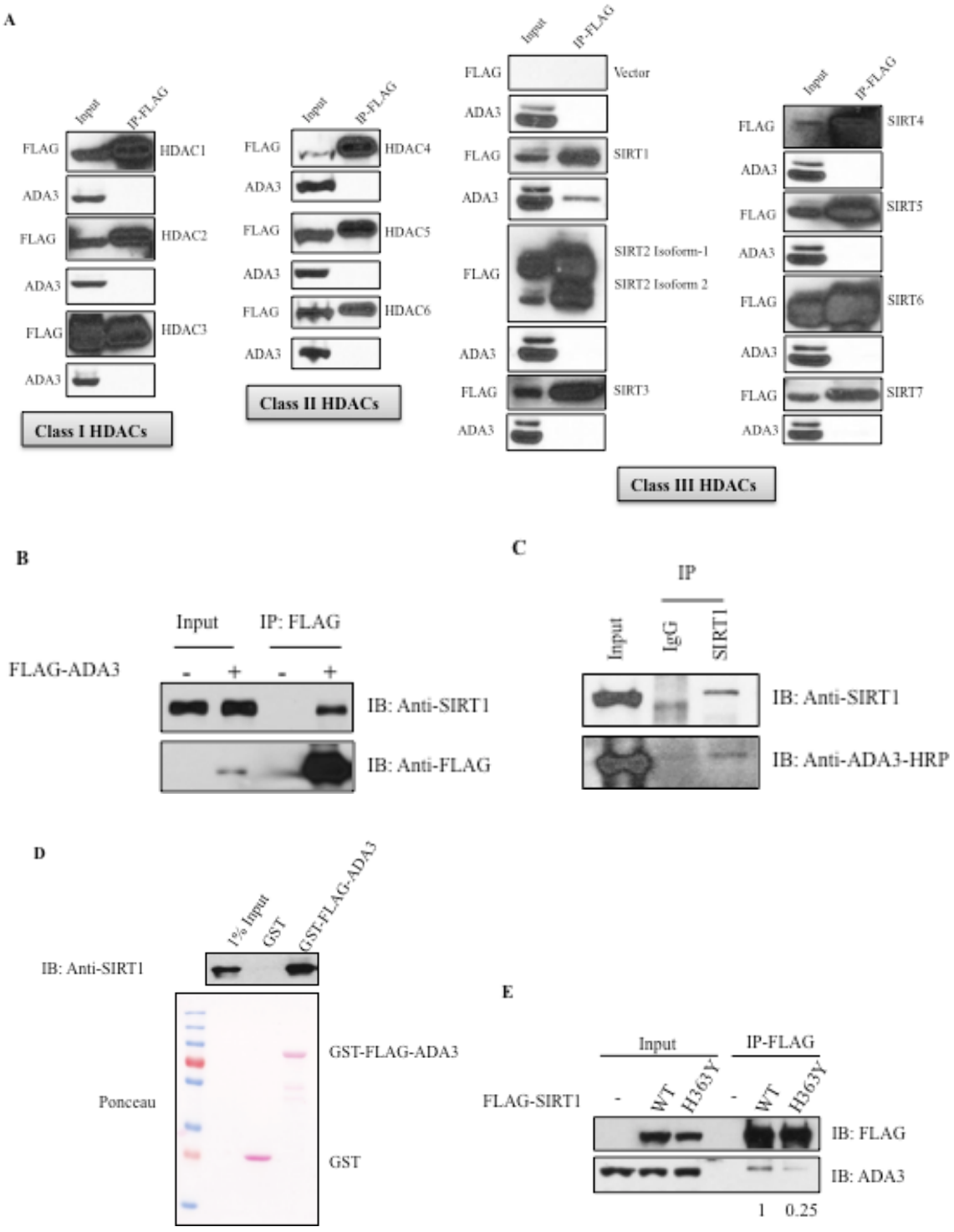


Figure 3.4 ADA3 exclusively interacts with SIRT1 among class I, -II and -III deacetylases. **(A)** HEK-293T cells were transfected with empty vector or various FLAG tagged HDAC and SIRT constructs. 48 h after transfection whole cell lysates were subjected to immunoprecipitation by M2 FLAG agarose beads, eluted with 3X FLAG peptide and immunoblotted with anti-ADA3 or FLAG-HRP antibodies. **(B)** HEK-293T cells were transfected either with empty vector or FLAG-ADA3. 48 h transfection whole cell lysates were subjected to immunoprecipitation by M2 FLAG agarose beads and immunoprecipitates were immunoblotted with anti-SIRT1 or FLAG-HRP antibodies. **(C)** HEK-293T cell lysates were subjected to immunoprecipitation using anti-SIRT1 antibody and immunoprecipitates were analyzed by western blotting using anti-ADA3-HRP and SIRT1 antibodies **(D)** *In vitro* GST pulldown assay; 300 ng recombinant SIRT1 was incubated with 1 µg glutathione bound GST or GST-FLAG-ADA3 followed by immunoblotting with anti-SIRT1 antibody. **(E)** Whole cell lysates of HEK293T cells transfected with FLAG-SIRT1 wild-type or its catalytically inactive mutant H363Y, were immunoprecipitated as in (A), followed by immunoblotting with anti-ADA3 and FLAG-HRP antibodies. The values represent ratio of signal intensities of ADA3 bands over that of total SIRT1 immunoprecipitated as calculated using ImageJ.

SIRT1 deacetylates ADA3 in cells and *in vitro*

To assess if SIRT1 functions as a deacetylase towards ADA3, we examined the acetylation of ADA3 in HEK-293T cells in the presence or absence of SIRT1 specific inhibitor EX-527, which inhibits SIRT1 activity by occupying the binding site for its co-factor NAD+ (31). Immunoprecipitation of FLAG-ADA3 followed by immunoblotting for acetylated lysine showed an increase in ADA3 acetylation in the presence of EX-527 (Figure 3.5A). Interestingly, SIRT1 levels did not change significantly after EX-527 treatment, which strongly suggests that increased ADA3 acetylation with the inhibitor treatment, reflects inhibition of SIRT1 activity (Figure 3.5A). In a complementary approach, we co-transfected HEK-293T cells with FLAG-ADA3 along with scrambled or *SIRT1*-specific siRNA and then examined ADA3 acetylation. Similar to the effect of EX-527, we observed enhanced ADA3 acetylation upon *SIRT1* knockdown (Figure 3.5B). Finally, to directly test the ability of SIRT1 to deacetylate ADA3, we subjected recombinant purified ADA3 to *in vitro* acetylation using GCN5 or p300 as HATs, and then subjected this acetylated ADA3 for an *in vitro* deacetylation in the presence of purified SIRT1 wild-type or its catalytic inactive mutant (H363Y). Consistent with observations in cells, a dramatic decrease in ADA3 acetylation was observed upon incubation with wild-type SIRT1 but not with catalytically inactive SIRT1-H363Y mutant (Figure 3.5C and D). Taken together, these results establish SIRT1 as a deacetylase for acetylated ADA3. The ability of SIRT1 to deacetylate ADA3 that was acetylated by either GCN5 or by p300 suggests that SIRT1 does not discriminate between ADA3 acetylation imparted by different HATs.

Figure 3.5: SIRT1 deacetylates ADA3 in cells and *in vitro*

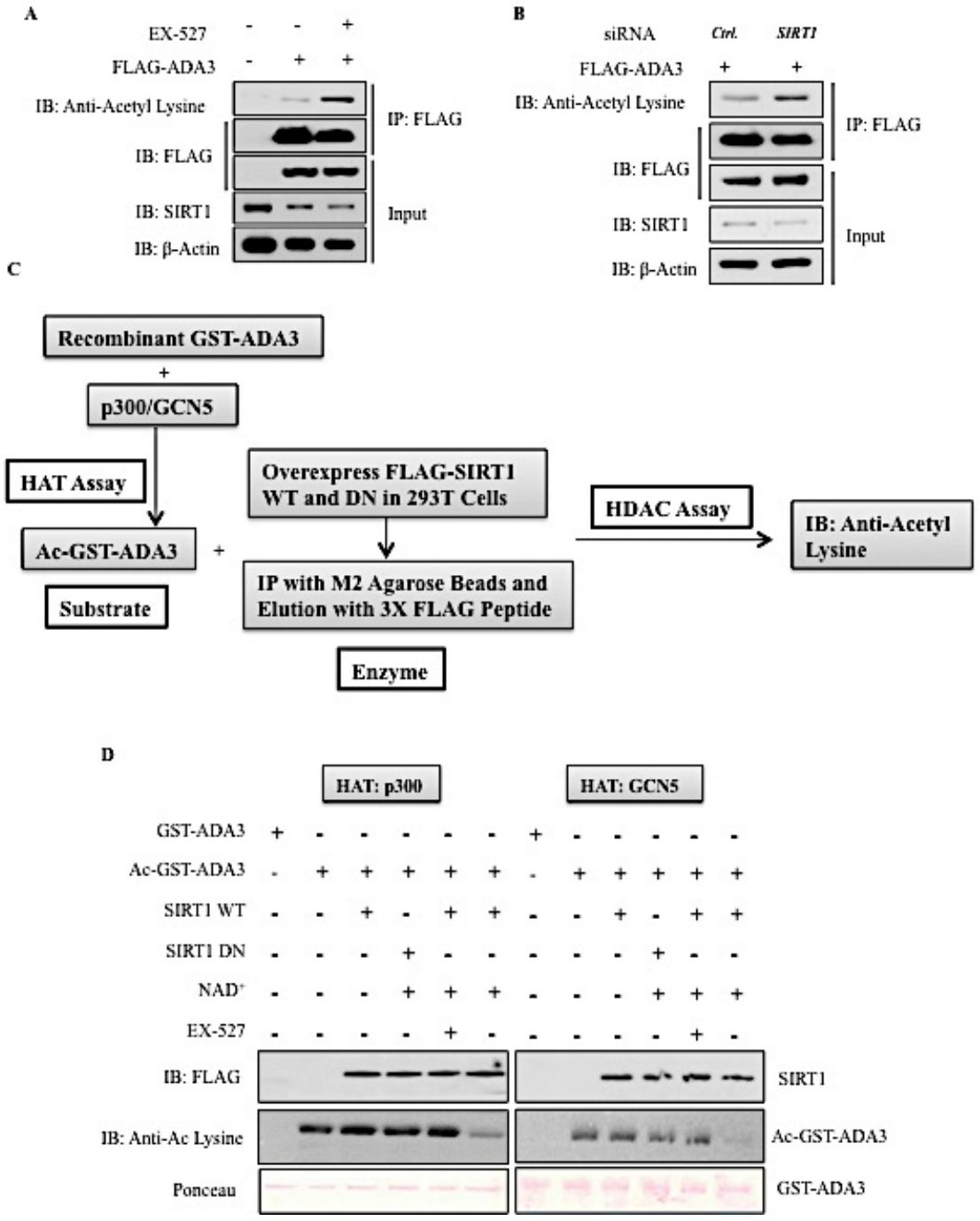


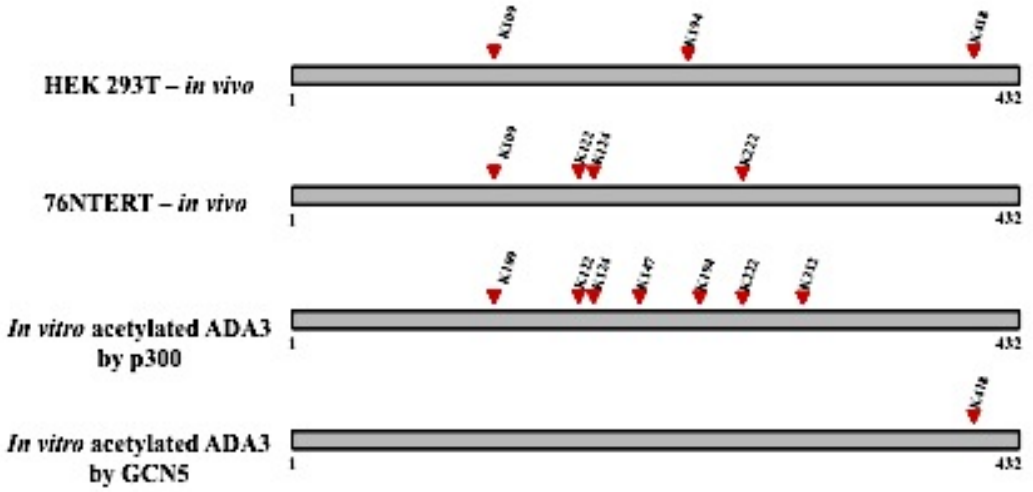
Figure 3.5 - SIRT1 deacetylates ADA3 in cells and *in vitro.* (A) HEK-293T cells were transfected with FLAG-ADA3 or empty vector. 30 h after transfection cells were treated either with DMSO or with 10 μM EX-527, a SIRT1 specific inhibitor, for 16 h. Whole cell lysates were then subjected to immunoprecipitation by M2 FLAG agarose beads and immunoblotted with either anti-acetyl lysine or FLAG-HRP antibodies **(B)** HEK-293T cells were co-transfected together with FLAG-ADA3 and control or siRNA against *SIRT1*. 48 h transfection, ADA3 acetylation was analyzed by immunoprecipitation followed by immunoblotting as in A. Whole cell extracts were also immunoblotted with anti-SIRT1 and anti-β-actin antibodies to examine *SIRT1* knockdown. **(C)** Schematic depicting the strategy used for *in vitro* deacetylation assay of ADA3 **(D)** Bacterially purified 1 μg ADA3 was *in-vitro* acetylated by either recombinant p300 HAT domain (25 ng) or recombinant GCN5 HAT domain (50 ng). Following this, acetylated ADA3, as a substrate, was subjected to *in-vitro* deacetylation assay. FLAG tagged SIRT1 wild-type or catalytic inactive mutant SIRT1 H363Y was overexpressed in HEK293T cells, immunoprecipitated by FLAG M2 agarose beads and eluted by 3X FLAG peptide. 20 ng of these elutes were used as enzyme in presence or absence of SIRT1 cofactor NAD^+ or SIRT1 inhibitor EX-527. Following the enzymatic reaction, the reaction mixtures were subjected to SDS PAGE and immunoblotted with anti-acetyl lysine antibody.

Mass spectrometry-based identification of lysine residues in ADA3 acetylated by GCN5 and p300

To delineate specific lysine residues in AD3 that are modified by acetylation by GCN5 or p300 in intact cells, we utilized a mass spectrometry approach. HEK-293T or 76N-TERT cells transiently or stably overexpressing FLAG-ADA3, respectively, were treated with TSA and NAM and FLAG-AD3 was purified using anti-FLAG beads. The anti-FLAG bead bound FLAG-ADA3 was eluted using a tandem triple FLAG peptide, resolved by SDS-PAGE, and the band corresponding to FLAG-ADA3 was excised and subjected to mass spectrometry analysis after digestion with trypsin or chymotrypsin. This analysis revealed three ADA3 acetylation sites in HEK293T cells (K109, K194 and K418) and four sites in 76NTERT cells (K109, K122, K124 and K222) with K109 site common in both cell lines (Figure 3.6A-J). To determine if any of these lysine residues were specifically acetylated by GCN5 or p300, we performed *in vitro* acetylation of purified recombinant GST-ADA3 with either GCN5 or p300, followed by mass spectrometric analysis after trypsin or chymotrypsin digestion. This analysis demonstrated that GCN5 acetylated a single lysine residue on ADA3 (K418) whereas p300 acetylated seven distinct lysine residues (K109, K122, K124, K147, K194, K222 and K312) (Figure 3.6A-J). Notably, all but two (K147 and K312) acetylation sites identified on *in vitro* acetylated ADA3 were observed in one of the two cell types examined, supporting the idea that the identified ADA3 lysine residues are bona fide acetylation sites. Notably, the identified lysine residues are preserved in all mammals, and a majority are conserved among vertebrates (Figure 3.6K), supporting a likely functional role of ADA3 acetylation.

Figure 3.6: Mass spectrometry-based identification of lysine residues in ADA3 acetylated by GCN5 and p300

A



B

Lysines colored in red were identified as acetylated lysines and the underlined sequences indicate the regions of ADA3 covered by mass spectrometry

(i)

Trypsin, TripleTOF: 67.1% sequence coverage

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PKSKNLQPKIQEYEFTDDPIDVPRIPKNDAPNRFWASVEPYCADITSEEVRTLEELLKPP
EDEAEHYKIPPLGKHYSQRWAQEDLLEEQKDGARAAAVADKKKGLMGPLTELDTKDVDAL
LKKSEAQHEQPEDGCPFGALTQRLLQALVEENIISPMEDSPIPDMSGKESGADGASTSPR
NQNKPFSVPHTKSLESRIKEELIAQGLLESEDRPAEDSEDEVLAELRKRQAELKALSAHN
RTKKHDLLRLAKEEVSRQELRQRVRMADNEVMDAFRKIMAARQKKRTPTKKEKDQAWKTL
KERESILKLLDG

(ii)

Chymotrypsin, TripleTOF: 73% sequence coverage

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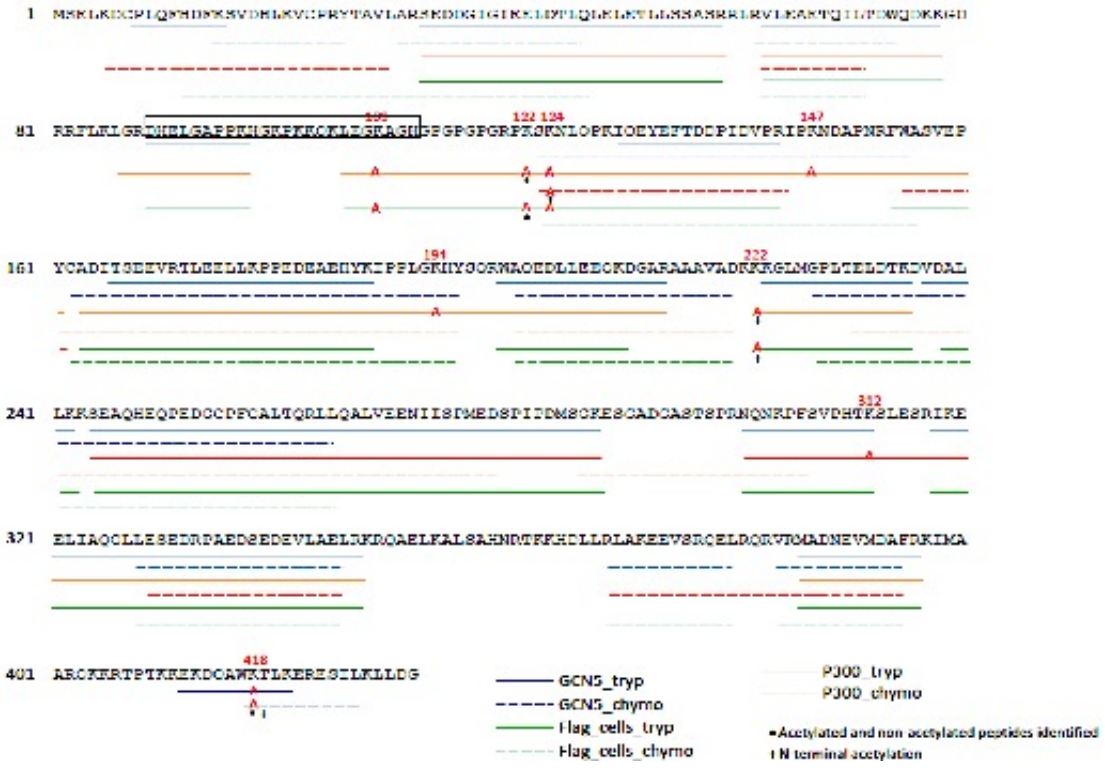
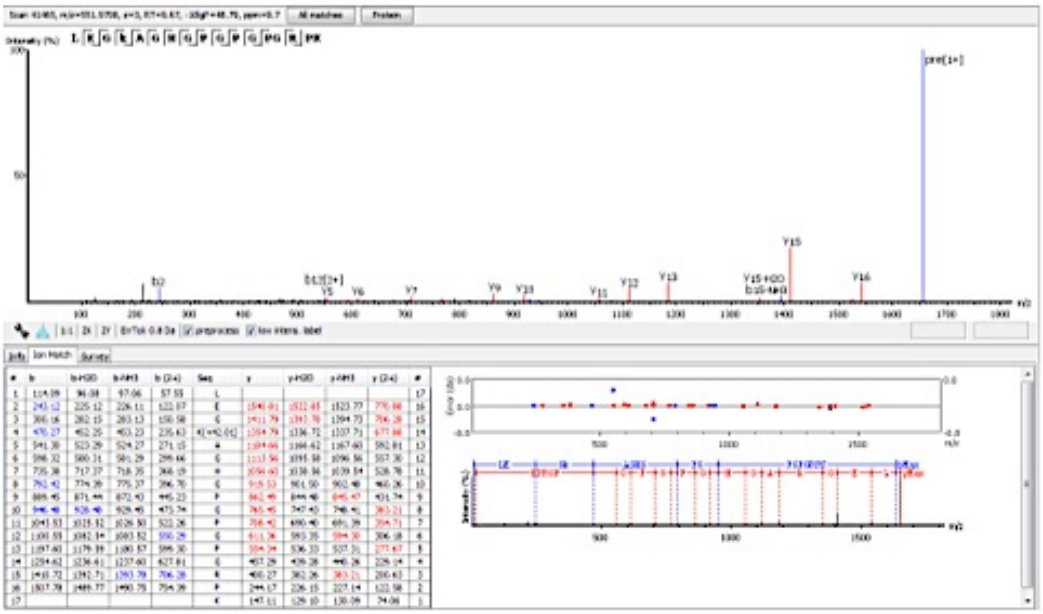

GCN5_tryp
GCN5_chymo
Flag_cells_tryp
Flag_cells_chymo
P300_tryp
P300_chymo
*Acetylated and non acetylated peptides identified
↑N terminal acetylation

D



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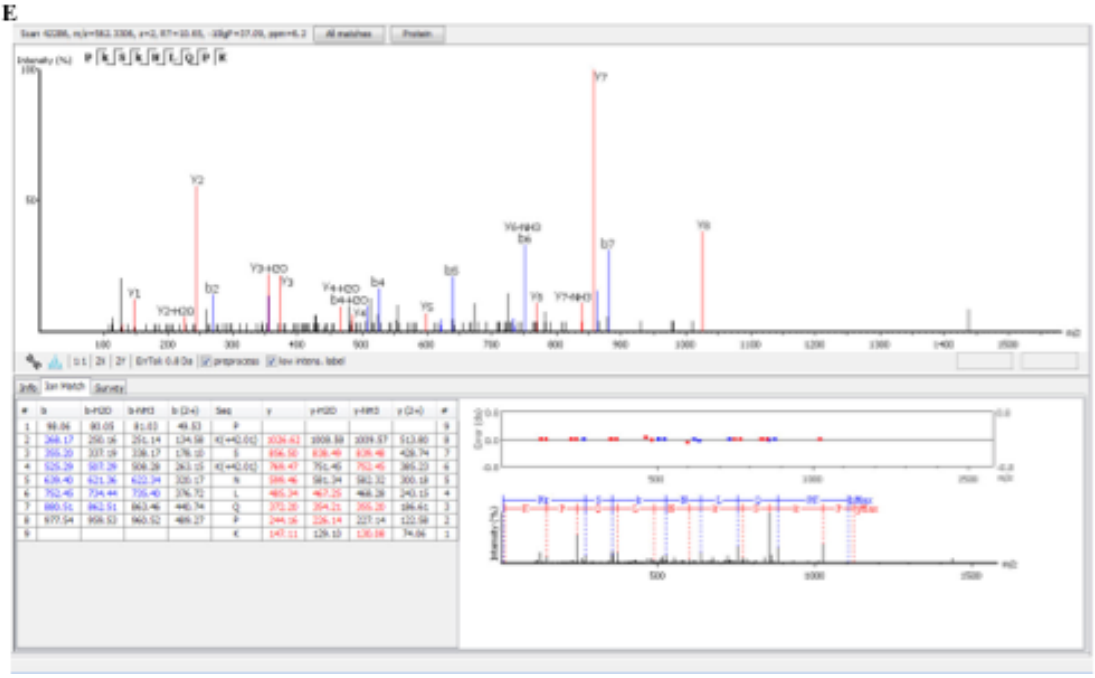

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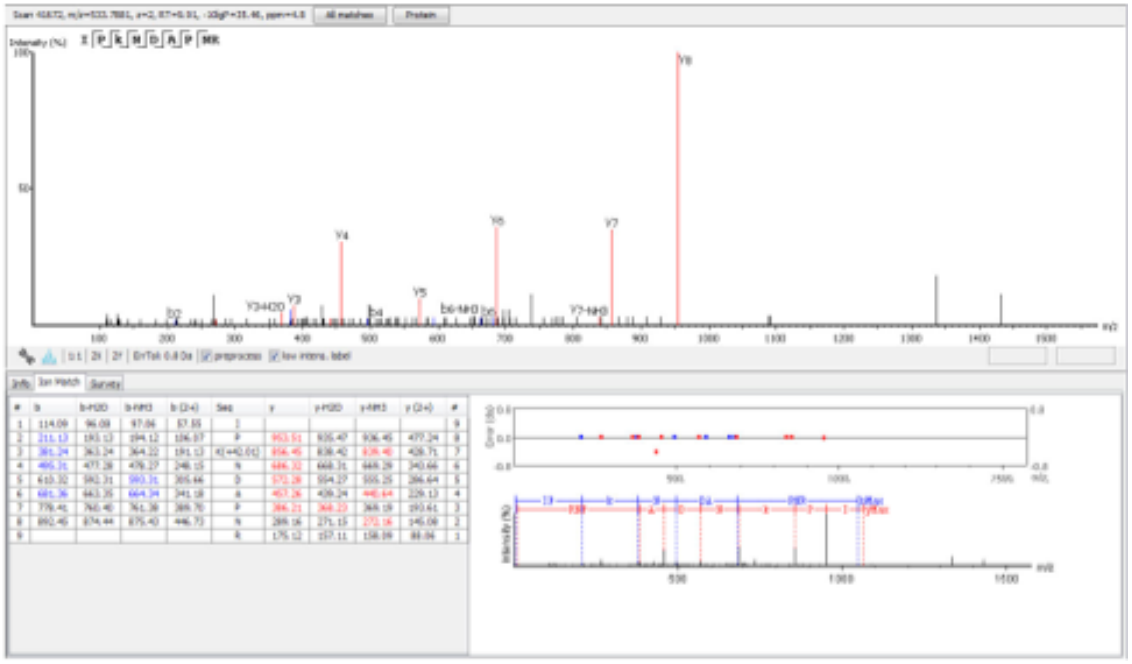

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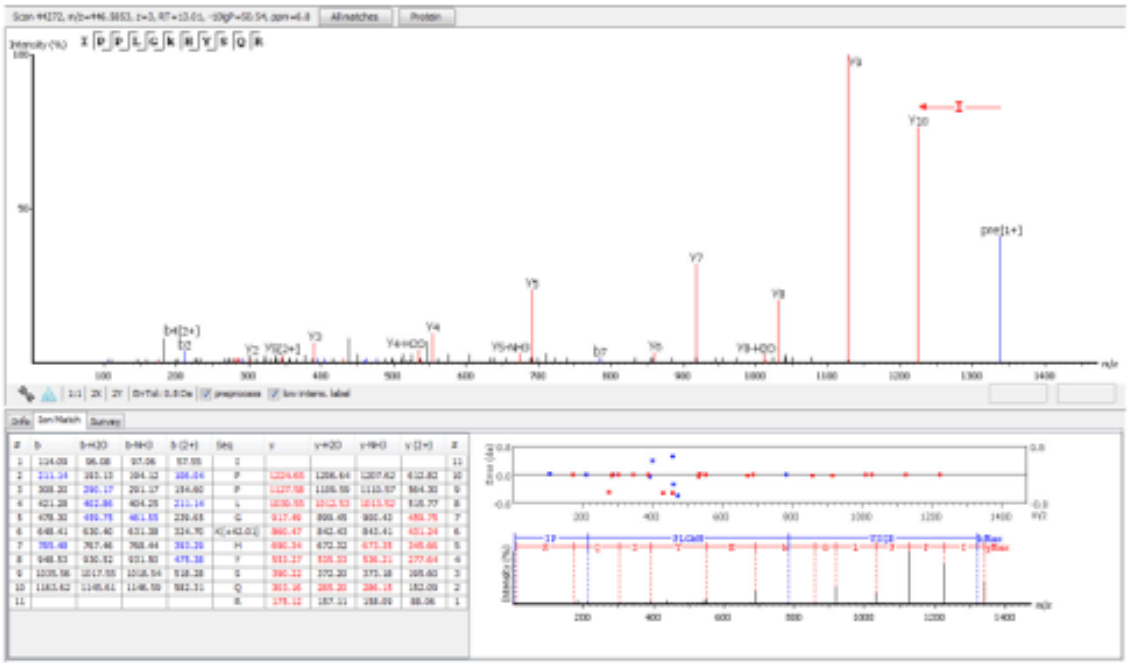

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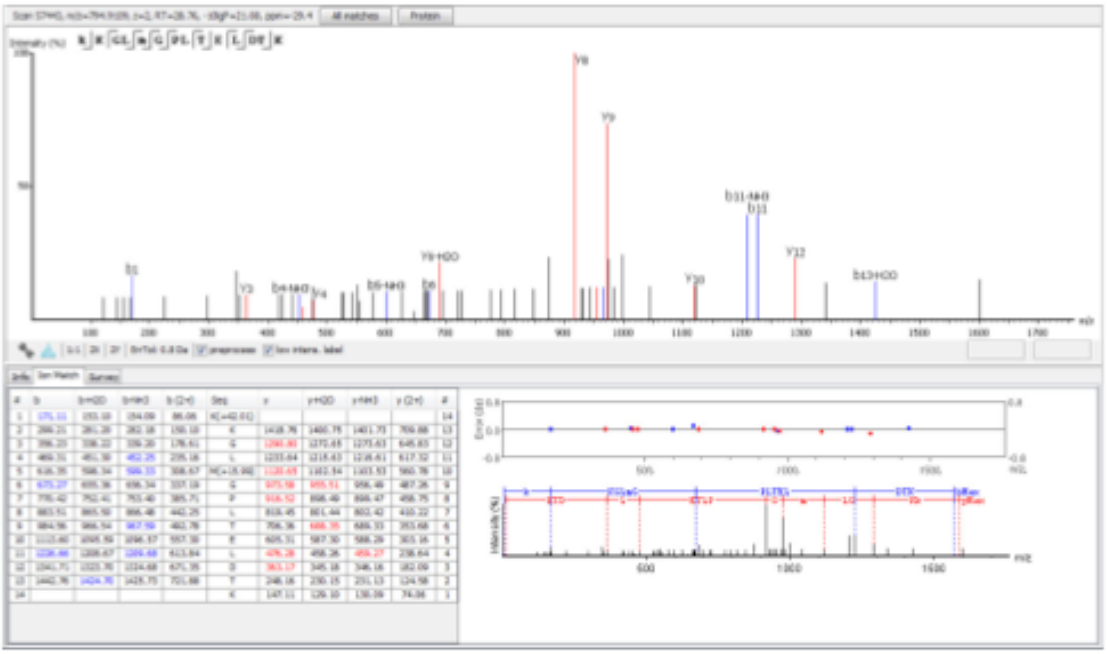

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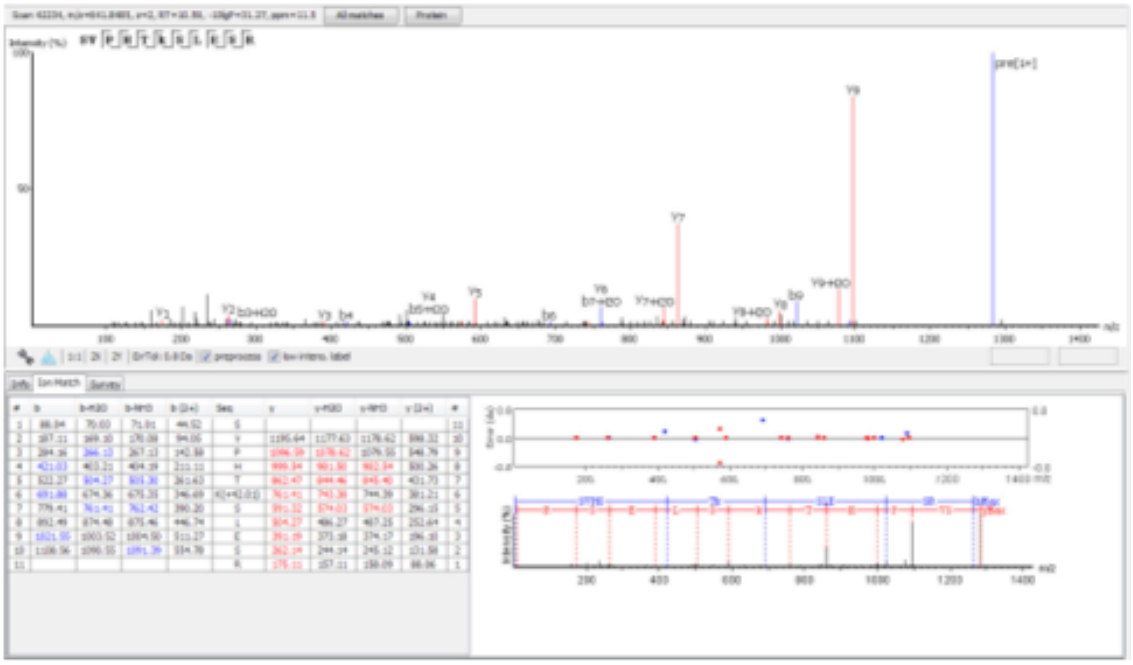

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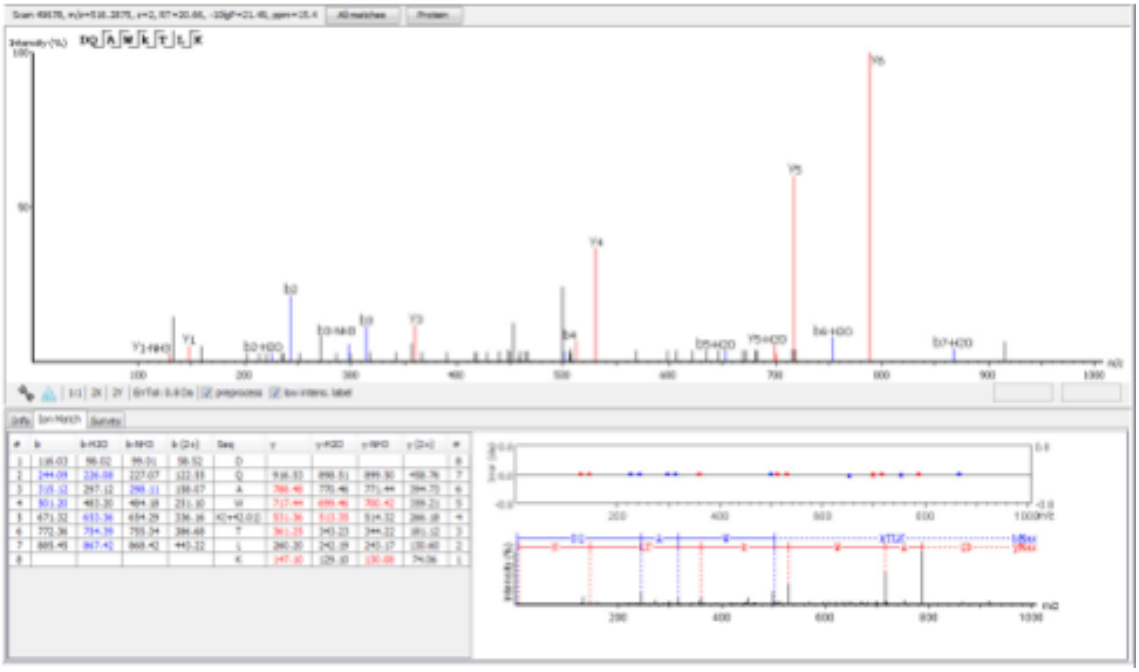


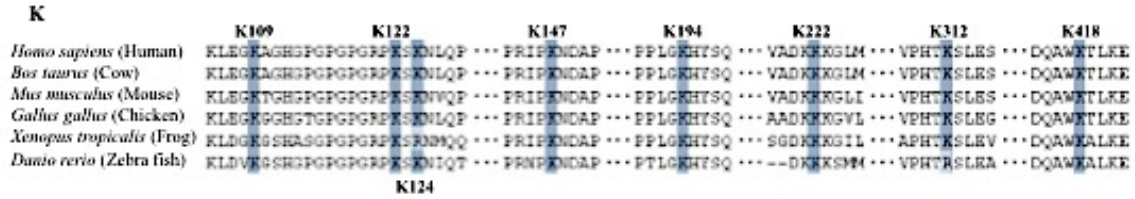


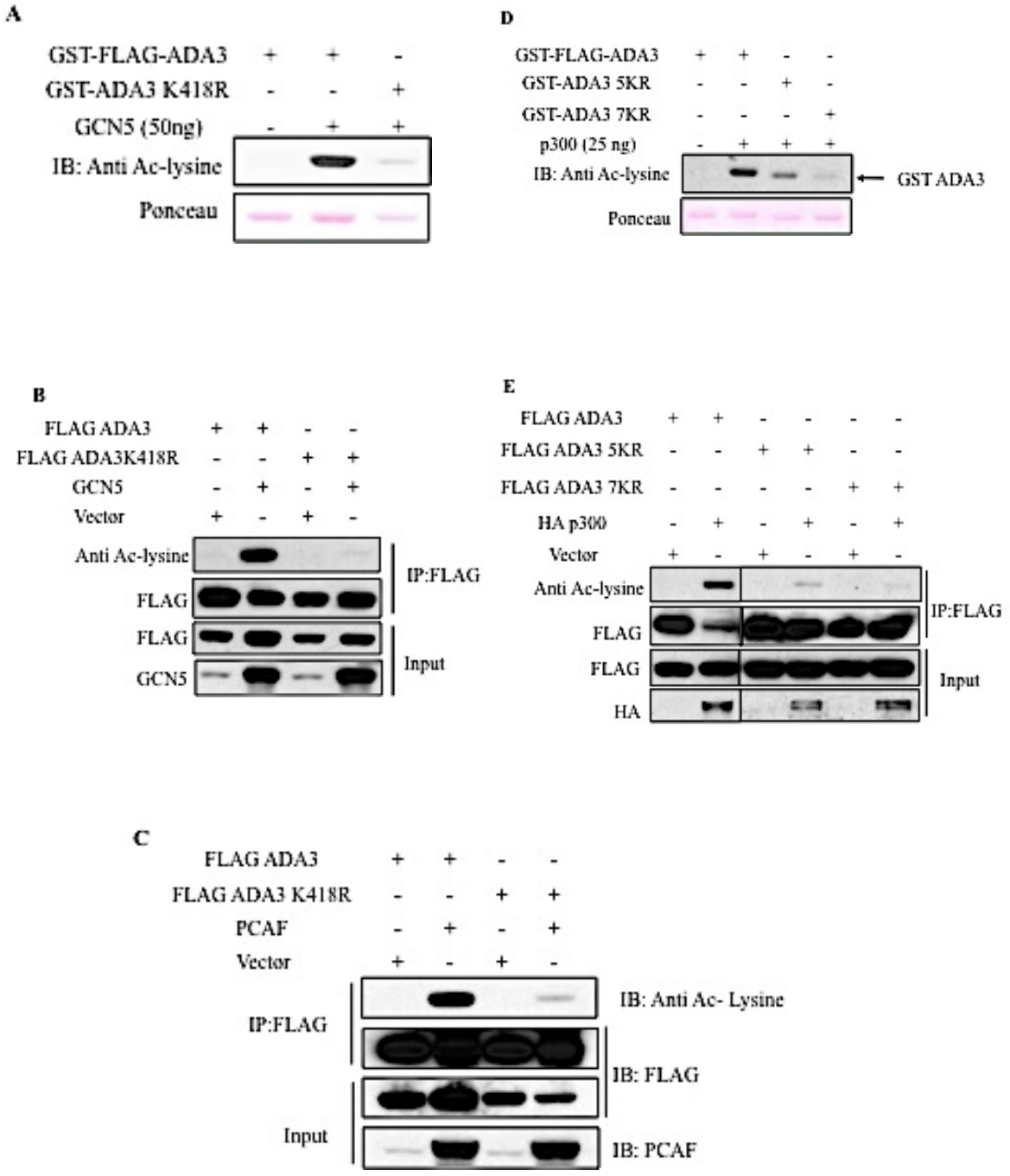
Figure 3.6 – Mass spectrometry-based identification and validation of lysine residues in ADA3 acetylated by GCN5 and p300. (A) Summary of various acetylation sites identified on ADA3 in various experimental settings, as indicated, using mass spectrometry. **(B & C)** Summary of coverage by mass spectrometry and acetylated lysines identified in ADA3 by mass spectrometry of both trypsin and chymotrypsin digested samples immunoprecpitated from (B) HEK293T, (C) 76NTERT or (C) *in vitro* acetylated by GCN5 or p300. (**D-J)** Identification of various acetylated lysine residues on ADA3 by mass spectrometry – (D) K109 (E) K122, K124 (F) K147 (G) K194 (H) K222 (I) K312 and (J) K418 **(K)** Sequence alignment of various ADA3 vertebrate sequences reveals high conservation of acetylated lysine residues.

Validation of GCN5- and p300-mediated acetylation sites on ADA3 *in vitro* and in intact cells

To further validate the GCN5- and p300-mediated acetylation sites on ADA3, we used site-directed mutagenesis to mutate various lysine (K) residues to arginine (R) residues, to preserve their positive charge but render them acetylation-incompetent. Based on results presented above, a single K418R substitution was made to assess the GCN5-mediated ADA3 acetylation while we made two overlapping mutants to assess the p300-mediated acetylation: (1) K>R mutation of K109, K122, K124, K194 and K222 (5KR), or (ii) K>R mutation of all seven lysine residues identified above (K109, 122, 124, 147, 194, 222, 312; denoted 7KR). These mutants were expressed as recombinant protein *in vitro* or in cells to assess their ability to serve as substrates of acetylation by GCN5 or p300. The *in vitro* acetylation assay demonstrated that K418 was the major site of acetylation by GCN5 as the level of acetylation upon incubation with GCN5 was greatly reduced compared to wild-type ADA3 (Figure 3.7A). When co-transfected in HEK-293T cells, GCN5 efficiently acetylated the wild-type ADA3 but not the K418R mutant (Figure 3.7B). A similar defect in the K418R mutant acetylation was observed with PCAF (Figure 3.7C). Taken together, these results demonstrate that both GCN5 and PCAF acetylate ADA3 at lysine 418. Next, we analyzed the 5KR and 7KR mutants of ADA3 *in vitro* to assess the impact on p300-mediated acetylation. The 5KR mutation of ADA3 greatly reduced its p300-mediated acetylation, whereas the 7KR mutation almost completely abrogated ADA3 acetylation (Figure 3.7D). These results were confirmed by co-expressing wild-type ADA3, ADA3-5KR or ADA3-7KR with HA-tagged p300 in HEK-293T cells (Figure 3.7E). Next, we generated an ADA3 mutant with K>R substitutions of all eight lysine residues (those acetylated by either GCN5 or p300; referred to as 8KR), and compared the level of acetylation of wild-type ADA3 vs. its K418R, 7KR and 8KR mutants expressed in HEK-293T cells upon treatment with TSA and NAM. Under these conditions, the 8KR mutant showed complete abrogation of acetylation (Figure 3.7F). Altogether

these results validate the eight lysine residues identified by mass spectrometry as the major acetylation sites on ADA3 that are modified by its interacting HATs.

Figure 3.7: Validation of GCN5- and p300-mediated acetylation sites on ADA3 *in vitro* and in intact cells



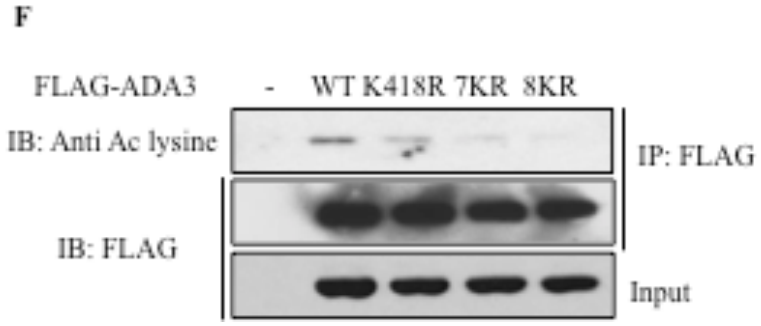


Figure 3.7: Validation of GCN5- and p300-mediated acetylation sites on ADA3 *in vitro* and in intact cells (A & D) *In-vitro* HAT assay was performed using GCN5 (A) or p300 (D) catalytic domains as acetyl transferases and recombinant GST-FLAG-ADA3, GST-ADA3K418R, -5KR or -7KR as substrates. The assay was followed by immunoblotting with anti-acetyl lysine antibody. **(B, C & E)** 293T cells were transfected with FLAG-ADA3 wild-type, -K418R, -5KR or -7KR with or without GCN5 (B), PCAF (C) and p300 (E). 48 h after transfection whole cell lysates were subjected to immunoprecipitation with M2 agarose FLAG beads and immunoblotted with indicated antibodies. **(F)** 293T cells were transfected with indicated plasmids. 42 h after transfection cells were treated with TSA (1 μM) and NAM (5 mM) for 6 h. Whole cell extracts were subjected to immunoprecipitation with M2 agarose. Immunoprecipitates were then eluted with 3X FLAG peptide and immunoblotted with indicated antibodies.

ADA3 acetylation levels change during cell cycle progression

Having demonstrated that ADA3 acetylation is dynamically regulated by its associated HATs (GCN5, PCAF and p300) and the deacetylase SIRT1, we asked if the levels of ADA3 acetylation are regulated under particular physiological conditions. Since we have established that ADA3 plays an important role in cell cycle progression (13, 23) we reasoned that its acetylation might vary with cell cycle progression. To assess if this is the case, we arrested 76N-TERT immortal mammary epithelial cells in G_1 phase of cell cycle by growth factor deprivation, released the cells from G_1 block by adding growth factor-containing medium and analyzed cells at various time points during cell cycle progression (based on FACS analysis) for levels of acetylation on immunoprecipitated ADA3 (26, 32). FACS analysis confirmed that a majority of cells were G_1-arrested upon growth factor deprivation followed by robust S and G2/M phase entry by 16 h and 20 h, respectively, after culture in growth factor rich medium (Figure 3.8A). Western blotting of whole cell extracts showed that total ADA3 levels remained relatively invariant during cell cycle progression (Figure 3.8B). As antibodies recognizing specific ADA3 acetylation sites are not available, ADA3 was immunoprecipitated from cell lysates followed by immunoblotting with pan acetyl-lysine reactive antibody. Compared to acetylation levels at time 0 (G_1-assrested cells), we observed an initial peak of ADA3 acetylation at 4 h followed by a second peak that persisted throughout S- and G_2/M phases (Figure 3.8C). Notably, these dynamic changes in ADA3 acetylation are consistent with previous reports of increase in GCN5 protein levels as cells enter the S-phase (33) and a requirement of the HAT activity of p300 for G_1-S transition (34-38). Taken together, our results demonstrate that ADA3 acetylation is cell cycle-dependent and raised the possibility that acetylation of ADA3 could regulate its function in cell cycle progression.

Figure 3.8: ADA3 acetylation levels change during cell cycle progression

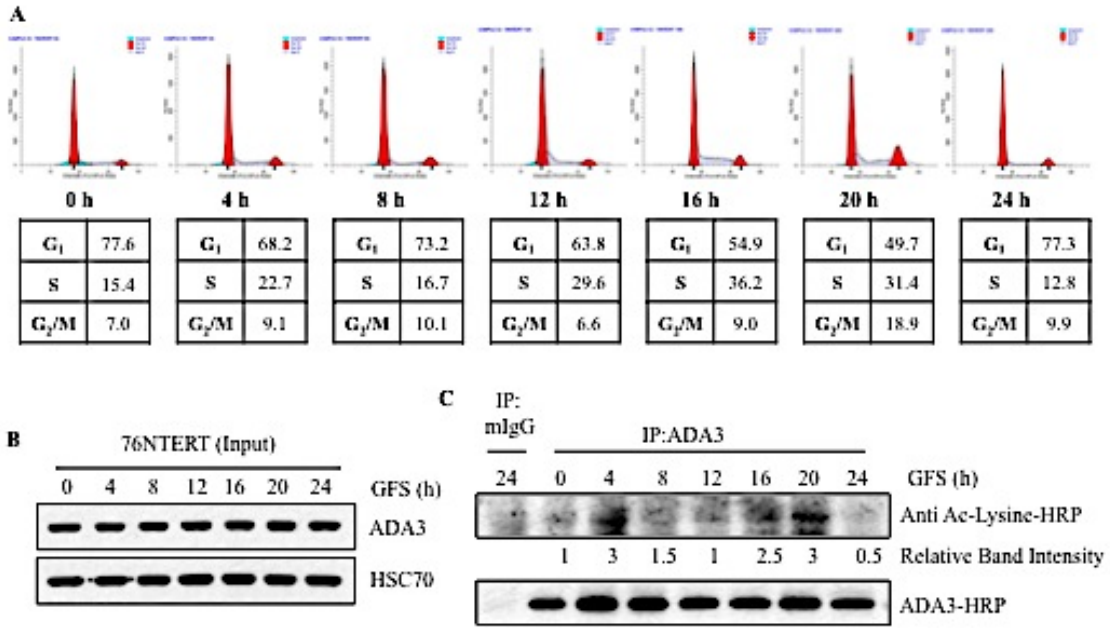


Figure 3.8 - ADA3 acetylation levels change during cell cycle progression. 76NTERT cells were growth factor deprived in DFCI-3 medium for 72 h and then stimulated with growth factors containing DFCI-1 medium for indicated time points. **A**. Cells were fixed in 70% ethanol, stained with propidium iodide and then subjected to FACS analysis. **B.** Whole cell extracts (Input) were immunoblotted either with anti-ADA3 or anti HSC70 antibodies. **C.** Equal amount of whole cell extracts from each time point were immunoprecipitated with normal mouse IgG or anti-ADA3 antibody. Immunoprecipitates were then immunoblotted with HRP labeled pan acetylated or anti-ADA3 antibodies. ADA3 acetylation band intensities were quantified using ImageJ software and normalized against 0 h which are represented underneath the blot.

Acetylation-defective mutants of AD3 fail to rescue the block in cell proliferation imposed by *Ada3* deletion in *Ada3*$^{FL/FL}$ MEFs

To investigate the functional importance of ADA3 acetylation, we assessed the ability of acetylation-defective ADA3 mutants vs. the wild-type (WT) ADA3 to rescue the block in cell proliferation and/or the defective histone acetylation observed upon induced *Ada3* deletion in *Ada3*$^{FL/FL}$ MEFs (13). For these experiments, we generated stable *Ada3*$^{FL/FL}$ MEF cell lines expressing either vector, WT FLAG-ADA3 or one of its acetylation-defective mutants, K418R, 7KR or 8KR. Western blotting confirmed that the expression of ectopic WT ADA3 or its mutants were comparable (Figure 3.9N). As expected (13), deletion of endogenous *Ada3* by adenovirus-Cre infection led to a severe proliferation defects in vector-expressing *Ada3*$^{FL/FL}$ MEFs, whereas cells expressing wild-type FLAG-ADA3 showed unperturbed cell proliferation demonstrating a functional rescue (Figure 3.9A and B). Notably, partial proliferation defects were seen upon endogenous *Ada3* deletion in cells expressing K418R or 7KR mutants (Figure 3.9C and D). More significantly, a severe proliferative block was seen upon *Ada3* deletion in MEFs expressing the 8KR mutant, suggesting that both GCN5- and p300- mediated acetylation is important for ADA3 function in cell proliferation (Figure 3.9E).

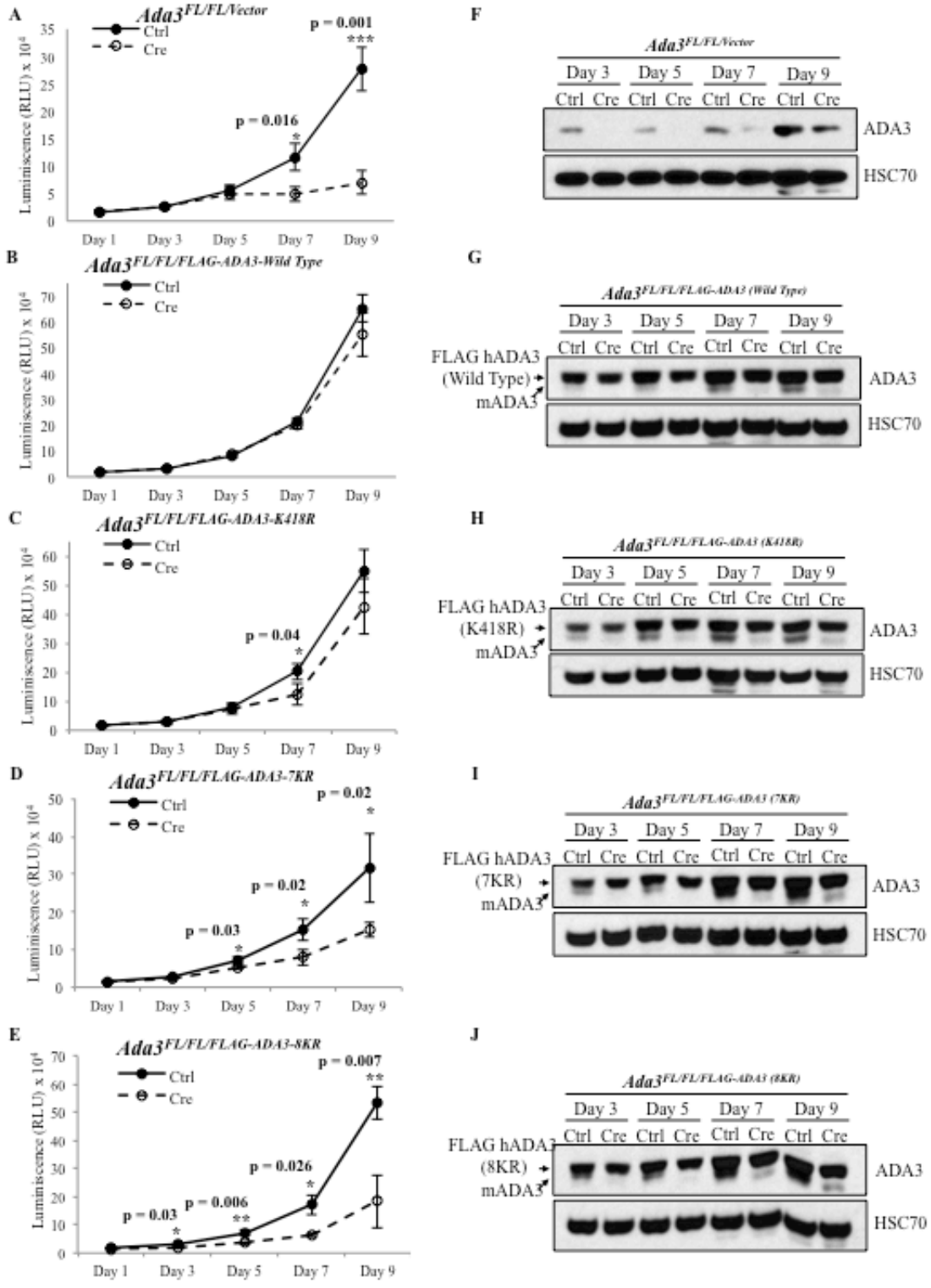
We confirmed the deletion of endogenous mouse *Ada3* in the Adeno-Cre transduced MEFs by immunoblotting at various time points (Figures 3.9F-J). While, a significant endogenous ADA3 depletion was seen in each cell line until Day 7; the recovery in the expression of endogenous mouse ADA3 at Day 9 reflects the outgrowth of cells in which *Ada3* was not deleted. We calculated the percentage rescue in proliferation (see materials and methods) for WT ADA3 or each mutant at various time points after Adeno-Cre transduction (Figure 3.9K). A significant defect in the rescue of cells from proliferation block was observed with each acetylation-defective ADA3 mutant compared to WT ADA3 at Day 7 after *Ada3* deletion (Figure 3.9K). At Day 9, the defect in rescue with K418R or 7KR was not significant but was significant

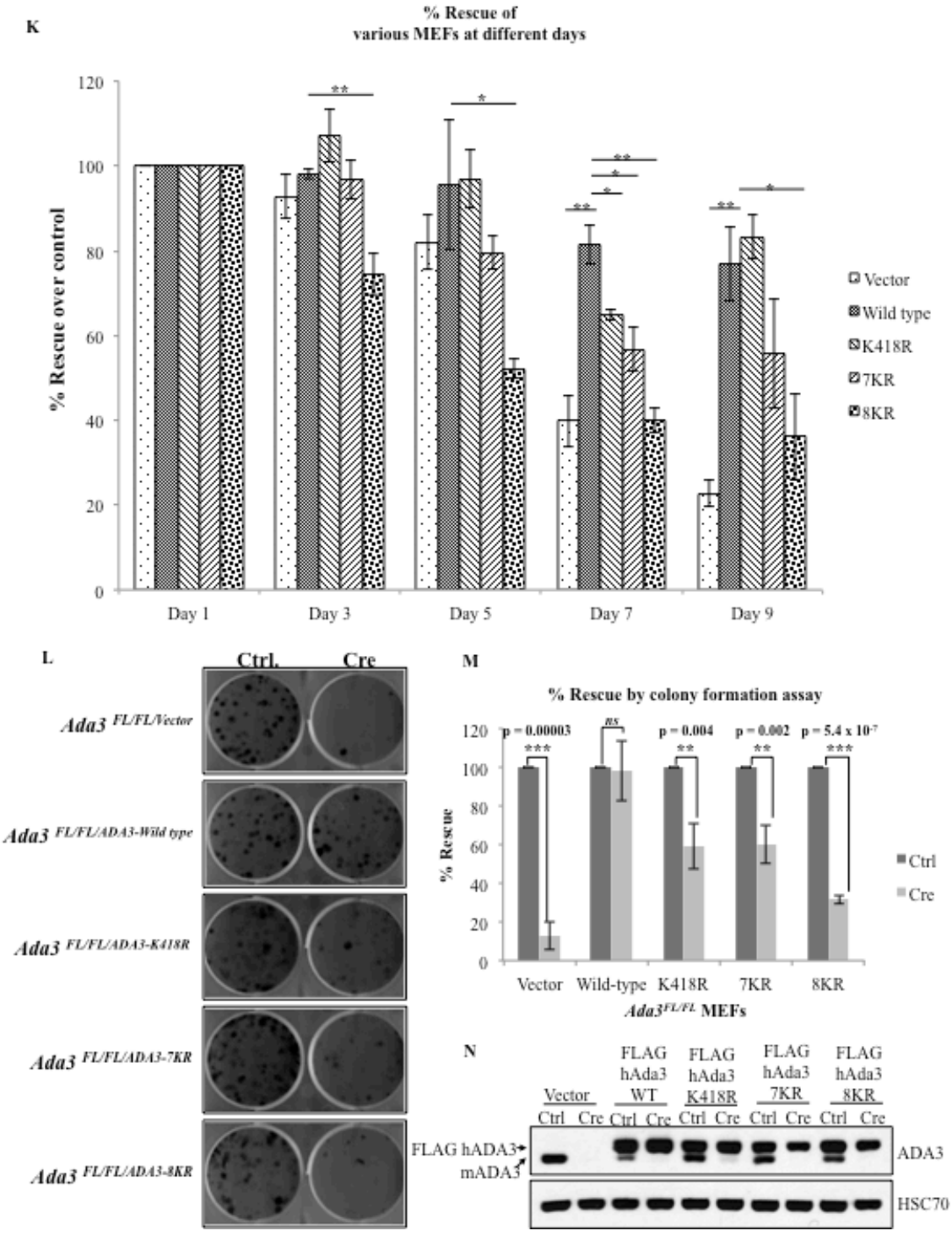
with the 8KR mutant as also for the vector-alone expressing cells (Figure 3.9K), likely due to the outgrowth of MEFs in which endogenous *Ada3* was not deleted, as suggested by the results of Western blotting (Figure 3.9H & I).

We also observed similar defect in the ability of mutant ADA3 proteins to rescue proliferation defects in *Ada3*-deleted MEFs using an independent colony formation assay (Figure 3.8L & M). In this assay, while vector-expressing cells showed about 90% defect in rescue, both ADA3 K418R- and 7KR-expressing cells showed about 40% defect, whereas 8KR showed about 70% defect in rescue. Taken together, we demonstrate that both GCN5- and p300-mediated acetylation of ADA3 is required for its function in cell proliferation.

To test the possibility that the observed defect in cell cycle rescue is due to the mislocalization of mutants, we analyzed the localization of WT and acetylation-defective mutants 5 days after control or Cre adenovirus infection of $Ada3^{FL/FL}$ MEFs expressing these mutants. As expected, the endogenous ADA3 in vector control cells was exclusively localized to the nucleus; however, the overexpressed WT ADA3 was localized to both nucleus and cytoplasm even after deletion of endogenous ADA3 (Figure 3.9O). Similar to WT ADA3, all acetylation-defective mutants of ADA3 were present in both the nucleus and cytoplasm (Figure 3.9O). Thus, the acetylation defective mutants are competent at nuclear entry, excluding the possibility that their defective ability to rescue ADA3-depleted MEFs from a proliferation block might be due to exclusion from the nucleus. Taken together, our results conclusively show that acetylation of ADA3 is pivotal for its function to promote cell cycle progression.

Figure 3.9 - Acetylation-defective mutants of AD3 fail to rescue the block in cell proliferation imposed by *Ada3* deletion in *Ada3*$^{FL/FL}$ MEFs.




K
% Rescue of
various MEFs at different days
% Rescue over control
120
100
80
60
40
20
0
Day 1
Day 3
Day 5
Day 7
Day 9
Vector
Wild type
K418R
7KR
8KR
L
Ctrl.
Cre
Ada3 FL/FL/Vector
Ada3 FL/FL/ADA3-Wild type
Ada3 FL/FL/ADA3-K418R
Ada3 FL/FL/ADA3-7KR
Ada3 FL/FL/ADA3-8KR
M
% Rescue by colony formation assay
p = 0.00003
ns
p = 0.004
p = 0.002
p = 5.4 x 10-7
% Rescue
Vector
Wild-type
K418R
7KR
8KR
Ada3FL/FL MEFs
Ctrl
Cre
N
FLAG hAda3
Vector
WT
K418R
7KR
8KR
Ctrl
Cre
FLAG hADA3
mADA3
ADA3
HSC70

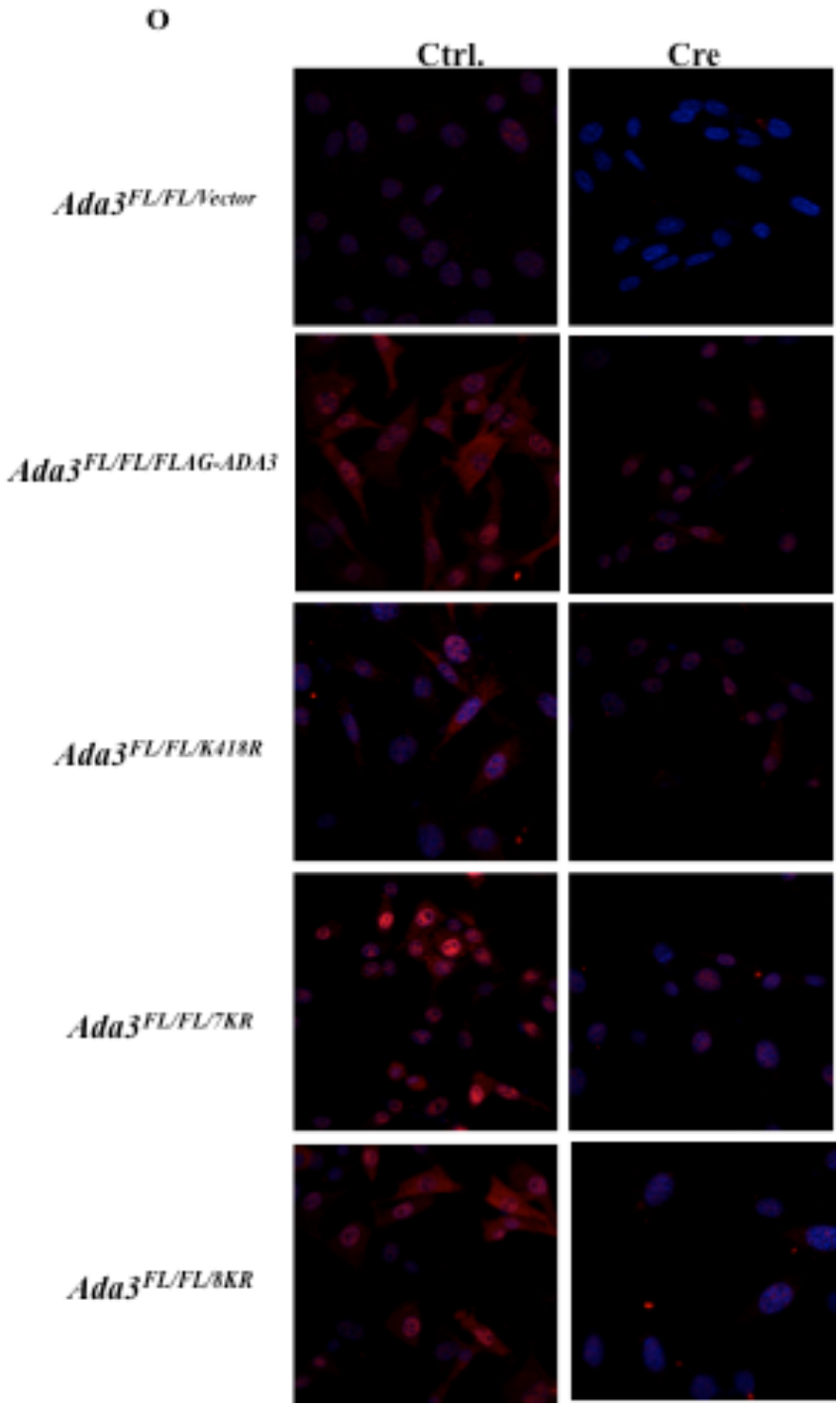

O
Ctrl.
Cre
Ada3FL/FL/Vector
Ada3FL/FL/FLAG-ADA3
Ada3FL/FL/K418R
Ada3FL/FL/7KR
Ada3FL/FL/8KR

Figure 3.9 - Acetylation-defective mutants of AD3 fail to rescue the block in cell proliferation imposed by *Ada3* deletion in *Ada3*$^{FL/FL}$ MEFs. *Ada3*$^{Fl/FL}$MEFs stably expressing either empty vector, *ADA3 WT* or acetylation defective mutants were infected either with only GFP or GFP-Cre expressing adenovirus followed by CellTitre-Glo, western blotting or colony formation assays were performed in these cells. **(A-E)** Relative luminescence units of various *Ada3*$^{FL/FL}$MEFs at various days after the infection are shown. RLU shown here are mean ± S.D. from three independent experiments each done in three replicates and *p* values were computed using Student's *t* test. **(F-J)** Western blots showing the expression of mADA3 and hADA3 WT or mutant proteins. HSC70 was used as a loading control. **K**. A composite computation of % rescue with respect to day 1 **(L-N)** Colony formation assay was performed 7 days after the infection and % rescue was calculated as described in Materials and Methods. Expression of mADA3 and hADA3 WT or mutants was examined by western blotting also with HSC70 as a loading control. **O.** *Ada3* deleted MEFs stably expressing ADA3 WT or various acetylation defective mutants were fixed in 4% PFA and immunostained with DAPI and anti-ADA3.

Acetylation defective mutants retain the ability to interact with various HATs and other HAT complex components, yet fail to rescue the histone acetylation defects globally and at *c-Myc* enhancer

Endogenous *Ada3* deletion in *Ada3*$^{FL/FL}$ MEFs leads to defects in global histone acetylation and a reduction in the levels of various HATs, and these defects can be rescued by ectopic WT human *ADA3* (13). As defective histone acetylation is likely to be the basis for functional defects associated with ADA3 depletion, we reasoned that the functional impairment of acetylation-defective ADA3 mutants may arise from impaired histone acetylation due either to their inability to help assemble the HAT modules or to promote HAT activity. To test these possibilities, WT FLAG-ADA3 or its acetylation-defective mutants expressed in HEK-293T cells were examined for their associations with endogenous HATs and other HAT complex components by co-immunoprecipitation. Notably, all three acetylation defective mutants were as efficient as wild-type ADA3 in their ability to co-immunoprecipitate endogenous GCN5, PCAF or p300 (Figure 3.10A & B). In addition, these mutants retained a strong association with other STAGA complex subunits TRRAP and ADA2b, and the ATAC-specific subunit ADA2a (Figure 3.10A). Importantly, while the vector cells showed dramatic decrease in the levels of various HATs upon *Ada3* deletion, the HAT levels remained unaltered in cells reconstituted with acetylation-defective ADA3 mutants, similar to those expressing the WT ADA3 (Figure 3.10C). These results ruled out the possibility that acetylation-defective mutants may be defective in their assembly into HAT complexes or have diminished interaction with HATs.

Next, we examined if the HAT complexes formed by the acetylation-defective ADA3 mutants are functionally active by assessing global chromatin histone acetylation. As expected from our previous study (13), a dramatic decrease in H3K9 and H3K56 acetylation was observed upon *Ada3* deletion in *Ada3*$^{FL/FL}$ cells. However, this defect was rescued by exogenous WT ADA3 (Figure 3.10D). Notably, the acetylation-defective mutants ADA3-K418R and ADA3-

7KR showed only approximately 50% rescue of global H3K9 acetylation in comparison with wild-type ADA3 (Figure 3.10D), similar to the level of rescue of the proliferation block (about 60%) with these mutants. While ADA3 K418R and 7KR mutants did not show any significant defect in the rescue of global H3K56 acetylation, the 8KR mutant showed about 70% defect in the rescue of both H3K9 and H3K56 acetylation (Figure 3.10D), again comparable to the deficit in its ability to rescue the proliferation block (about 70%).

Given that H3K9 acetylation mark is important in gene transcription and is known to be present at active gene enhancers/promoters (39), we used chromatin immunoprecipitation (ChIP) to examine the relative rescue of the locus-specific H3K9 acetylation by WT vs. acetylation-defective ADA3 mutants in *Ada3*$^{FL/FL}$ MEFs subjected to deletion of the endogenous *Ada3*. We chose *c-Myc* enhancer to assess the H3K9 acetylation status, as c-Myc is indispensable for cell proliferation and is a known target of ADA3 containing HAT complexes (13, 15, 18). We observed about a four-fold enrichment in H3K9 acetylation at the *c-Myc* enhancer in WT ADA3-expressing *Ada3*-deleted MEFs as compared to those expressing the vector control (Figure 3.10E). By contrast, H3K9 acetylation at the *c-Myc* enhancer was substantially less robust in cells reconstituted with ADA3 K418R or 7KR mutants, while acetylation levels in cells expressing the 8KR mutant were essentially comparable to vector control cells (Figure 3.10E). Taken together these findings support the notion that ADA3 acetylation is essential for global and gene-specific histone acetylation by ADA3-containing HAT complexes, and that this activity, independent of ADA3's role to facilitate the assembly of HAT complexes, may be key to ADA3's role in cell cycle progression through histone acetylation at proliferation-associated genes such as *c-Myc* (Figure 3.10F).

Figure 3.10 Acetylation defective mutants retain the ability to interact with various HATs and other HAT complex components, yet fail to rescue the histone acetylation defects globally and at *c-Myc* enhancer

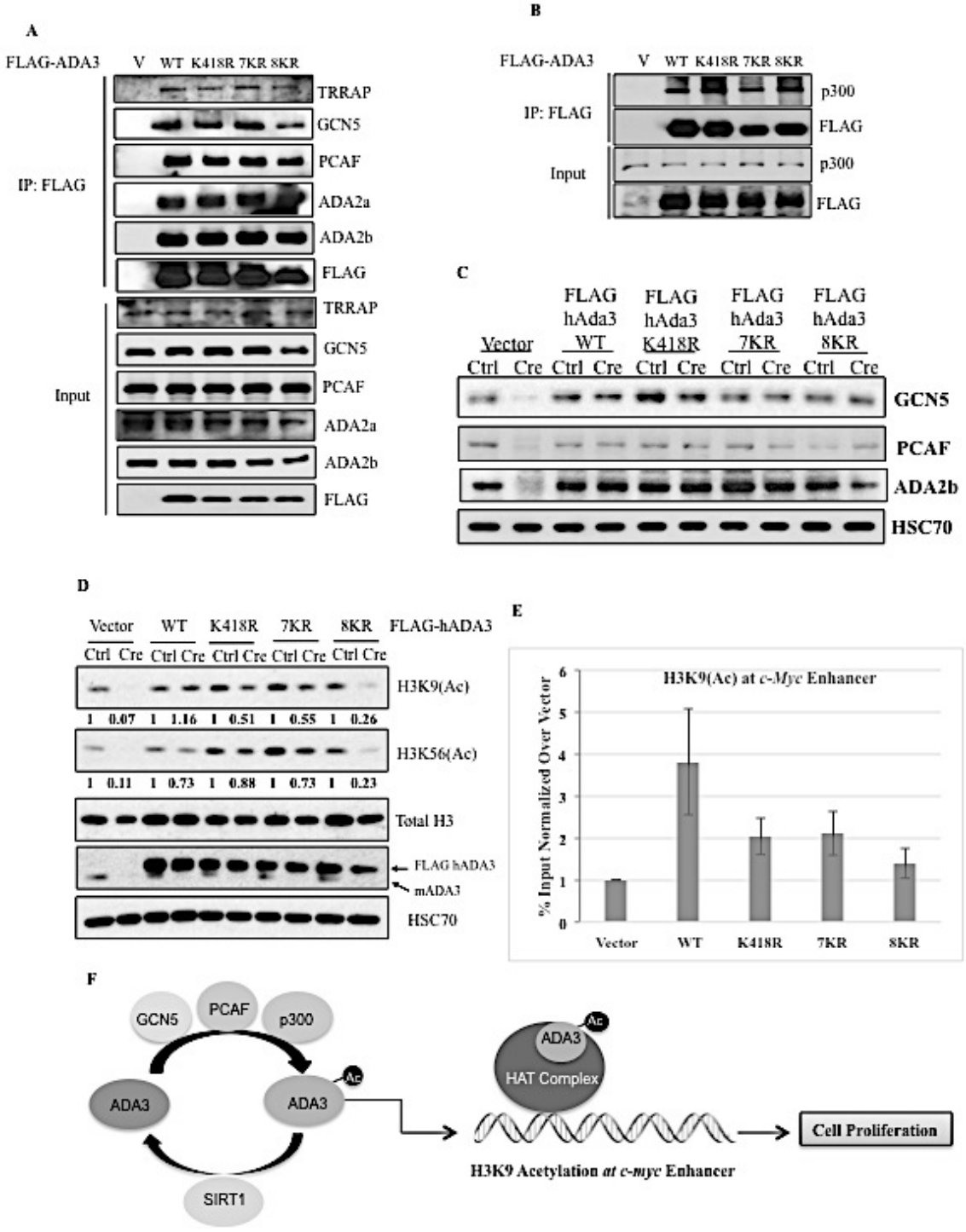


Figure 3.10: Acetylation defective mutants retain the ability to interact with various HATs and other HAT complex components, yet fail to rescue the histone acetylation defects globally and at *c-Myc* enhancer. (A) HEK-293T cells were transfected with empty vector, ADA3 WT or various acetylation defective mutants. 48 h after transfection whole cell extracts were subjected to immunoprecipitation by M2 agarose FLAG beads, eluted with 3X FLAG peptide and then immunoblotted with indicated antibodies. **(B)** HEK-293T cells were transfected with empty vector, ADA3 WT or various acetylation defective mutants. 48 h after transfection whole cell extracts were subjected to immunoprecipitation by M2 agarose FLAG beads and immunoblotted with indicated antibodies. **(C)** Whole cell lysates of Day 5 from cell cycle rescue experiment were immunoblotted anti-GCN5, -PCAF, -ADA2b or HSC70 antibodies. **(D)** Whole cell lysates of Day 7 from cell cycle rescue experiment were immunoblotted with indicated antibodies. The numbers underneath the blots indicate the band intensities computed from imageJ normalized over total H3 with respect to Ctrl **(E)** A ChIP quantitative PCR of H3K9(Ac) signals at *c-Myc* enhancer in *Ada3* deleted MEFs overexpressing *ADA3 WT or* various acetylation defective mutants. Y-axis shows enrichment as % of input normalized over signals in vector cells. Data represents the mean $\pm$ SD of three different experiments **(F)** Model showing the role of ADA3 acetylation in cell proliferation.

Discussion

ADA3 is an evolutionarily-conserved protein that functions as a transcriptional co-activator and forms a core component of the multi-subunit HAT complexes (3). Previous studies by us and others have shown that ADA3 associates with GCN5, PCAF and p300, HATs found in ADA3-containing complexes (8-10). Thus, ADA3 is ideally positioned to regulate the function of its associated HATs. How ADA3 carries out its function in this regard is unknown. In a previous study (13), we noted that ADA3 could be acetylated by p300 *in vitro* raising the possibility that acetylation could regulate ADA3 function in cells. The present study, for the first time, establishes that ADA3 is dynamically regulated by acetylation mediated by its associated HATs GCN5, PCAF and p300 and deacetylation by SIRT1; that ADA3 acetylation is required for the histone-modifying activity of ADA3-containing HAT complexes and that ADA3 acetylation is essential for its function in cell cycle progression.

To gain more insights into ADA3 acetylation, we used a mass spectrometry approach to define a single lysine residue, K418, that is acetylated by GCN5 (and PCAF), and seven distinct lysine residues (K109, -122, -124, -147, -194, -222 and -312) that can be acetylated by p300 (Figure 3.6A). Using site-directed mutagenesis followed by *in vitro* acetylation assays or expression in cells, we validated the lysine residues in ADA3 identified through proteomics to be the major sites of acetylation. As p300 is not an integral component of the HAT modules of STAGA or ATAC complexes (3), the seven distinct lysine residues acetylated by p300 are of considerable interest, suggesting that either ADA3 serves to recruit p300 as an accessory HAT into these complexes or that ADA3 functions together with p300 in a STAGA/ATAC-independent manner. Our previous biochemical fractionation analyses (8), which showed that both GCN5 and p300 could be purified as components of ADA3-containing complexes in human cells support the former possibility, although more in depth analyses will be needed to determine if one or both of these models are operational in mammalian cells.

The K>R point mutants of the GCN5-targetd, p300-targeted or both sets of lysine residues allowed us to determine the functional importance of ADA3 acetylation. Our previously established *Ada3*$^{FL/FL}$ MEFs provided a system where any functional deficits of the K>R mutants could be established by assessing their abilities to rescue the cells from a proliferative block and associated biochemical defects upon Cre-induced deletion of endogenous mouse *Ada3* (13). Importantly, analyses of cell proliferation or colony-forming ability showed that mutation of either GCN5-dependent (K418R) or p300-dependent (7KR) acetylation sites led to partial deficits in ability of the mutants to complement the loss of endogenous ADA3, with mutations at both the GCN5- and p300-mediated acetylation sites, essentially abrogating the ability of ADA3 to sustain cell proliferation (Figure 3.9K & M). These results underscore the critical functional importance of ADA3 acetylation in its function as a component of HAT complexes. The functional importance of ADA3 acetylation by two distinct HATs may reflect the possibility that acetylation by the two HATs occurs at discrete steps during cell proliferation or regulates discrete functional activities that are part of the complex process of cell proliferation. Notably, a previous study showed that ADA3-associated ATAC complex and p300 regulate the expression of distinct set of genes (40).

Previously, we have established that deletion of ADA3 causes a dramatic deficit in global H3K9 and H3K56 acetylation on chromatin (13). By further analyzing the K>R mutants of ADA3 in the context of endogenous *Ada3* deletion in MEFs, we established that ADA3 acetylation is required for the role of ADA3 in promoting global as well as locus-specific acetylation of chromatin-associated histones. Notably, while mutations of the GCN5-dependent acetylation site (K418R) or p300-dependent acetylation sites (7KR) led to a significant deficit in global H3K9 acetylation in comparison with wild-type ADA3 (Figure 3.10D), these mutants did not affect the global H3K56 acetylation; on the other hand, the 8KR mutant, which eliminates acetylation by both GCN5 and p300, showed about 70% defect in the rescue of H3K9 and H3K56 acetylation (Figure 3.10D). The extent of the deficit in histone acetylation correlated with the

extent to which the corresponding mutants were defective in cell cycle rescue in *Ada3*-deleted MEFs (Figure 3.9K & M). Furthermore, analysis of *c-Myc* enhancer associated histone acetylation showed that ADA3 is key to chromatin modification at key genes that function as master controllers of cell cycle progression and other functions.

That ADA3 acetylation is critical for HAT complexes to promote global and locus-specific histone acetylation could arguably arise from a defect in the ability of mutant ADA3 proteins to be assembled into HAT complexes. By examining the association of the ADA3 K>R mutants used in our biochemical and functional analyses with components of HAT complexes, we established that acetylation of ADA3 is dispensable for its association with HATs (p300, GCN5 and PCAF) and other components that define major ADA3-containing HAT complexes such as STAGA and ATAC (Figure 3.10 A&B). Thus, the requirement of ADA3 acetylation to promote histone acetylation and cell cycle progression does not reflect the requirement of such acetylation in HAT assembly, but must reflect a discrete function. The important question remains is how ADA3 acetylation regulates HAT complex activity despite the fact that it does not affect the overall composition of the complex. One possibility is while the acetylation of ADA3 is not required for its association with HATs/HAT complex components, it remains possible that subunits are not incorporated in correct stoichiometry leading to defect in the overall activity of the complex. Alternatively, ADA3 acetylation might play an important role in chromatin recognition. Future biochemical studies are warranted to better understand the role of ADA3 acetylation in this regard.

Notably, detection of ADA3 acetylation in cells was facilitated by the incorporation of HDAC inhibitors (Figure 3.1A & B), suggesting the possibility that ADA3 acetylation status is bi-directionally-regulated by the action of HATs and deacetylases. By screening the members of various HDAC families for their interaction with ADA3, we identify SIRT1 as an ADA3 partner and establish that it helps regulate the low steady state level of ADA3 acetylation (Figure 3.4A). That ADA3 acetylation is determined by opposing actions of HATs and a deacetylase strongly

supports the potential importance of this post-translational modification in the functional regulation of ADA3-containing HATs. Our *in vitro* deacetylation assays (Figure 3.5D) showed that SIRT1 was able to deacetylate ADA3 regardless of whether ADA3 was acetylated by p300 or GCN5. Future biochemical and cell-based studies are needed to further establish if SIRT1 is indeed a global deacetylase for ADA3 or if other deacetylases may be involved under specific scenarios. The functional consequence of ADA3-SIRT1 interaction could be manifold. A recent study showed that STAGA complex DUB module component USP22 associates with and is deacetylated by SIRT1 (41). However, the study did not identify any direct SIRT1-binding partner in STAGA complex. It remains possible that ADA3-mediated recruitment of SIRT1, aside from ADA3 deacetylation, also promotes deacetylation of USP22 in the DUB module, allowing ADA3 to indirectly regulate the function of HAT complexes.

Our studies also suggest that the balance of ADA3 acetylation vs. deacetylation in cells is a regulated process, as we show the level of ADA3 acetylation to fluctuate during cell cycle progression. ADA3 acetylation increases early upon entry into cell cycle, followed by a decline in the late G_1 phase and then re-accumulates as cells enter the S phase, persisting through the G_2/M phase. Such dynamic regulation during cell cycle progression further supports the functional role of ADA3 acetylation, suggesting regulation at the levels/activities of HATs/HDACs targeting the acetylation/deacetylation of ADA3. The transient earlier peak and more sustained delayed ADA3 acetylation during cell cycle progression may reflect the relative activities of GCN5 and p300, a possibility consistent with the reported increase in GCN5 levels from G_1 to early S-phase (33) and the requirement for p300 HAT activity for progression through S phase (34-38). If established in future studies, such a scheme will support distinct roles for GCN5/PCAF vs. p300-mediated acetylation of ADA3. Consistent with our observations, acetylation of yeast ADA3 was found to increase when quiescent cells (comparable to G_0 phase of mammalian cell cycle) transited to growth phase (comparable to S phase of mammalian cell cycle), with persistent acetylation through the cell division phase (comparable to G_2/M phase of mammalian cell cycle)

and a decline as the cells re-entered the next cell cycle (24). Future analyses of the dynamics of site-specific ADA3 acetylation during cell cycle progression and in other physiological/pathological scenarios will therefore be of great interest as acetylation site-specific antibodies become available. In conclusion, we establish that mammalian ADA3 is acetylated at distinct sites by its associated HATs, GCN5/PCAF and p300, and deacetylated via a novel interaction with SIRT1, and further demonstrate that ADA3 acetylation is essential for its physiological function in promoting histone acetylation and cell cycle progression in mammalian cells. These studies should provide a basis for future cell-based and/or animal-based knock-in of acetylation site mutations to examine the *in vivo* roles of ADA3 acetylation in chromatin modifications, and potentially other physiological processes. As we have shown that ADA3 is overexpressed and mislocalized in human cancers, correlating with poor patient survival (42), future studies of ADA3 acetylation in relation to its role in oncogenesis will be of substantial significance.

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CHAPTER 4

LAPATINIB EXERTS ITS CELL CYCLE INHIBITORY EFFECT THROUGH A NOVEL AKT-p300-ADA3 SIGNALING PATHWAY

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(Manuscript to be submitted)

Introduction

Breast cancer is one of the most common occurring cancers among women in the United States and it is the second leading cause of cancer related deaths following lung cancer (53) . Based on molecular profiling of patient derived tumors breast cancer is classified into various subtypes (54) . Among breast cancer molecular subtypes, HER2 (human epidermal growth factor receptor 2) is either amplified or overexpressed in 25-30% of total breast cancer and represents one of the most aggressive subtypes (55, 56) . HER2 belongs to epidermal growth factor family of tyrosine kinases that regulates cell proliferation and transformation leading to cancer (57) . The other members of this family are EGFR/HER1, HER3 and HER4, which contain an extracellular ligand-binding region, single membrane spanning region, and a cytoplasmic tyrosine kinase-containing domain. Upon binding to a specific ligand they either homodimerizes or heterodimerizes and activate several downstream signaling pathways. HER2 however, does not have a specific ligand and is activated by heterodimerization with other family members when bound to their ligands. The important downstream signaling pathways activated by HER2 are mitogen-activated protein kinase MAPK and the phosphatidylinositol 3-kinase (PI3K) −AKT pathway that function as mediators and essentially send the signals to effector proteins to regulate cell growth, differentiation and apoptosis. Since *HER2* is a potent oncogene, much attention has been paid to target HER2. Of note, monoclonal antibodies and small molecule tyrosine kinase inhibitors (TKIs) against HER2 has been emerged as potential therapeutic approaches against HER2 (57, 58) .

Lapatinib (GW572016, GlaxoSmithKline) is a dual EGFR/HER2 tyrosine kinase inhibitor that interrupts downstream cell proliferation and survival signaling by targeting PI3K/AKT and MAPK pathways (59) . In 2007 the U.S. Food and Drug Administration approved for treatment of patients with advanced HER2-positive breast cancer by lapatinib in combination with capecitabine (60, 61) . Previous studies have revealed that treatment of lapatinib in cultured cells lead to cell cycle arrest in G_0/G_1 phase and thereby inhibiting cancer

cell proliferation. However, the molecular basis of lapatinib induced cell cycle arrest remains poorly understood.

The Alteration/Deficiency in Activation-3 (ADA3) is an evolutionary conserved component of several lysine acetyltransferase (KAT) complexes. We have earlier shown that ADA3 is required for normal cell cycle progression and its deletion in MEFs causes severe cell cycle arrest by stabilization of CDK inhibitor p27 (28) . In this study we show that lapatinib targets ADA3 for degradation. We further show that ADA3 acetylation stabilizes the protein by preventing its ubiquitination and lapatinib mediated degradation in total ADA3 levels was attributed to abrogation in its acetylation. Our extensive experiments uncover a novel signaling pathway in HER2 positive breast cancers i.e. HER2 -AKT-p300-ADA3. Finally, we discover various stimuli that cause ADA3 acetylation and experimentally proved that ADA3 acetylation is induced by EGF and TNF-alpha. Taken together, our results demonstrate ADA3, a critical cell cycle regulator, as a target of lapatinib and uncover a novel mode of action of dual kinase inhibitor lapatinib. Inhibition of AKT-p300-ADA3 pathway by lapatinib certainly highlights the importance of ADA3 as a marker for treatment efficacy and a promising therapeutic target.

Materials and Methods

Cell Lines and Cell Culture

HEK-293T was cultured in DMEM, SkBr3 cells in RPMI 1640, UACC812 in MEM-alpha supplemented with 1 μg/ml hydrocortisone and 12.5 ng/ml EGF. All the cell lines were cultured in media supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1x minimal non-essential amino acids, 10 μg/ml gentamycin, 1 μg/ml insulin and 10% fetal bovine serum. For serum starvation experiments cells were cultured in media without glutamine, minimal non-essential amino acids, insulin and fetal bovine serum.

Reagents

Lapatinib (L-4899) was purchased from L.C. laboratories. EGF (E9644), TNFα (T0157), Cycloheximide (C7698), MG-132 **(**M7449**),** Acetyl co-enzyme A sodium salt (A2056), garcinol (G5173), HA peptide (I2149) and FLAG peptide (F4799) were purchased from Sigma.

Plasmids and Transient Transfection

Generation of FLAG tagged ADA3 wild type or 5K/R mutant were described previously (62) . HA-AKT1 wild type (plasmid# 9004-903) and K179M (plasmid# 9007-904) were purchased from Addgene. HA-p300 WT, S1834A and S1834E were generous gift from Dr. Denise Galloway's laboratory. For transient transfection experiments the indicated plasmids were transfected using X-tremeGene HP transfection reagent (Roche # 06366236001) according to manufacturer's protocol. For *ADA3* knockdown experiments, cells were transfected with 50 nM of control (sc-37007, Santa Cruz Biotechnology) or *ADA3* siRNA (sc-78466, Santa Cruz Biotechnology), using the DharmaFECT Transfection Reagent (T-2001-03, Dharmacon).

Immunoprecipitation

For immunoprecipitation, cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM Na_4VO_3, 1 mM NaF, and protease inhibitor mixture, 2 μM TSA and 10 mM NAM for acetylation experiments) and whole cell extract were subjected to immunoprecipitation using appropriate antibodies overnight at 4°C. Beads were then washed five times at 5000 rpm for 1 minute with lysis buffer. For elution by FLAG and HA peptide, generally the immunoprecipitated FLAG/HA tag proteins were eluted with 0.25 μg/μl peptide (Sigma) into lysis buffer. The elutes were subjected to SDS PAGE and analyzed by immunoblotting as indicated.

Antibodies

Generation of anti-hADA3 mouse monoclonal antiserum has been described previously (28) . Antibodies against HSC-70 (sc-7298), pHER2 (sc-12352), HER2 (sc-284), pAKT (sc-7985), AKT (sc-5298), p300 (sc-584 & sc-585) were purchased from Santa Cruz Biotechnology. Agarose conjugated anti-FLAG (A2220) and anti-HA (A2095); anti-ADA3 rabbit polyclonal (HPA042250), FLAG (A8592), β-actin (A5441) were purchased from Sigma. Histone H3 (06-755) was purchased from EMD Millipore, p27 (610241) from BD Biosciences, pH3 (S10) (ab-14955) was from Abcam, and pp300 (S1834) (PA5-12735) was purchased from Thermo Scientific. Anti-acetyl lysine (9681), anti-acetyl lysine-HRP (6952) and anti-HA (2999) were purchased from Cell Signaling Technologies.

***In vitro* KAT Assay**

For in vitro acetylation reactions, HA-p300 WT, S1834A or S1834E were ectopically expressed and immunoprecipitated from HEK293T cells. The immunoprecipitates were eluted with HA peptide (I2149, Sigma) and a fraction of it was analyzed on SDS-PAGE by CBB staining along

with known amounts of BSA. 1 µg GST-ADA3 or purified histone H3 were incubated with 20 ng HA-p300 WT, S1834A or S1834E in KAT buffer (50 mM Tris HCl pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1mM DTT, 2 µM TSA, 50 µM Acetyl co-enzyme A sodium salt and 1 mM PMSF) at 30°C for 30 minutes. The reaction was stopped by adding 6x SDS sample buffer and products were subjected to SDS-PAGE analysis and immunoblotted with the indicated antibodies.

ADA3 Half-Life Experiments

HEK293T cells grown in p60 were transfected with wild type or acetylation deficient FLAG-ADA3 constructs. 24 hours post transfection, cells were trypsinized and plated in 6 well plates. After overnight growth, cells were treated with 50 µg/ml cycloheximide ± 20 µM MG132 and harvested at indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. To examine effect of lapatinib on half-life of endogenous ADA3 in SkBr3 or UACC812 cells, the 8 µg/ml cycloheximide was used and rest of the protocol remained the same as described above. Densitometry analysis was carried out on scanned images using ImageJ software.

***In vivo* Ubiquitination Assays**

For *in vivo* ubiquitination assays, HEK293T cells were transfected with 1 µg wild type or acetylation defective FLAG ADA3 construct with or without 2 µg pcDNA3.1-HA-Ubiquitin construct. 40 hours post transfection, cells were treated with 20 µM MG132 for 5 hours followed by lysate preparation and immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-HA-HRP antibody.

RESULTS

ADA3 knockdown recapitulates the cell cycle inhibitory effects of lapatinib HER2 positive cells

We have previously demonstrated that conditional deletion of *Ada3* in MEFs leads to accumulation of CDK inhibitor p27 and severe cell cycle arrest through G_1 to S phase (28) . In order to test the effect of ADA3 on cell cycle progression in HER2 cells, we specifically knocked down *ADA3* in SkBr3 cells and examined the levels of p27. Consistent with the effect of *Ada3* deletion in MEFs, we observed marked increase in p27 levels in SkBr3 cells after *ADA3* knockdown (Figure 4.1A). In addition to p27, we also assessed the levels of phospho-H3(S10), a hallmark of mitosis and found that *ADA3* knockdown in SkBr3 leads to dramatic reduction in phospho-H3(S10) clearly suggesting the importance of ADA3 for the cell cycle progression in HER2 positive cells (Figure 4.1A). It has been established that lapatinib also induces an increase in p27 levels and cell cycle arrest (60, 63) , an effect similar to ADA3 knockdown and therefore hypothesize that lapatinib functions through ADA3 to manifest its cell cycle inhibitory role. In order to test this, we examined the effect lapatinib on ADA3 and interestingly found that lapatinib treatment leads to downregulation of ADA3 (Figure 4.1B). Furthermore, this decrease in ADA3 levels was consistent with remarkable increase in p27 levels and reduction in mitotic index as shown by phospho-H3(S10) (Figure 4.1B). Next, we asked whether the observed downregulation in ADA3 protein levels is due to decrease in mRNA levels. We treated SkBr3 cells with lapatinib and analyzed *ADA3* mRNA levels by Q-RTPCR. However, we did not observe any change in *ADA3* mRNA levels (data not shown), Additionally previous microarray studies highlighting the effect of lapatinib on gene expression profile do not reveal *ADA3* (64, 65) clearly indicating the involvement of a posttranslational regulatory mechanism in lapatinib induced ADA3 downregulation.

Figure 4.1: ADA3 knockdown recapitulates the cell cycle inhibitory effects of lapatinib HER2 positive cells

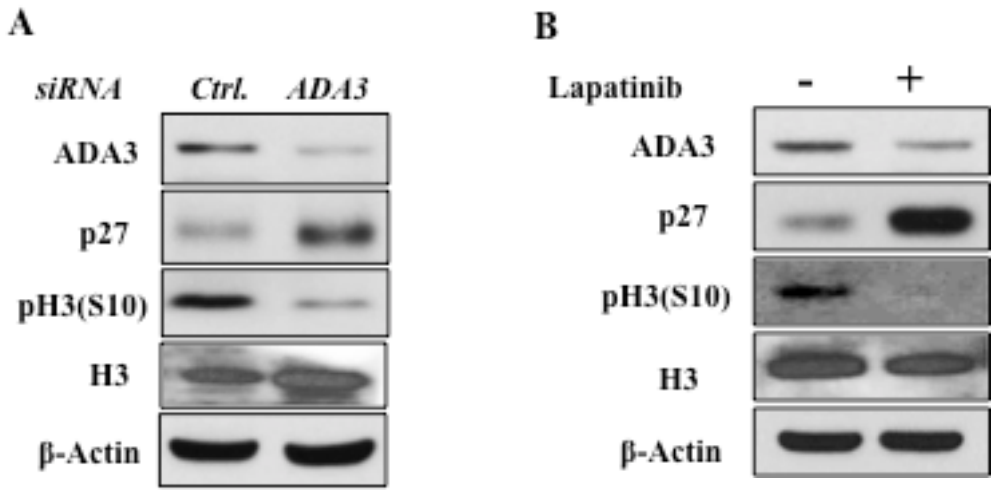


Figure 4.1: *ADA3* knockdown recapitulates the cell cycle inhibitory effect of lapatinib. (A) SkBr3 transiently transfected with Ctrl or ADA3 siRNA for 48 h and whole cell extracts were immnublotted with indicated antibodies. **(B)** SkBr3 cells were treated with either DMSO or 1 μM lapatinib and whole cell extracts were immnublotted with indicated antibodies.

Acetylation of ADA3 plays an important role in maintaining its stability

Since we demonstrate that lapatinib downregulates ADA3 protein levels, we set out to explore what regulates ADA3 protein stability. We have recently shown that KAT3B/p300 acetylates ADA3 (62) and acetylation of several proteins has been shown to regulate their stability. This is in part because both acetylation and ubiquitination covalently modify the same ε-amino group of lysine residues. Thus, if both modifications occur on the same lysine residue, it would create a competition for the acetylation or ubiquitination of the lysine involved. As a consequence, acetylation of the protein would prevent its ubiquitination and subsequent proteasomal mediated degradation. This phenomenon has been demonstrated for several key cellular proteins, including, but not limited to SMAD7, p53, FOXP3, SREBPs, ER-α, RelA and ATP-citrate lyase (66-72) . Given that ADA3 is acetylated by KAT3B/p300 (62) , we wished to examine if acetylation of ADA3 also plays a role in preventing its ubiquitination and thus, stabilizing the protein. To investigate this, we treated 76N-TERT hMEC cells with various doses of p300 inhibitor, garcinol for 18 hours and analyzed ADA3 protein levels by immunoblotting. We observed dramatic decrease of ADA3 protein levels upon garcinol treatment suggesting that p300 mediated acetylation of ADA3 may play an important role in conferring stability to ADA3 protein (Figure 4.2A). To elucidate, if the degradation of ADA3 upon inhibition of p300 mediated acetylation, occurs through ubiquitin-proteasomal machinery, we also treated cells with proteasomal inhibitor MG132 along with garcinol. Interestingly, we observed inhibition of ADA3 degradation when we administered garcinol treated cells with MG132 substantiating our hypothesis that inhibition of p300 mediated ADA3 acetylation leads to degradation of ADA3 through ubiquitin-proteasomal pathway (Figure 4.2A).

Based on the above findings, we hypothesized that if acetylation of ADA3 stabilizes the protein, then we would observe a decrease in half-life of acetylation defective mutants. Through mass spectrometry studies we recently have identified 5 potential lysine residues (K109, -122, -

124, -194 and -222) in ADA3 that are acetylated by p300 (62) and generated an *ADA3* mutant in which these 5 lysines are mutated to acetylation deficient arginine referred to as 5KR mutant. We then analyzed the half-life of wild type and acetylation-deficient ADA3 5KR mutant by cycloheximide chase experiment in HEK293T cells. The half-life of exogenous wild type ADA3 appeared to be about 4 hours from these experiments (Figure 4.2B). To our surprise, 5KR acetylation mutant was highly stable and exhibited virtually no degradation during the course of cycloheximide treatment (Figure 4.2C). This result could be explained based on the aforementioned phenomenon of competition between acetylation and ubiquitination for the same lysine residues. Thus, if both acetylation and ubiquitination modify the same lysine residues in ADA3, then mutation of these lysines to arginine residues will not only abrogate ADA3 acetylation, but also prevent ubiquitination of ADA3 rendering the protein highly stable.

To test the above-mentioned proposition, that the 5KR acetylation deficient mutant is also prevented from ubiquitination, we performed ubiquitination experiments in cultured cells. We co-transfected HA-Ubiquitin together with wild type or 5KR FLAG-ADA3 constructs in HEK-293T cells. 40 hours after transfection, we treated cells with MG132 for 5 hours and harvested the cells. This was followed by immunoprecipitation with anti-FLAG and immunoblotting with anti-HA to detect ubiquitination of wild type and 5KR ADA3. Interestingly, we observed a remarkable decrease in the ubiquitination of ADA3-5KR mutant versus wild type ADA3 corroborating our earlier findings that ADA3-5KR mutant is extremely stable (Figure 4.2D). These findings provide conclusive evidence that acetylation and ubiquitination compete for the same lysine residues on ADA3. Thus, p300 mediated acetylation of ADA3 should prevent its ubiquitination leading to ADA3 protein stabilization.

Figure 4.2: Acetylation of ADA3 plays an important role in maintaining its stability

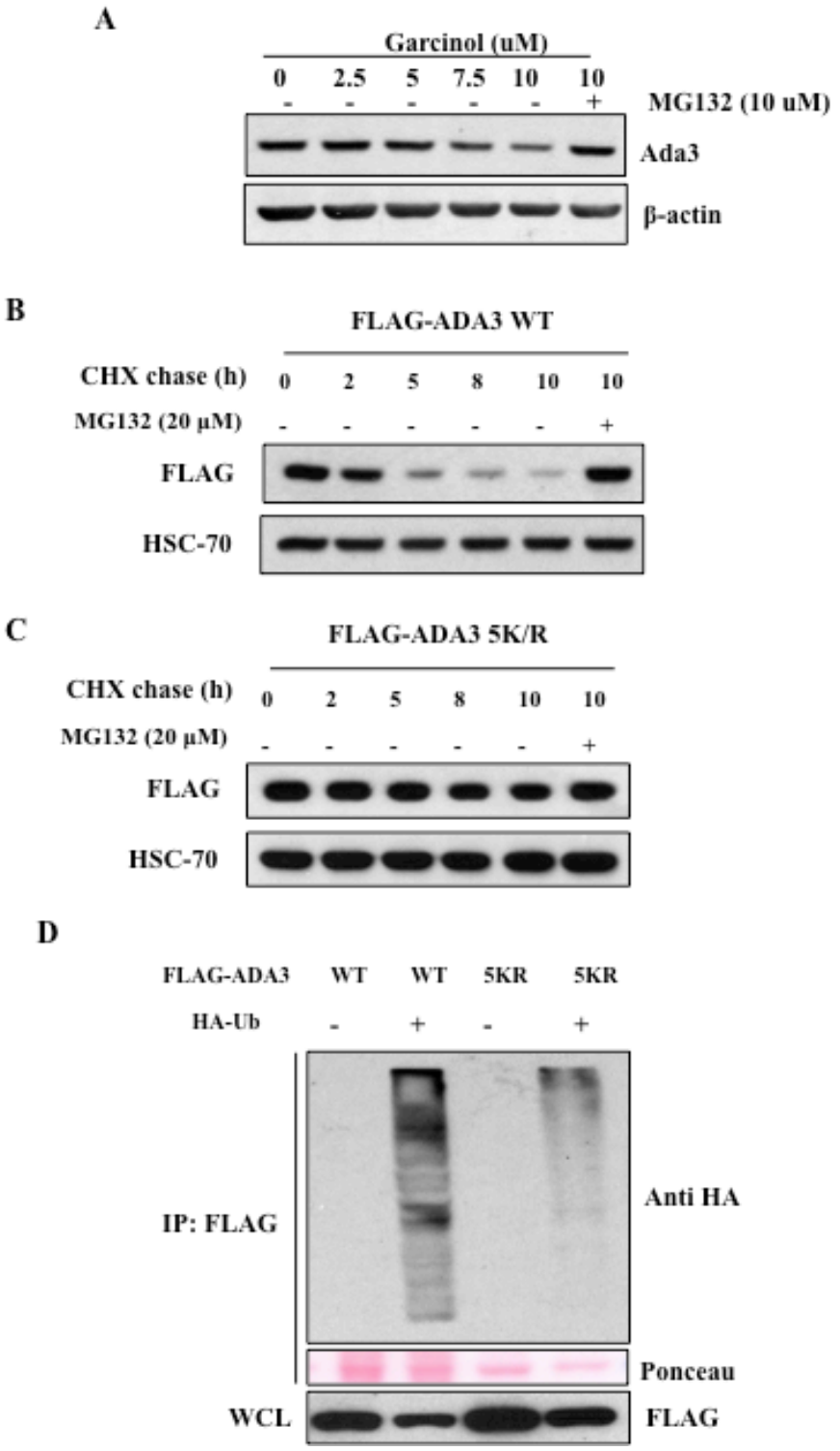


Figure 4.2: Acetylation of ADA3 plays an important role in maintaining its stability. **(A)** 76NTERT hMECs were treated with various doses of HAT inhibitor Garcinol for 18 h and the lysates harvested were subjected to immunoblotting with inidicated antibodies. **(B)** 76NTERT hMECs were treated with various doses of HAT inhibitor Garcinol ± MG132 for 12 h and the lysates harvested were subjected to immunoblotting with inidicated antibodies. **(C & D)** 36 hours after transfection of FLAG-ADA3 wild type (C) or 5KR mutant (D) in HEK-293T, cells were treated with 50 μg/ml cycloheximide ± 20 μM MG132 and harvested at the indicated time points, and blotted with indicated antibodies.

Lapatinib inhibits the AKT mediated phosphorylation of p300 and targets ADA3 for degradation

As mentioned above, lapatinib treatment in SkBr3 and UACC-812 caused ADA3 protein downregulation but not mRNA, we examined the effect of lapatinib on ADA3 protein half-life in these cells. For the purpose, SkBr3 and UACC-812 cells were treated with protein synthesis blocker cycloheximide in presence or absence of lapatinib and analyzed ADA3 degradation over regular intervals for 12 hours (Figure 4.3A & B). To our surprise we found that ADA3 is a very stable protein and its levels remained almost the same even after 12 hours of cycloheximide treatment. However, in presence of lapatinib a gradual decrease in ADA3 levels were observed (Figure 4.3A & B) which indicates that HER2 signaling pathway plays a major role in maintaining ADA3 stability.

So far we have established that both lapatinib and garcinol downregulates ADA3. This observed phenomenon generates two possibilities i.e. either lapatinib and garcinol might be regulating ADA3 protein levels through different pathways or there could be crosstalk involved that affects the ADA3 levels. In order to investigate these possibilities, we examined the effect of lapatinib on downstream signaling pathways and its potential connection with p300. Interestingly we found that p300 has been shown be phosphorylated by AKT at S-1834 residue (73) and lapatinib is known to inhibit HER2 downstream PI3K/AKT pathway (59) . Therefore, if lapatinib inhibits the phosphorylation of AKT, it must also bring about decrease in phosphorylation of p300 at S-1834 residue. We tested this phenomenon in two HER2 positive cell lines, namely SkBr3 and UACC-812 and we indeed observed the lapatinib reduced the phosphorylation p300 at S1834 which was correlated with inhibition of phospho-HER2 and phospho-AKT. Consistent with our previous experiments, in this case also we observed ADA3 downregulation upon lapatinib treatment (Figure 4.3C & D).

Figure 4.3: Lapatinib inhibits the AKT mediated phosphorylation of p300 and targets ADA3 for degradation.

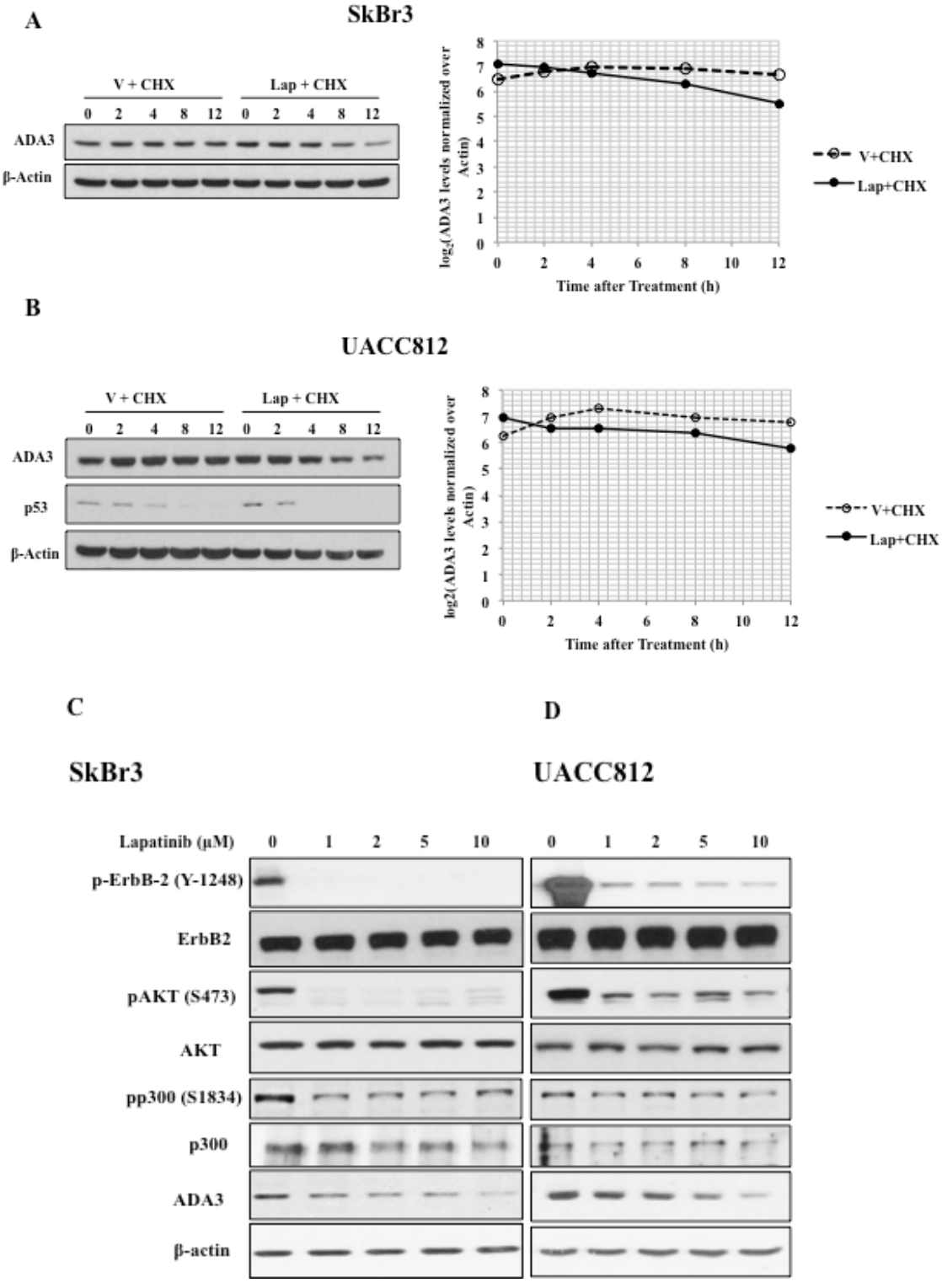


Figure 4.3: *Lapatinib inhibits the AKT mediated phosphorylation of p300 and targets ADA3 for degradation. (A & B)* SkBr3 (A) and UACC-812 (B) cells were treated with 8 μg/ml cycloheximide in presence or absence of 1 μM lapatinib. Cells were harvested at indicated time points after the treatment and whole cell extracts were immunoblotted with indicated antibodies. The graph below the western blot shows the log band intensity of ADA3 normalized over β-actin as computed from imageJ software. (C & D) SkBr3 (C) and UACC-812 (D) cells were treated with increasing concentration of lapatinib for 4 hours and whole cell extracts were immunoblotted with indicated antibodies.

The phospho defective mutant p300 S1834A has reduced ability to acetylate ADA3

If phosphorylation of p300 at S-1834 regulates its KAT activity then prevention of p300 phosphorylation must abrogate the acetylation of p300 targets including ADA3. In order to test whether p300 phosphorylation indeed regulates the acetylation status of ADA3, we tested the abilities of a phospho defective S1834A or mimic S1834E mutant of p300 to acetylate ADA3 by variety of assays. For our *in vitro* KAT assay, we first transfected HA-p300 wild type or S1834A/E mutants in HEK293T cells, and eluted HA-p300 wild type or mutant proteins using HA peptide (see materials and methods for detail) (Figure 4.4A). These eluted p300 proteins were used as lysine acetyltransferase and recombinant GST-ADA3 as substrate in our *in vitro* KAT assay. As expected p300 wild type was found to be efficient enough to acetylate ADA3 whereas acetylation of ADA3 was markedly reduced when the phospho defective mutant S1834A was used as acetyltransferase. More importantly, the phospho mimic mutant S1834E was as efficient as wild type in order to acetylate ADA3; supporting our hypothesis that phosphorylation of p300 at S1834 regulates the acetylation of ADA3 (Figure 4.4B). We also used recombinant histone H3 as positive control and observed that both p300 and S1834E mutant was potent enough to acetylate histone H3 whereas the phospho defective mutant S1834A remains inefficient in this process that further substantiate our hypothesis (Figure 4.4C). Next, to examine the effect of p300 phosphorylation in cellular system, we transfected HA-p300 wild type or S1834A mutant along with FLAG-ADA3, We then immunoprecipitated FLAG-ADA3 and by immunoblotting with pan acetylated lysine antibodies, we observed that while wild type p300 was able to acetylate ADA3, the phospho defective mutant was incompetent to acetylate ADA3 (Figure 4.4D). Taken together our *in vitro* and in cell assays demonstrates that phosphorylation of p300 at S1834 regulates acetylation of ADA3.

Figure 4.4: The phospho defective mutant p300 S1834A has reduced ability to acetylate ADA3

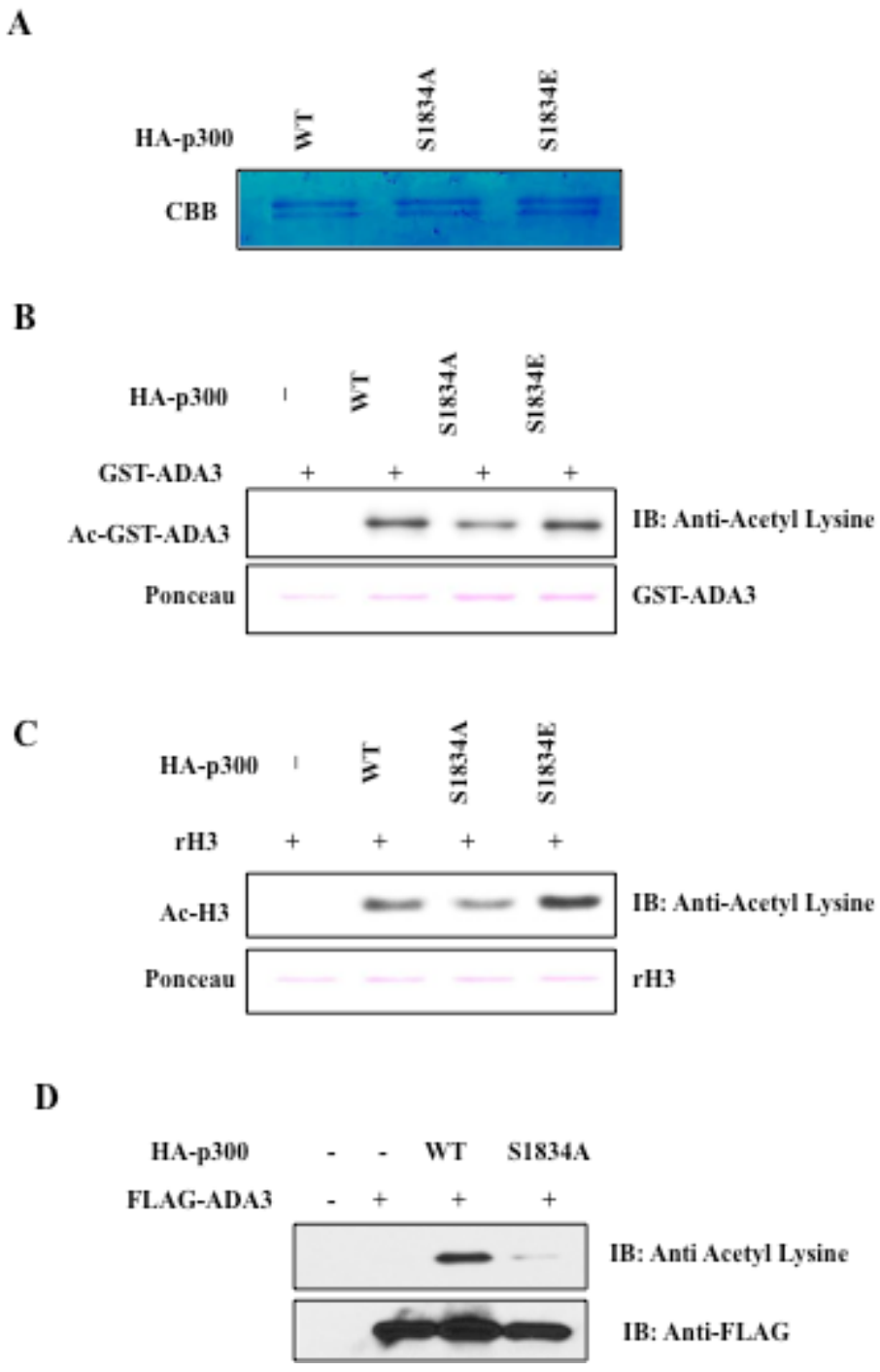


Figure 4.4: The phospho defective mutant p300 S1834A has reduced ability to acetylate ADA3. (A) HEK-293T cells were transfected with HA-p300 wild type, S1834A/E mutants. 48 h after transfection, whole cell extracts were subjected to immunoprecipitation by agarose conjugated anti-HA beads. Immunoprecipitates were then eluted with HA peptide and visualized on SDS gel by CBB staining. **(B & C)** *In vitro* KAT assay using 20 ng HA-p300 wild type or S1834A/E enzymes obtained from A, and 1 μg recombinant histone H3 (B) or GST-ADA3 (C) as substrates. The assay was then followed by immunoblotting with anti-acetyl lysine antibody. **(D)** HEK-293T cells were co-transfected with FLAG-ADA3 and HA-p300 wild type or S1834A mutant. 48 h after transfection whole cell extracts were immunoprecipitated with M2 agarose and immunoprecipitates were eluted by 3X FLAG peptide. Elutes were then immunoblotted with indicated antibodies.

AKT overexpression or activation induces ADA3 acetylation

If ADA3 acetylation is regulated by p300 phosphorylation status, the activation of upstream kinase AKT should also cause ADA3 acetylation. In order to test this, we first overexpressed FLAG-ADA3 along with HA-AKT wild type or a dominant negative AKT mutant (K179M). Our FLAG-ADA3 immunoprecipitation followed by immunoblotting with pan anti acetyl lysine antibody demonstrated an increase in ADA3 acetylation where AKT wild type was overexpressed. More importantly, in presence of AKT dominant negative mutant (K179M), ADA3 acetylation was markedly abrogated (Figure 4.5A). Here it should be noted that albeit in this experiment although p300 was not overexpressed, we noticed a slight increase in ADA3 acetylation in presence of overexpressed AKT that indicates the involvement of AKT in ADA3 acetylation.

Next, in order to delineate the whole signaling event that induces ADA3 acetylation in cells, we serum starved the SkBr3 and UACC812 cells for 48 hours followed by stimulation with EGF for 15 and 30 minutes to induce AKT phosphorylation. We subjected a small fraction of whole cell lysate to western blotting and as expected we noticed the induction of AKT phosphorylation at S473 as early as 15 minutes that persisted 30 minutes after EGF stimulation (Figure 4.5B & C, Input). We also examined the phosphorylation of p300 and consistent with AKT phosphorylation induction, p300 phosphorylation was also triggered (Figure 4.5B & C, Input). Now, in order to test whether activation of AKT followed by p300 phosphorylation induces ADA3 acetylation, we immunoprecipitated the endogenous ADA3 from rest of the lysate. Our immunoblotting analysis with pan anti-acetyl lysine antibody demonstrated no acetylation signal under serum-starved conditions however, after EGF stimulation there was an induction in ADA3 acetylation which was consistent with AKT and p300 phosphorylation (Figure 4.5B & C). Next, we hypothesized that if AKT phosphorylation induces ADA3 acetylation, any other factor other than EGF that causes AKT and p300 phosphorylation subsequently, must also have the

same effect on ADA3 acetylation. For the purpose, we took A459, a lung cancer cell line, and serum starved it similar to SkBr3. Instead of EGF, this time we stimulated the cells with TNFα for 30 and 60 minutes. Similar to our previous experiment, we first analyzed the induction of AKT and p300 phosphorylation in input fraction and not observed that indeed these phosphorylation events were induced after TNFα treatment. Now, in order to examine ADA3 acetylation, we immunoprecipitated endogenous ADA3 for immunoblotting with pan anti-acetyl lysine antibody. Consistent with our previous result, this time also we found that ADA3 acetylation was induced after TNFα stimulation, which was concomitant with AKT and p300 phosphorylation (Figure 4.5D). Taken together, our results dissect a novel signaling event that involves pAKT-pp300-Ac-ADA3 (Figure 4.6)

Figure 4.5: AKT overexpression or activation induces ADA3 acetylation

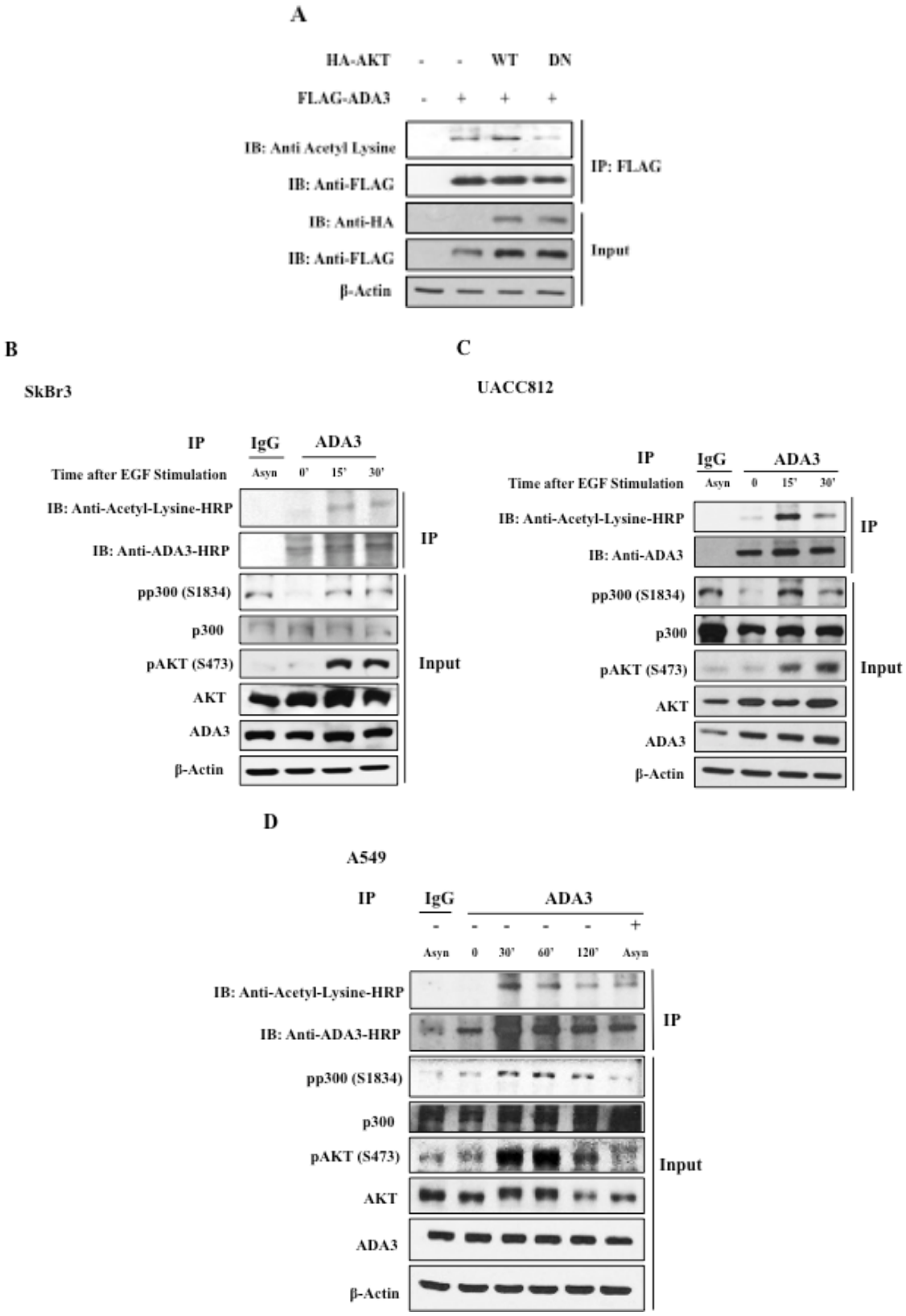
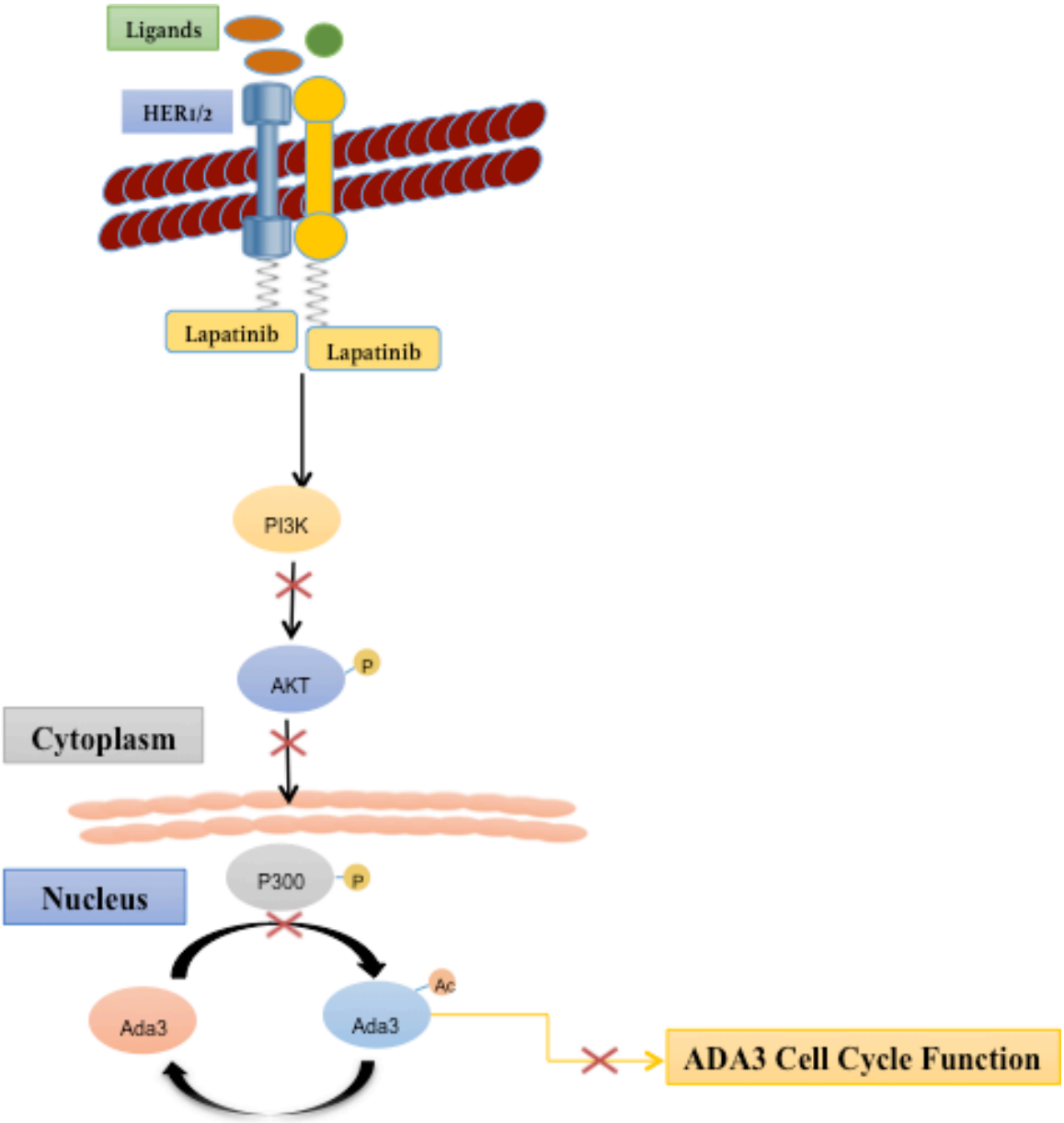


Figure 4.5. AKT overexpression or activation induces ADA3 acetylation (A) HEK-293T cells were co-transfected with FLAG-ADA3 and HA-AKT wild type or dominant negative (K179M) mutants. 48 h after transfection whole cell extracts were immunoprecipitated with M2 agarose and immunoprecipitates were eluted by 3X FLAG peptide. Elutes were then immunoblotted with indicated antibodies. **(B, C & D)** SkBr3, UACC812 and A549 cells were serum starved for 48 h and stimulated with EGF (B & C) or TNFα (D) as indicated. Whole cell extracts from indicated time points were subjected to immunoprecipitation with normal IgG or anti-ADA3 antibodies. Immunoprecipitates and input fractions were then immunoblotted with indicated antibodies.

Figure 4.6: Model showing pHER2-pAKT-pp300-AcADA3 pathway



Discussion

Cell cycle is a tightly regulated sequence of events that maintain the genomic stability and deviation from normal cell cycle control leads to detrimental diseases including cancer (74) . The cell cycle is orchestrated by many proteins that play their roles at different stages and among these players ADA3 has emerged as a key cell cycle regulator. Previous studies from our laboratory and others' have shown that ADA3 regulates G_1 to S phase transition and mitosis by regulating chromosome segregation (28, 48, 75) . Despite such an important role of ADA3 in maintaining the genomic stability its role had not been explored in cancer until we found its correlation with various clinicopathological parameters in breast cancer specimens. ADA3 was found to be mislocalized/overexpressed in aggressive subtypes i.e. HER2+ breast cancer specimens and correlated with poor patient outcomes (50) . In this study we attempted to explore the role of ADA3 in HER2+ breast cancers with a focus on signaling pathways that ADA3 is involved in. We observed a decrease in ADA3 levels by inhibiting the phosphorylation of HER2 and thus found ADA3 as a downstream target of HER2.

We have recently reported that ADA3 is posttranslationally modified by acetylation by its associated KATs however under what physiological conditions its acetylation is induced remained largely unknown (62) . One intriguing finding from current study is that EGF induces ADA3 acetylation. Typically, EGF is considered to be phosphorylation inducing agent and in our study we found it as an acetylation triggering stimuli albeit not directly. That EGF stimulates ADA3 acetylation through the phosphorylation of HER2-AKT-p300 pathway indicates the existence of potential crosstalk between various posttranslational modifications in response to an extracellular stimulus in cell.

Another striking finding from our study is competition between acetylation and ubiquitination of ADA3. How ADA3 acetylation dominates over ubiquitination still remains to be explored. One possibility could be ADA3 is constantly deubiquitinated that renders lysine residue available for acetylation. In-fact ADA3 associated STAGA complex also harbors a deubiquitinase

USP22 that has also been identified as 11-gene signature of poor prognosis of many cancers (76) . Whether USP22 deubiquitinates ADA3 that leads to it hyperacetylation is a subject of future studies.

Given that AKT is frequently activated in HER2+ breast tumors (77) and the presence of pAKT of in HER2+ cells, it is reasonable to speculate hyper-phospho-p300 and consequentially hyperacetylated ADA3 in these cells as compare to normal hMEC. Since acetylation of ADA3 stabilizes the protein and regulates its function in cell proliferation, HER2+ mediated oncogenesis could be mediated by ADA3 acetylation. In fact such studies are currently underway in our laboratory using ADA3 acetylation specific antibodies to consolidate the correlation between pAKT, phospho-p300 and acetylated ADA3. While navigating the role of ADA3 in HER2+ downstream signaling pathways, in the present study we mainly focused on PI3K/AKT and other mediators such as ERK were untouched. Clearly, future studies are warranted in order to elucidate the role of ADA3 in HER2+ signaling cascades in more comprehensive manner

In current study we used lapatinib, a HER2/EGFR dual kinase inhibitor, to study the involvement of ADA3 in HER2 signaling pathways, which has been known to suppress cancer cell proliferation and to promote G_1 arrest by increasing p27 (60) . Here we not only demonstrated that lapatinib indices cell cycle arrest at G_1 but also dramatically lowers the mitotic index as manifested by pH3(S10). Interestingly, *ADA3* knockdown in HER2+ cells mimicked the cell cycle inhibitory effects of lapatinib suggesting that lapatinib might act through ADA3. This finding together with downregulation of ADA3 by lapatinib certainly highlights the importance of ADA3 as a marker for treatment efficacy and a promising therapeutic target.

One potential missing part of this study is that we showed cell cycle inhibition upon *ADA3* knockdown but not with the acetylation defective mutants. If we speculate that hyper proliferation in HER2+ cells is attributed to hyper acetylated ADA3, future studies must be directed to demonstrate that inhibition in HER2+ phosphorylation indeed leads to decrease in

ADA3 acetylation and ADA3 acetylation defective mutants have the same cell cycle inhibitory effect to that of gene knockdown.

The entire HER2 signaling pathway is extremely intricate that involves a great redundancy both at mediator and effector level. For example, here the observed increase in p27 levels after *ADA3* knockdown could most likely be due to the inhibition of its proteasomal by ubiquitin ligase SKP2, that is known to be regulated by ADA3 (28). However, both p27 and SKP2 has been shown to be the direct substrates of AKT for phosphorylation that leads to their degradation and activation respectively (78-81). Moreover, through a positive feedback loop, SKP2 also activates AKT by mediating its non-proteolytic ubiquitination that further increases the complexity of the signaling events (82). Taken together, ADA3 certainly appears to be one of those numerous players that regulate HER2 mediated oncogenesis and certainly much is still to be learned about the interplay between these players and their regulation at transcriptional and post-translational levels.

Based on our results ADA3 can certainly be regarded as a downstream effector molecule of HER2 signaling. Although we used lapatinib as a tool to inhibit the HER2 activation in the present study, one could expect the similar downregulation of ADA3 by other HER2 targeting agents such as herceptin. A potential translation of our findings could be to increase the treatment efficacy or to control therapy resistance that remains a challenge in the treatment of cancer in question (83). Resistance to RTK inhibitors commonly arises due to direct activation of mediator signaling cascades such as MAP kinase or PI3K/AKT and efforts have already been made to test the inhibitors against these mediators to overcome the resistance (84, 85). However, due the occurrence of redundant signaling events inhibitor efficacy is one of the major problems and toxicity associated with combinatorial treatment that may have greater effects, further limits effective treatment option. Therefore in such cases the use of inhibitors against effectors such as ADA3 might be a smart strategy (83). Nonetheless, as indicated above the ADA3 could just be

one of those numerous effectors and therefore redundancy at effector level must be taken into account while addressing therapy resistance.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

ADA3 is a conserved component of many HAT complexes, which plays an important role in transcription regulation. When I started out my thesis work, the primary focus of the laboratory was to explore the role of ADA3 in cell cycle and cancer. Shortly afterwards, studies from our laboratory reported the critical role of ADA3 in cell cycle progression and in maintenance of genomic integrity. We demonstrated that the deletion of *Ada3* from MEFs leads to severe cell proliferation block, delay in G_1/S and G_2/M phase transitions, formation of abnormal nuclei, mitotic defects and spontaneous chromosomal aberrations (28, 86) .

Whereas the delay in G_1/S phase transition was attributed to increased levels of CDK inhibitor p27, the mechanism behind the delay in G_2/M transition and mitotic defects remained understudied. In order to understand the role of ADA3 in mitosis at molecular level, Dr. June Wang of our laboratory performed a ChIP seq analysis and observed that ADA3 is recruited at centromere region of most of the chromosome. Given the vital role of centromeres in chromosome segregation, we speculated that ADA3 might regulate the mitosis through centromere, which indeed turned out to be the case later on, and the detailed study is the part of this thesis as Chapter 2.

Another important and major portion of my thesis work focuses on posttranslational modification of ADA3 that basically stemmed from two serendipitous findings that ADA3 is subjected to acetylation by its associated HATs (p300 and GCN5) during the course of chromatin/histone acetylation (24, 28) . Our finding that ADA3 remains in equilibrium of acetylation and deacetylation and that its acetylation is required for histone acetylation and cell proliferation uncovers a novel mode of regulation of ADA3 function. This particular study is detailed in Chapter 3 of the thesis.

Being such an important cell cycle regulator a parallel focus of the laboratory was to investigate the role of ADA3 in cancer. To better understand the implication of ADA3 in oncogenesis our laboratory had examined the expression of ADA3 in 900 breast cancer tissue specimens with known clinicopathological parameters and found that ADA3 was

mislocalized/overexpressed in HER2+ or EGFR+ subtypes (50) . The study suggested us that ADA3 could potentially be involved in HER2+/EGFR+ mediated oncogenesis and I became interested in exploring the role of ADA3 in HER2+ signaling cascade. Through my extensive experiments I discovered a novel-signaling pathway and found that EGF stimulation leads to ADA3 acetylation by activating (phospho-HER2)- (phospho-AKT)-(phospho-p300) cascade. More details of this study are illustrated in Chapter 4 of the thesis.

Since in our studies we found that ADA3 is mislocalized/overexpressed in HER2+ breast cancer cell lines, it will be of paramount importance to study the ADA3 associated HAT complexes in these cells. It is likely that other components of complex are also mislocalized/overexpressed. In fact, the STAGA complex component USP22 has been identified as part of 11-gene signature causing death from cancer (76) . As far as ADA3 associated HAT complex components are concerned, most of the studies were either performed in yeast or HeLa cells (3, 24) and so far the complex has not been studied in detail in breast cancer cells, therefore it would be interesting to examine the subunits organization in these cells as compare to normal mammary epithelia and its relevance to cancer.

Unstable genome and uncontrolled cell proliferation are the fundamental features of cancer (87) . My thesis work indicates ADA3 acetylation as a major determinant to regulate its function in cell proliferation. Our finding that p300 mediated ADA3 acetylation and ubiquitination competes with each other for the same lysine residues further substantiate that acetylation of ADA3 is required to maintain its stability and therefore may be critical for its function. If ADA3 acetylation is crucial for cell proliferation, what role does it play in the context of centromere function through CENP-B? The truncation mutation analysis of ADA3 revealed that first 110 amino acids are crucial for ADA3-CENP-B interaction and normal cell proliferation. In fact the first 110 amino acids harbor a Lys-109, which is a potential acetylation site and it will be interesting to examine whether or not ADA3-CENP-B interaction and as a consequence cell proliferation and chromosome segregation depends on acetylation at Lys-109.

From my studies knockdown of *ADA3* in HER2+ cells led to decreased pH3(S10) and increased levels of CDK inhibitor p27, the two key cell cycle regulatory marks. Is it the total or acetylated ADA3 that regulates G_1 to S phase transition and mitotic index should be investigated thoroughly. Indeed the future studies should be directed to examine the role of acetylated ADA3 in HER2 mediated oncogenesis using better system such as cell lines in which ADA3 acetylation defective mutants are knocked in.

One seminal finding that emerged from my thesis work is that *c-Myc* enhancer acetylation is contingent upon ADA3 acetylation status (Chapter 3). Whereas in previous studies, loss of ADA3 led to decreased *c-Myc/MYC* transcripts, in our study ADA3 acetylation defective mutants resulted in decreased *c-Myc* enhancer acetylation (28, 49, 62) . *c-MYC* is a key regulatory gene for cell proliferation and growth that has been reported to be amplified or overexpressed in breast cancers (88) . *c-MYC* amplification has been shown to be significantly associated with *HER2* amplification and tumors with *c-MYC/HER2* co-amplification have worse prognosis than those having either one of the two amplified (89, 90) . Moreover, *c-MYC* mediated transcription has not only been shown to be regulated by PI3K/AKT (91) , c-MYC also complements the PI3K/AKT mediated cellular transformation ((92, 93) and proliferation. Thus there appears to be a close nexus among HER2, AKT, c-MYC and ADA3 and clearly the interplay between these players' remains to be determined with regards to oncogenesis. Given the significant redundancy at molecular level among various types of cancers, the findings from my studies can serve as a foundation learn more about the role of ADA3 in other subtypes of breast cancer or cancer in general. In fact in this direction we recently discovered that ADA3 is required for normal and ER+ tumor cell proliferation through c-MYC (Griffin et al., manuscript under revision in BCR) and also in our previous study the expression/localization patterns of ADA3 was also found to be correlated with EGFR status (50) , another subtype of breast cancer with poor clinical outcomes.

Although the results from my studies are described thoroughly in previous chapters of the thesis and two of the chapters are published, following are the important results from my thesis work:

1. ADA3, through its N terminus directly interacts with centromeric protein CENP-B and
2. ADA3 is recruited at HOR region of the centromere, which is also the occupancy site of CENP-B.
3. Loss of ADA3 diminishes CENP-B recruitment at HOR region and leads to chromosome missegregation.
4. ADA3 protein remains in the equilibrium of acetylation and deacetylation. GCN5, PCAF and p300 acetylate ADA3, whereas it is deacetylated SIRT1.
5. ADA3 acetylation and its interaction with CENP-B appear to be important for normal cell proliferation.
6. ADA3 acetylation is dispensable for its interaction with HAT complex components however, the same is important for global and locus specific (*c-Myc* enhancer) histone acetylation.
7. p300 mediated ADA3 acetylation competes with its ubiquitination and prevents its degradation.
8. Inhibition of p-HER2 by lapatinib causes decrease in total ADA3 levels through the inhibition of p-AKT and p-p300.
9. Activation of p-AKT and p-p300 by EGF induces ADA3 acetylation.
10. *ADA3* knockdown in HER2+ cells have the same cell cycle inhibitory effect as lapatinib as manifested by reduced mitotic index and increased levels of p27.

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