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APOPTOSIS INHIBITOR 5 (Api5) ACETYLATION: ROLE IN APOPTOSIS

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A thesis submitted in partial fulfillment of the requirements

for the BS-MS dual degree programme in IISER Pune

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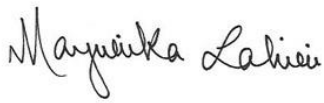
Certificate

This is to certify that this dissertation entitled “Apoptosis Inhibitor 5 (Api5) acetylation: role in apoptosis” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Meera Bessy K.A at IISER Pune under the supervision of Dr. Mayurika Lahiri, Associate Professor, Biology Division, IISER Pune during the academic year 2015-2016.



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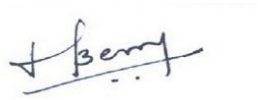
Declaration

I hereby declare that the matter embodied in the thesis entitled ‘Apoptosis Inhibitor 5 (Api5) acetylation: role in apoptosis’ are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Mayurika Lahiri, Associate Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.



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Abstract

Apoptosis Inhibitor 5, Api5 is a nuclear protein which inhibits apoptosis upon growth factor deprival and is up regulated in various cancers. Api5 levels have been shown to be cell cycle regulated. Role of Api5 in cellular processes is not completely understood, especially it's regulation by post translational modifications is least explored. Api5 lysine-251 acetylation, only post translational modification known to occur on Api5, was shown to negatively regulate apoptosis induced by serum starvation. Here, we tried to understand the mechanism through which acetylation regulates DNA damage induced apoptosis. In the process of standardizing dose regimen for the apoptotic assays, we observed that varying doses of camptothecin treatment did not induce DNA fragmentation. However, UV damage induced time dependent activation of caspase-9 and PARP-1 in MCF-7 cells. Further, it was observed that UV damage also induced pre-apoptotic nuclear morphology and cytoplasmic localization of lamin B1. Previous studies in our lab demonstrated that Api5 interacts with TopBP1, a mediator protein in single stranded break induced DNA damage. Further, using deletion constructs it was showed that lysine 251 lies in that region of Api5 which interacts with TopBP1. Therefore, we also tried to investigate its role in interaction with TopBP1 *in vivo*. Towards this end, we have successfully generated siRNA resistant HA-Api5 and Api5-mVenusC1 acetylation mutants (an uncharged mutant, acetylation-deficient and a constitutive acetylation mimic). Also, our preliminary experiments suggested that Api5 is indispensible for cell survival. Additionally, this study also focused on the degradation pathway of Api5. We observed that Api5 levels were not altered by MG-132 (proteasome inhibitor) treatment, suggesting that Api5 may not be undergoing degradation through proteosomal pathway. In summary, with further investigation, this study could potentially give new insights in understanding the role of posttranslational modifications on Api5 in carrying out its function.

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List of Abbreviations

IAP	Inhibitors of Apoptosis
BIR	Baculoviral IAP repeat
Api5	Apoptosis Inhibitor 5
TopBP1	Topoisomerase (DNA) II Binding Protein 1
NLS	Nuclear Localization Signal
E2F	E2-promoter binding factor
FGF2	Fibroblast Growth Factor
EJC	Exon Junction Complex
CPT	Camptothecin
PARP-1	Poly (ADP-ribose) Polymerase 1
XRCC	X-ray repair cross complementing protein
PTM	Posttranslational modifications
EDTA	Ethylenediaminetetraacetic acid
DTE	1,4-Dithioerythritol
EGTA	Ethylene Glycol Tetraacetic acid
DMEM	Dulbecco’s Modified Eagle Medium

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Research experience would not have been so much fun and exciting if I had not joined the Genome Integrity Lab at IISER, Pune. It gives me immense pleasure to express my love and gratitude to all my colleagues who had been a great support and encouragement throughout my stay in the lab.

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I am thankful to Vijay Vittal and IISER Pune microscopy facility. I express my gratitude to Biology Department at IISER Pune for the facilities it has provided for the successful execution of my project. I would also like to thank INSPIRE for providing me financial support.

This acknowledgement would be incomplete without thanking those people who have special place in my heart. No words to extend my love to my friends especially Catherine and Aparna who were like my shadow during my good and hard times.

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I owe deep sense of gratitude to my parents for their immense support and blessings they have showered on me all throughout my life.

Introduction

Apoptosis is a highly evolutionarily conserved physiological cell suicidal program, which plays a vital role in development, tissue homeostasis and to prevent the spread of infections (de Almagro and Vucic, 2012; Deveraux and Reed, 1999). Deregulation in the pathway has been implicated in a variety of clinical disorders (Deveraux and Reed, 1999). Excessive apoptosis results in various diseases such as neurodegenerative disorders while conditions such as cancer and autoimmune diseases results from insufficient apoptosis (Grutter, 2000).

Suppressors of apoptosis are the Inhibitors of Apoptosis (IAP) family of proteins that are involved in cell death, cell cycle, migration and immunity (Lopez and Meier, 2010). IAPs were first discovered in baculoviruses as proteins, which inhibit apoptosis in host cells (Birnbaum et al., 1994; Crook et al., 1993). These proteins are characterized by the presence of at least one baculoviral IAP repeat (BIR) domain of 70 amino acids which is thought to be involved in its anti-apoptotic function (Deveraux and Reed, 1999). The exact targets of IAPs are not clearly known. But it is proposed that they interfere directly with the catalytic activity of caspases or inhibit procaspases and other proteins, which are necessary for the activation of caspases.

Apoptosis Inhibitor 5 or Api5 as the name suggest is a suppressor of apoptosis, which is an apoptosis inhibiting nuclear protein with no BIR domain (Han et al., 2012). This 60 kDa protein was first identified by Tewari *et al* as a cDNA which inhibited apoptosis upon growth factor withdrawal (Tewari et al., 1997). Api5 shows strong species conservation ranging from plants to flies to humans (Morris et al., 2006). Api5 was shown to be up regulated in various cancers. It's over expression has been implicated in tumor progression and poor prognosis in cervical cancer (Cho et al., 2014). This protein is also found to show no structural similarity with any of the known proteins. Api5 exhibits an all-helical structure with 19α helices and two 3_{10} helices. The protein has three distinct domains – an LxxLL motif which is expected to contribute to the stability of the protein, an unusual leucine zipper motif with no DNA binding domain and a nuclear localization signal (NLS) at the C-terminal region. The N-terminal half of the protein resembles the HEAT repeat and

the C-terminal half aligns with the ARM repeats which are considered as protein-protein interacting domains. These protein interaction modules could act as scaffold for multi-protein complexes. There are contradictory reports regarding the nature of the Leucine Zipper domain in Api5. In 2009, Rigou et al has shown that the Leucine Zipper Domain of Api5 homodimerizes (Rigou et al., 2009). But later in 2012, Han et al showed that Api5 has an unusual Leucine Zipper with no DNA binding domain. It does not involve in the homodimerization of the protein but is implicated to heterodimerize with its interacting partners under specific cellular conditions.

Various interacting partners of Api5 have been identified. Api5 has been shown to specifically interact with high molecular mass Fibroblast Growth Factor-2 (HMM FGF-2), a nuclear protein, which has also been reported to inhibit apoptosis upon stress conditions (Van den Berghe et al., 2000). This interaction explains one of the mechanisms through which Api5 may carry out its anti-apoptotic function. Api5-mediated resistance to apoptosis is through the up regulation of FGF2 secretion, which results in the degradation of pro-apoptotic protein, BIM via extracellular signal-regulated kinase (ERK) pathway (Noh et al., 2014). Api5 is also involved in the suppression of E2-promoter binding factor (E2F) 1-dependent apoptosis (Morris et al., 2006). In 2009, Rigou *et al* showed that Api5 interacts with and regulated Acinus mediated DNA fragmentation. Api5 interacts directly with Acinus and prevents its cleavage by caspase 3 to its active fragment, essential for DNA fragmentation during apoptosis (Rigou et al., 2009). Homolog of Api5 in rice is shown to interact with RNA helicases regulating programmed cell death in tapetum during male gametophyte development (Li et al., 2011). The human homolog of these helicases is involved in pre-mRNA splicing and mRNA nuclear export (Shen, 2009). They are also components of exon junction complex (EJC) where Acinus is one of the components (Le Hir and Andersen, 2008; Michelle et al., 2012). This could imply the functional link between Api5 and EJC. Api5 has been shown to interact with a chromatin remodeling enzyme ALC1, that has been shown to interact with various proteins involved in DNA repair mechanism (Ahel et al., 2009). This suggests that Api5 could possibly mediate protein-protein interactions in DNA damage surveillance pathway. In corroboration with this prediction, studies in our lab have shown an interaction

between Api5 and TopBP1, a mediator protein in the DNA damage response pathway, both *in vitro* and *in vivo* (unpublished data).

TopBP1 was first identified as a protein that interacted with Topoisomerase II-β in a yeast two-hybrid screen (Yamane et al., 1997). Apart from its role in initiation of replication, it also plays an important role in the activation of DNA damage and replication checkpoints (Hashimoto and Takisawa, 2003; Makiniemi et al., 2001). The transcriptional regulation of TopBP1 is also well known. TopBP1 interacts with E2F1 during normal cell cycle as well as during damage response and promotes its repression state to prevent apoptosis during replication and repair (Lin et al., 2001; Liu et al., 2013; Liu et al., 2003).

Posttranslational modifications (PTM) increase the functional diversity of the proteome by the covalent addition of functional groups to specific residues on the protein. Lysine residue is a target for many PTMs which are mutually exclusive thus generating greater potential for cross regulation (Yang and Seto, 2008).

Lysine acetylation was first identified in histones which play a significant role in chromatin structure and dynamics and thereby gene transcription regulation (Singh et al., 2010). Intensive research in the field put forward non-histone proteins also to be targets of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Reversible acetylation in these proteins has been implicated in various cellular metabolisms such as protein and mRNA stability, protein subcellular localization and degradation, protein-protein interaction and DNA-protein interactions (Glozak et al., 2005; Singh et al., 2010). The founding member of non-histone acetylation target is the tumor suppressor and transcription factor protein p53 (Gu and Roeder, 1997). Acetylation of p53 has been shown to activate its ability to bind DNA and thus leading to the activation of its response genes (Ito et al., 2002).

Another protein E2F1 has been shown to be acetylated which increases its DNA binding ability and thereby E2F1 mediated transcriptional activation (Martinez-Balbas et al., 2000; Marzio et al., 2000). Ku70, a protein involved in the non-homologous end joining DNA repair pathway was shown to be regulated by acetylation. Upon DNA damage, the levels of Ku70 increase and inhibit apoptosis by directly interfering

with the activation of pro-apoptotic factor Bax. Ku70 was shown to bind Bax and thus avoids the translocation of Bax into mitochondria. Acetylation of two lysine residues in Ku70 disrupts its interaction allowing Bax to localize to the mitochondria and initiate apoptosis (Cohen et al., 2004).

Taken together, these studies highlight the distinct roles of acetylation like protein stability, protein-protein interactions and DNA-protein interactions (as summarized in Figure 1).

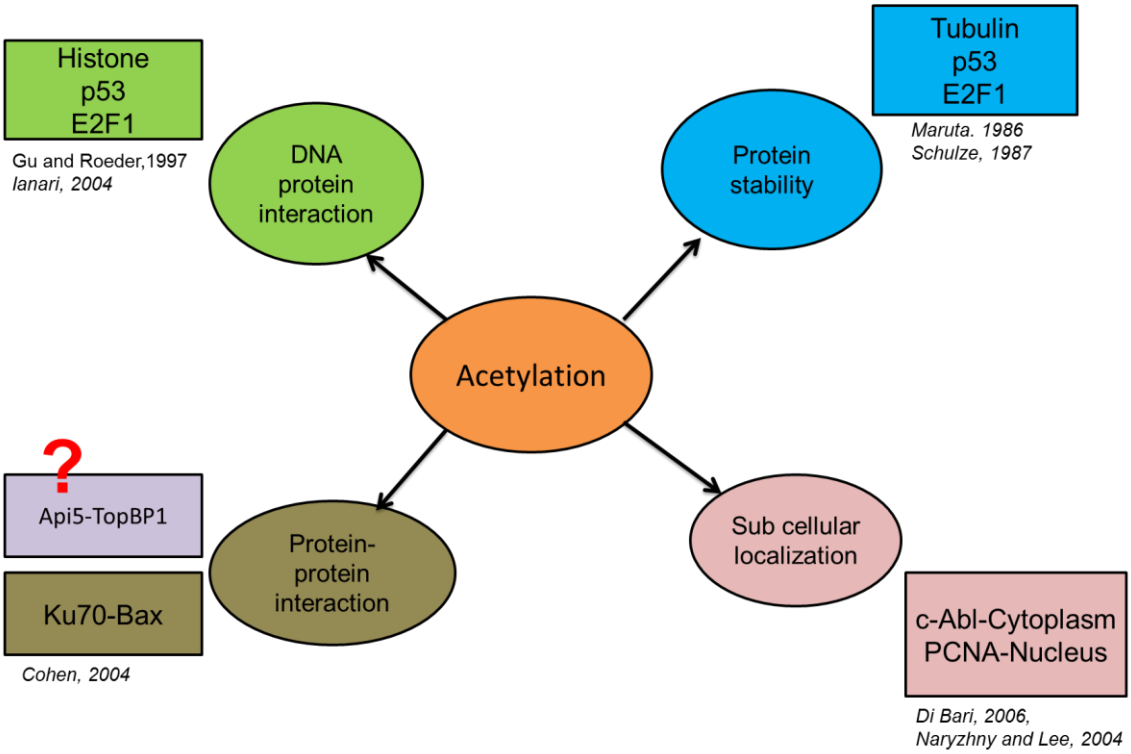

Figure 1: Various roles of Acetylation post-translational modifications in proteins. Histone, p53 and E2F1 when acetylated stabilize the protein and increase its DNA binding ability. Acetylation of tubulin α-subunit stabilizes the microtubules. c-Abl, a protein involved in myogenic differentiation upon acetylation accumulates in cytoplasm to carry out its function whereas PCNA upon acetylation localizes to the nucleus. Acetylation of Ku-70 abrogates its interaction with Bax, signaling for apoptosis. We are interested to study the effect of Api5 acetylation in its interaction with TopBP1.

Rationale of the study

A global acetylome analysis performed by Choudhary *et al* has shown that Api5 is acetylated at lysine-251 (Choudhary et al., 2009). The fact that lysine-251 residue is highly conserved across different species as shown in Figure 2 (Morris et al., 2006), has prompted Han and his colleagues to understand the role of K251 acetylation in mediating Api5 function. In their study, it was shown that unlike wild type Api5, over-expression of a constitutive acetylation mimic (Api5 K251Q) failed to inhibit apoptosis induced by serum starvation (Han et al., 2012). However, they could not answer how and why acetylation of Api5 inhibits its anti-apoptotic function.

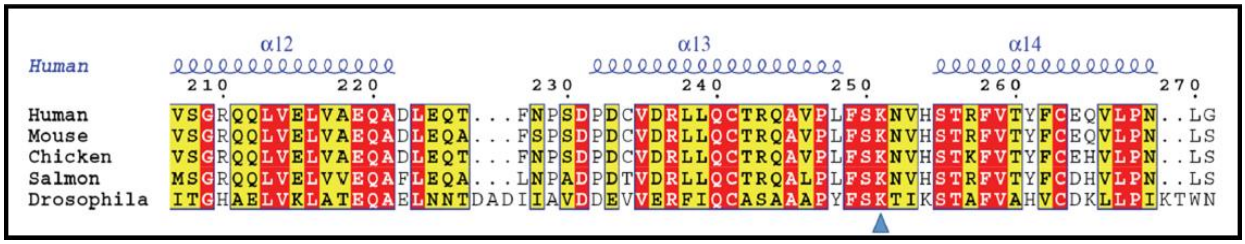

Figure 2: Conservation of Lysine 251 across various species (Han et al., 2012).

In the present study, we asked whether acetylation of Api5 sensitize cells in DNA-damage induced apoptosis as well. If yes, we would like to decipher the mechanism through which K251 acetylation inhibits anti-apoptotic function of Api5.

Also, Api5 is found to interact with a chromatin remodeling enzyme ALC1 which has been reported to interact with various DNA repair proteins like DNA-PKCs, Ku, XRCC (X-ray repair cross complementing protein) (Ahel et al., 2009). This implies that Api5 could mediate protein-protein interactions in DNA damage response pathway. Thus it would also be interesting to look at the role of Api5 in DNA damage response pathway.

On the other hand, in 2004 Liu *et al* and Morris *et al* in 2006 have shown that both TopBP1 and Api5 inhibit apoptosis independently (Liu et al., 2004; Morris et al., 2006). Preliminary results in lab demonstrate DNA damage-dependent enrichment of Api5 in full length GST-TopBP1 pull-down. Later it was shown that the BRCT 7 and 8 domains of TopBP1 interacted with N-terminal region of Api5 which includes Lysine 251 residue by far western blotting. Interaction of full length TopBP1 with Api5 has

also been confirmed *in vivo* (unpublished data). Hence we would like to investigate the role of Api5 K251 acetylation on its interaction with TopBP1. Preliminary studies in the lab, shows Api5 interaction with TopBP1 to be independent of its acetylation *in vitro*. In the current study, we would like to investigate the same *in vivo.*

Api5 is a cell cycle regulated protein, whose level peaks in cells at the end of G1 phase of their cell cycle and is minimal in M phase (Garcia-Jove Navarro et al., 2013). However it is not known how Api5 levels are regulated in a cell cycle dependent manner. This study is one of the reasons that prompted us to study the degradation pathway of Api5. Hence we were interested in understanding how the protein levels are regulated in a cell cycle dependent manner. Also in 2012, Han *et al* proposed how the turnover of Api5 is maintained in cells. According to them, Api5 protein is immediately acetylated at lysine 251 after its synthesis which stabilizes the protein. Upon deacetylation, Api5 protein becomes activated but unstable and carries out its function before undergoing degradation. This is yet another reason which prompted us to understand the degradation pathway.

Materials and Methods:

Chemicals and antibodies

1,4-Dithioerythritol (DTE), Acrylamide, bromophenol blue, ethylenediaminetetraacetic acid (EDTA) N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), sodium azide, Trizma® base, N,N,N′,N′-tetramethylethylenediamine (TEMED), Tween 20, sodium acetate, sodium pyrophosphate, sodium orthovanadate, sodium deoxycholate, dimethyl sulfoxide (DMSO), RNase A and polybrene were obtained from Sigma-Aldrich. Formaldehyde, glycerol, glycine, hydrochloric acid, methanol, potassium chloride, sodium chloride, sodium hydroxide, acetic acid, Isoamyl alcohol, and chloroform were bought from Fisher Scientific. Agarose, ethylene glycol tetraacetic acid (EGTA) and Phenol were purchased from Lonza, Amresco and Merck respectively. Triton-X-100 was purchased from USB Corporation. 3B prestained protein ladder was procured from Abcam. Polyclonal Api5 antibody (1:5,000) was purchased from Abnova. HA antibody (1:1,000), PARP-1 (1:100), β-catenin (1:2,000), GAPDH (1:40,000) were bought from Millipore, Calbiochem, BD Biosciences and Sigma respectively. Caspase-9 (1:1,000), Caspase-3 (1:1000) and Lamin B1 (1:500) were obtained from Abcam. Peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgG (H+L) and were acquired from Jackson Immuno Research. Alexa Fluor 488 Goat anti-rabbit IgG (H+L), Alexa Fluor 568 Phalloidin and Hoechst 33342-trihydrochloride trihydrate, were procured from Invitrogen. Bethyl laboratories provided the rabbit IgG used for immunoprecipitation studies. Ethanol was purchased from Honyon International Incorporation, China. Proteinase-K was a kind gift from Dr. Aurnab Ghose (Indian Institute of Science Education and Research, Pune, India). MG-132 was a generous gift from Dr. Manas Kumar Santra (National Centre for Cell Science, Pune, India) XX-155 UV Bench Lamp was purchased from Upland, CA, USA.

Cell lines and culture conditions

HeLa and MCF-7 cell lines were procured from European Collection of Cell Cultures (ECACC). HEK 293T cell line was a generous gift from Dr. Jomon Joseph (National Centre for Cell Science, Pune, India). All the cell lines were grown in High Glucose Dulbecco's Modified Eagle Medium (DMEM; Lonza) comprising of L-glutamine

complemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen), and 50 units/mL penicillin-streptomycin (Invitrogen). All cell lines were cultured in 100 mm tissue culture treated dishes (Corning or Eppendorf) in humidified 5% CO_2 incubator (Eppendorf) at 37°C. For cryo preservation, cells were frozen in DMEM containing 10% DMSO and 20% FBS.

Immunofluorescence analysis

2 X 10^5 cells seeded on cover slip (Blue star microscopic cover glass) in a six well dish was incubated for 16 hours. Following the respective drug treatments, wells were washed twice with 1X PBS. Cells were fixed using freshly prepared 4% formaldehyde solution for 20 minutes in dark at room temperature (RT) following which they were washed twice with 1X PBS and once with 1X PBS-glycine (1X PBS with 100 mM glycine) for 10 minutes each. Cells were then permeabilized using ice-cold 0.5% Triton-X-100 (prepared in 1X PBS) for 5 minutes at 4°C. Coverslips were gently washed thrice with 1X PBS for 10 minutes each which was followed by blocking in 10% FBS in 1X IF Buffer (1X PBS containing 0.05% [w/v] sodium azide, 0.1% [w/v] BSA, 0.2% [v/v] Triton-X-100 and 0.05% Tween 20) for 1 hour at RT. After blocking, cells were incubated with primary antibody prepared in blocking solution overnight at 4°C. Next day, cells were washed with 1X IF buffer for 20 minutes and twice with 1X PBS for 10 minutes each. Secondary antibody along with Phalloidin (1:500) prepared in blocking solution was incubated for 1 hour at RT. Following washes with 1X IF buffer; nucleus was counter stained with Hoechst 33342 (0.5 µg/ml) for 5 minutes at RT and then cells were washed with 1X PBS for 10 minutes. Coverslips were mounted with mounting medium (Glycerol: PBS, 9:1) on glass slides (Micro-Aid, India) and sealed with transparent nail polish. Slides were stored in a humid chamber overnight at 4°C and imaged the next day using 63X oil-immersion objective under a Zeiss LSM 710 laser scanning confocal microscope.

Immunoblot analysis

Cell were lysed in 2X Laemmli sample buffer composed of (Tris, pH 6.8, (0.06 mM), bromophenol blue (0.006%), sodium dodecyl sulphate (SDS, 2%), glycerol (6%), and dithiotreitol (DTT, 0.1 M)) and stored at -40°C. A SE 260 mini-vertical gel electrophoresis unit (GE Healthcare) was used to cast sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE). 10% resolving gel consisting of acrylamide (9.86%), bisacrylamide (0.14%), Tris, pH 8.8 (475 mM), SDS (0.1%), APS (0.1%) and TEMED (0.04-0.08% (v/v) and 5% stacking gel comprising of acrylamide (4.93%), bisacrylamide (0.07%), Tris-pH 6.8 (125 mM), SDS (0.1%), APS (0.1%) and TEMED (0.1% [v/v]) was used. Samples were pre-heated at 95°C for 5 minutes and were centrifuged at 13,400 rpm for 1 minute at RT. Lysates were loaded on to the polymerized acrylamide gel and resolved at 130 V and subsequently were transferred onto Immobilon-P polyvinyldifluoride (PVDF) membranes (Millipore) at 250 mA for 3 hours. 5% (w/v) non-fat dried skimmed milk (SACO Foods, US) prepared in 1X TBS-T (Tris Buffered Saline (TBS; Tris-pH 7.6 (25 mM), NaCl (150 mM) and KCl (2 mM) comprising 0.1% Tween 20) was used to block the PVDF membrane for 1 hour at RT. Blots were then incubated in primary antibody for 3 hours at RT or overnight at 4°C in the rotor. Blots after incubation were washed three times with 1X TBS-T for 20 minutes each and were incubated with respective peroxidase-conjugated secondary antibody solution at a dilution of 1:10,000 prepared in 5% skimmed milk for 1 hour at RT with slow shaking. Blots were further washed thrice with 1X TBS-T and developed using Immobilon Western Detection Reagent kit (Millipore). Images were obtained using ImageQuant LAS4000 gel documentation system (GE Healthcare). Densitometry analysis was performed on the increment images acquired using Image J.

Lentivirus production (using calcium phosphate) and transduction

For virus production, $1X10^6$ HEK 293T cells were seeded on 60mm dishes coated with Matrigel®. Matrigel® diluted with chilled PBS (1:500) was spread uniformly on the surface of tissue-culture treated dishes (Corning) and incubated for 16 hours at RT. The dishes were washed with PBS thrice. Cells were transfected 24 hours after seeding. 10 µg of the vector (PLKO1.eGFP or PLKO1.eGFP-shApi5), 7.5 µg of packaging plasmid (psPax2) and 3 µg of envelope plasmid (pMD2) were mixed to make plasmid mix of 250 µl using ultrapure DNase RNase free water (Invitrogen). 250 µl of 2X HBS was added followed by drop-wise addition of 25 µl of 2.5 M $CaCl_2$ and mixed gently. The mixture was incubated for 20 minutes at RT. The calcium phosphate-DNA mix was then added to HEK 293T cells and mixed gently by swirling the plate. Cells were incubated for 16 hours following which the media was replaced

with fresh DMEM containing 10% FBS. Virus containing medium was collected post 48 hours, filtered through a 0.45 µm filter and was added to HeLa cells along with 4µg/ml of polybrene.

Lentivirus production (using lipofectamine) and transduction

1.5 X 10^6 HEK 293T cells were seeded on a 35 mm dish and were incubated for 16 hours in high Glucose containing DMEM at 37°C. Following day, cells were co-transfected with PAX2 (2 µg), pMD2 (1 µg) and shApi5 (2 µg) or PLKO1.eGFP (2 µg) using Lipofectamine 2000 transfection reagent. Lipofectamine (20 µl) mixed with plain OptiMEM I Reduced Serum Medium (Invitrogen) (480 µl) was incubated for 5 minutes at RT. Meanwhile, lentiviral vector containing shRNA or control vector and packaging plasmids were mixed with OPTIMEM to make up the equal volume as that of Lipofectamine-OPTIMEM mixture. OPTIMEM containing Lipofectamine was added onto the DNA-OPTIMEM mixture and was further incubated for 20 minutes. During this time, cells were pre-incubated with OPTIMEM (1 ml) for 20 minutes. OPTIMEM was aspirated from the dish and the DNA- Lipofectamine-OPTIMEM mix was added drop-wise on to the cells. The cells were incubated for 24 hours following which 2 ml of 30% FBS-containing DMEM media was added. The viral supernatant collected after 48 hours was filtered through 0.45 µM filter and was added to HeLa cells seeded at a density of 0.6 X 10^6 in a 35 mm dish (Corning) along with polybrene (20 µg/ml). The HeLa cells were then incubated for 48 hours for efficient transduction after which cells were fed with fresh media.

Site directed mutagenesis

Primers required for site directed mutagenesis of siRNA resistant Api5 K251 mutations were designed and obtained from Sigma-Aldrich. Target siRNA sequence of Api5 is as follows: 5'- GACCTAGAACAGACCTTCA-3'.
Forward primer: 5'- GCCGAACTGGATCAGACCTTCA-3'.
Reverse primer: 5'- TGAAGGTCTGATCCACTTCGGC-3'.
The sequencing primer used was
Api5 K251 mutchk-1: 5'-AACAGACCTTCAATCCCTCG-3'.
Pfu Turbo DNA Polymerase was purchased from Stratagene, dNTPs from MP Biomedicals and Dpn1 restriction enzyme was from New England Biolabs. Plasmids

used for the study were Api5 K251Q mVenusC1 (acetylation mimic), Api5 K251R mVenusC1 (acetylation deficient) Api5 K251A mVenusC1 (uncharged mutant), with kanamycin resistance, and Api5 K251Q HAC NLS, Api5 K251R HAC NLS, Api5 K251A HAC NLS with ampicillin resistance, siRNA resistant Api5 pCDNA3.1 HAC3 with Ampicillin resistance. Site directed Mutagenesis for six of the Api5 acetylation mutants (HA-Api5 K251Q/R/A and mVenus-Api5 K251Q/R/A) was performed using the PCR mixture and PCR cycle given in Table 1 and 2 respectively. To digest the parental plasmid, PCR product was further subjected to Dpn1 digestion for 3 hours at 37°C. The digested mixture was then transformed in to DH5α cells and bacteria were plated in a solid agar plate containing antibiotics. The plate was incubated at 37°C overnight. Colonies obtained in the presence of respective antibiotics was inoculated. The plasmid DNA was isolated using Miniprep Kit was sent for sequencing (1st Base, Singapore).

Table 1: PCR mixture for site directed mutagenesis.

Plasmid	2 µl
10X Pfu buffer	5 µl
5’ Primer (25 µM)	2 µl
3’ Primer (25 µM)	2 µl
dNTPs (10 mM)	0.5 µl
Pfu Turbo	1 µl
Water	38.5 µl

Table 2: PCR cycles for site directed mutagenesis.

95°C	30’’	
95°C	30’’	20 Cycles
55°C	1’	
68°C	20’	
4°C	Hold	

Plasmid isolation and purification was done using the manufacturers’ protocol provided in the QIAprep spin Miniprep Kit (QIAGEN) or NucleoSpin Plasmid Miniprep Kit from Machery-Nagel (MN). Plasmid DNA purification Midiprep Kit was provided from Machery-Nagel (MN).

Transfection of cells

HeLa cells were seeded at a density of 2 X 10^5 in a 12-well tissue culture treated dish and were incubated for 12-16 hours at 37°C. Transfection was performed the next day following the protocol described below: Lipofectamine (4 µl) was mixed with OPTIMEM (96 µl) and was incubated for 5 minutes. Meanwhile, DNA (1 µg) was mixed with OPTIMEM to make up the volume to 100 µl. Further, the Lipofectamine-OPTIMEM mix was added to equal volume of DNA-OPTIMEM, mixed well and incubated for 20 minutes. Meanwhile, cells were incubated with 1 ml of OPTIMEM media for 20 minutes. Once the incubation was over, OPTIMEM was added to DNA-Lipofectamine-OPTIMEM mix to make up the volume to 1 ml. The cells were then

incubated with the above mixture for 4 hours following which 1 ml DMEM containing 30% FBS was added to the wells. Cells were incubated with the transfection mix for around 18 hours after which the media was replaced by normal 10% FBS containing DMEM. Cell lysates were collected after 24 hours of transfection.

Drug Treatment and Time-course assays

MCF-7 cells were seeded at a density of 1×10^6 per well in 35 mm tissue culture treated dishes (Corning) and cultured at 37°C for 16 hours. For, DNA fragmentation assay, cells were treated with 10 µM Camptothecin (CPT) for 16 hours following which cells were harvested as mentioned in the protocol in the DNA Fragmentation assay section. For studying the activation of apoptosis pathway upon UV treatment, cells were irradiated with UV (50 J/m^2) and were incubated back for time course experiment ranging from 2 to 12 hours. At respective time points, cell lysates were collected and stored at -40°C. For immunofluorescence studies, cells were seeded at a density of 2×10^5 and were treated as previously mentioned. For the ubiquitination study of Api5, cells were seeded at a density of 0.75×10^6 cells per well and incubated for 16 hours at 37°C. Following day, cells were treated with 5 µM MG-132 by direct addition to the culture medium for 6 hours. Control cells were treated with equivalent volume of DMSO (drug solvent). After drug treatment, medium-containing drug was aspirated out and cells were washed gently with 1X ice-cold Phosphate Saline Buffer (PBS, Lonza). Cells were lysed in Laemmli sample buffer and stored at -40°C.

siRNA knockdown

siRNA duplexes targeted towards LacZ and Api5 were purchased from Dharmacon (Thermo Scientific). Sense sequences of the siRNA are: Api5, 5'-GACCUAGAACAGACCUUCAUU-3', LacZ, 5′-CGUACGCGGAAUACUUCGAdTdT-3′. MCF-7 cells were seeded at a density of 0.5×10^5 cells per well in 12-well tissue culture treated plates and grown overnight at 37°C. Transfections were performed using X-tremeGENE siRNA transfection reagent (Roche) with a final siRNA concentration of 100 nM diluted in plain OptiMEM I Reduced Serum Medium. 4 hours post-transfection, DMEM complemented with 30% FBS was added to wells to achieve a final FBS concentration of 10%. siRNA transfection was repeated for

each of the sets after 24 hours. Cells were cultured at 37°C for an additional period of 48 hours before cells were lysed as mentioned earlier.

DNA fragmentation assay

HeLa cells were seeded at a density of 4 X 10^6 in 100 mm tissue cultured treated dishes and maintained at 37°C for 12-16 hours. Cells were then treated with 10 µM CPT and incubated for 16 hours. Control cells were treated with equivalent volume of DMSO (drug solvent). After drug treatment, culture medium containing apoptotic cells were collected. Attached cells were trypsinized and collected together which was then centrifuged at 5,000 rcf to obtain the cell pellet. Pellet was washed with 1X PBS (pH 7.4) and centrifuged again at 5000 rcf. This was then subjected for overnight cell lysis in 150 µl of TES cell lysis buffer (EDTA (10 mM), SDS (0.5%), Trizma Base (10 mM), Proteinase K (0.5 µg/ml), pH 7.5). Following day, Proteinase K was deactivated by heating the lysed samples at 75°C for 15 minutes. Samples were then incubated with RNase A (0.5 µg/ml) at 37°C for 2 hours. DNA isolation was carried out using phenol-chloroform-isoamyl alcohol method. Equal volume of the phenol: chloroform: isoamyl alcohol (25:24:1) solution was added and mixed till emulsion is formed. The mixture was then centrifuged at 12000 rcf for 5 minutes at RT. The upper transparent aqueous phase was separated to a new micro centrifuge tube (Axygen, Corning). The process was repeated till no opaque protein interface was observed. Further, equal volume of chloroform was mixed with the aqueous phase and centrifuged at 12000 rcf for 5 minutes. Upper layer was pipetted out to a new centrifuge tube. To this, 1/10 volume of 3 M sodium acetate, pH 5.2 and 3 volumes of 100% ethanol were added. The mixture was frozen overnight at -20°C or at -80°C for 1 hour. The solution is then spun down at 15,000 rcf in a micro centrifuge at 4°C for 30 minutes. The pellet obtained was air-dried and re-suspended in 40 µl TE buffer (Tris-HCl, pH 7.5 (10 mM), EDTA (1 mM)). The total DNA solution was loaded onto a 2% agarose gel stained with EtBr and was electrophoresed at 110 V for 2-3 hours. The images were acquired using GeneSnap, Syngene.

Immunoprecipitation (IP)

Cells were seeded in 60 mm tissue culture treated dishes such that it attains 70% confluence the following day. Cells were lysed in 150 µl of 1X RIPA buffer or IP lysis buffer (Tris-HCl (20 mM), NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), sodium deoxycholate (1%), sodium pyrophosphate (2.5 mM), sodium orthovanadate (1 mM), NP40 (1%), 1X Protease Inhibitor (PI). Protein concentration was estimated using BSA Protein Assay Kit from Thermo Scientific. 500-800 µg of protein diluted in 400-700 µl IP lysis buffer along with 1-3 µg of protease inhibitor was incubated overnight with 2 µg of desired antibody (Api5 or IgG) at 4°C. 10 ml magnetic protein beads A/G (Ademtech) were equilibrated thrice with IP lysis buffer, followed by incubation of whole cell-lysate for 4 hours at 4°C. The beads were then washed thrice with the lysis buffer for 5 minutes at room temperature. Samples were eluted in 40 µl of elution buffer containing 0.1 M glycine, pH 2 and 0.2 M bromophenol blue. Eluted samples were boiled at 100°C for 5 minutes followed by placing on ice for 2 minutes. The samples were then stored at -40°C.

Results

Generation of siRNA resistant acetylation mutants (an uncharged mutant, acetylation-deficient and a constitutive acetylation mimic)

To study the effect of Api5 acetylation in its interaction and function, the three acetylation mutants of Api5 were used, Api5 K251Q- acetylation mimic, Api5 K251R- acetylation deficient, Api5 K251A- uncharged mutant. *In vitro* biochemical interaction studies were successfully performed using site directed mutagenesis of full length Api5 cloned into two mammalian vectors, HA and mVenusC1 at the lysine 251 site. However, for *in vivo* studies, it was important to express the acetylation mutants in an Api5 null background. Towards this, siRNA resistant acetylation mutants were generated. Thus site directed mutagenesis was performed to replace three of the bases in the target siRNA sequence in the three acetylation mutants cloned in the two mammalian vectors, HA and mVenusC1. The desired mutation containing primers were designed and the same was used for mutagenizing and amplifying the plasmids. The following mutants were successfully prepared.

HA-Api5	**Api5-mVenusC1**
K251Q	K251Q
K251A	K251A
	K251R

Table 3: Successfully generated siRNA resistant HA-Api5 and mVenus-Api5 acetylation mutants.

All the desired mutants except HA-Api5 K251R were successfully generated. HA-Api5 and Api5-mVenusC1 acetylation mutants will be used for immunoprecipitation and microscopic studies respectively.

siRNA resistant HA-Api5 and Api5-mVenusC1 mutant protein expression was confirmed.

Two of the siRNA resistant HA-Api5 acetylation mutants and three siRNA resistant Api5-mVenusC1 mutants that were generated using site directed mutagenesis were transfected into HeLa cells to check for its expression in mammalian system. Following transfection lysates were collected after 24 hours. Cells transfected with only transfection reagent were used as negative control. Previously cloned HA-Api5 was used as positive control. Expression of mutant Api5 was confirmed by probing using HA and Api5 antibodies in HA-Api5 and Api5-mVenusC1 transfections respectively (Figure 3a and 3b).

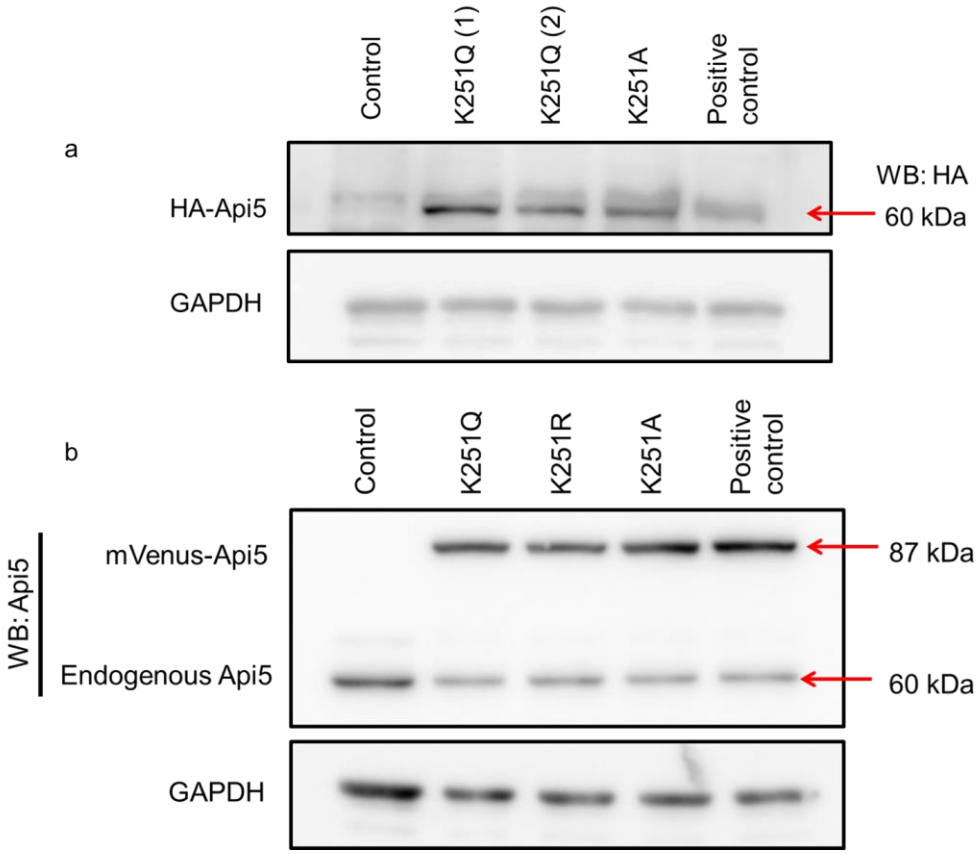

Figure 3: Expression of siRNA resistant HA-Api5 and Api5-mVenusC1 acetylation mutants was confirmed in HeLa cells. (a) HeLa cells were transfected with siRNA resistant HA-Api5 K251Q, acetylation mimic and HA-Api5 K251A, uncharged mutants. Lysates were collected after 24 hours and analyzed for the expression of HA-Api5 mutant protein expression by probing against HA-antibody. GAPDH was used as a loading control. **(b)** HeLa cells were transfected with the siRNA resistant Api5 acetylation mutants cloned in mVenus vector. Exogenous protein expression was confirmed by probing against Api5 antibody. Api5-mVenus protein was expressed at a higher molecular weight of 87 kDa. GAPDH was used as a loading control.

Api5 is indispensable for cell survival

To understand the functional significance of K251 acetylation, we sought to prepare stable Api5 knockdown cell line. For the same, shRNA for Api5 was cloned into PLKO1.eGFP lentiviral vector. For the viral production, PLKO1.eGFP empty vector or PLKO1.eGFP-shApi5 along with its packaging plasmids psPAX2 and pMD2 were transfected into HEK 293T cells. We had very little success in producing lentivirus using calcium phosphate method. After repeated failed trials with calcium phosphate method, we finally standardized the virus production method using lipofectamine transfection reagent.

Within 24 hours of transfection of plasmids using lipofectamine, HEK 293T cells were fluorescing GFP confirming the transfection of plasmids (Figure 4a). As shown in figure 4a, transfection efficiency was found to be around 95-100%. After 48 hours, the filtered viral supernatant collected was then used to transduce HeLa cells. Expression of GFP was not visible till 48 hours post transduction. These cells were then passaged, re-plated and allowed to grow. The cells started expressing GFP following the passage (Figure 4b). Thus expression of GFP observed after 48 hours confirmed the generation of stable Api5 knockdown cell line.

Interestingly, it was observed that shApi5 transduced cells underwent cell death within the next 24 hours and sparse number of cells were found to be attaching. The attached cells were not fluorescing GFP implying that knockdown of Api5 affects the viability of the cells. The experiment was repeated to confirm the results obtained. To further understand this phenomenon, various other viability and proliferation assays will be performed.

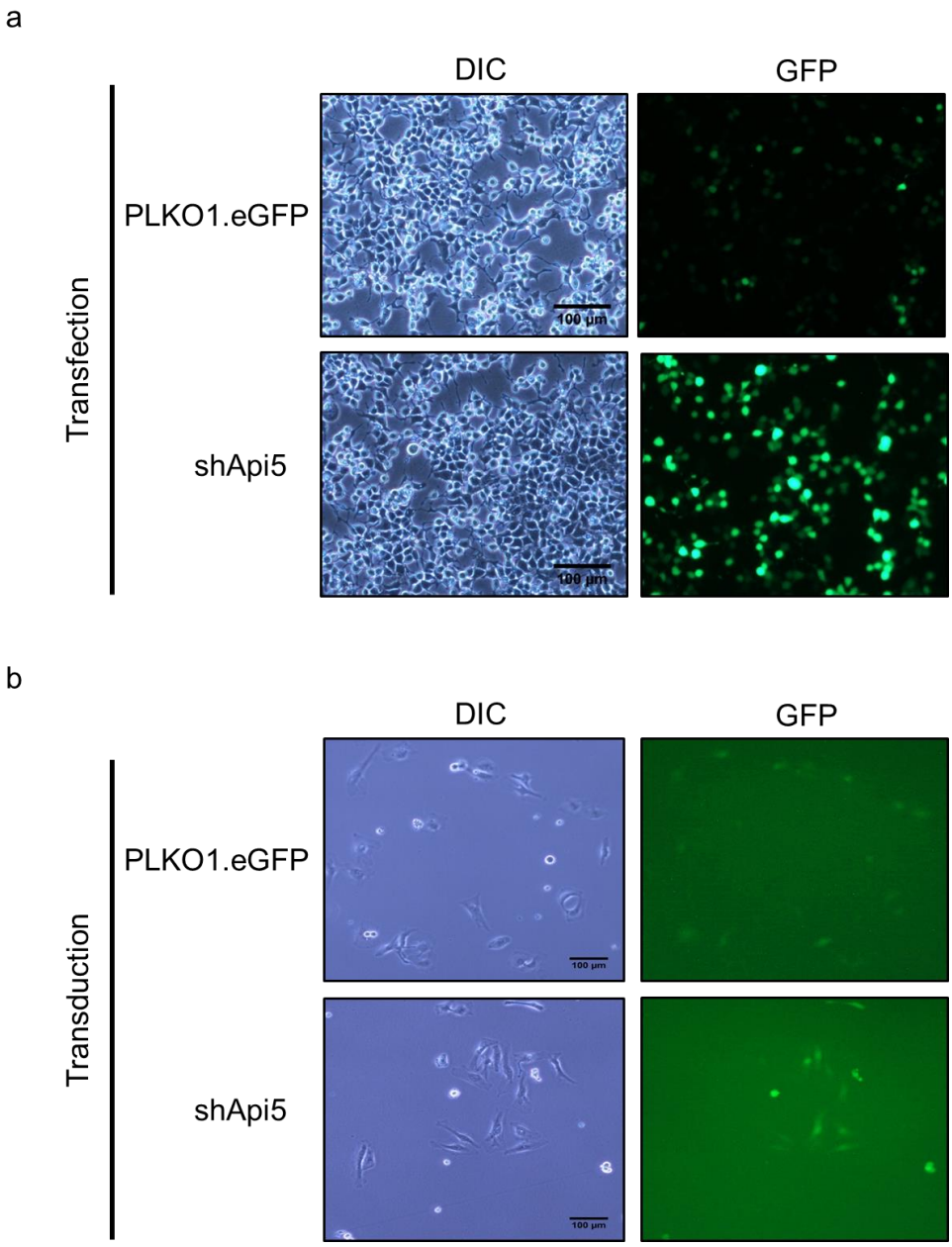

Figure 4: Api5 knockdown stable HeLa cell line was successfully generated. (a) HEK 293T cells were transfected with PLKO1.eGFP (Panel 1) or shApi5 (Panel 2) along with its packaging plasmids. **(b)** Viral supernatant collected from HEK 293T cells was used to transduce HeLa cells to produce cell line with stable knockdown of Api5. Upper panel shows HeLa cells expressing empty vector (control). Lower panel shows cells with Api5 knockdown. Phase contrast images were acquired using Nikon Microscope at 10X magnification.

Standardizing assays to decipher the role of Api5 acetylation in apoptosis

Api5 is known to inhibit apoptosis upon growth factor deprival (Tewari et al., 1997) as well as DNA-damage induced apoptosis (Rigou et al., 2009). Reports show that Api5 inhibits apoptosis through interacting with Acinus, a protein involved in DNA fragmentation (Rigou et al., 2009). Considering the possible role of acetylation on protein-protein interaction and the initial reports from Han *et al* 2012, (Han et al., 2012), We hypothesized that Api5 acetylation may affect the interaction with Acinus negatively thus sensitizing the cells to apoptosis leading to Acinus mediated DNA fragmentation. To address this, suitable drug and dose regimen and an apoptotic assay to assess the DNA fragmentation had to be standardized in the lab.

DNA Fragmentation

Camptothecin (CPT) is a widely known drug for its antitumor activity (Venditto and Simanek, 2010; Zeng et al., 2012). It binds to Topoisomerase I and inhibits nucleic acid synthesis resulting in single stranded breaks in mammalian DNA (Pommier, 2006). Down regulation of Api5 in the presence of CPT (10 μM for 16 hours) sensitizes U2OS cells to drug-induced cell death (Rigou et al., 2009). Also, studies in our lab have shown that this dose of CPT activates caspase-3. We thus decided to go ahead with this dosage of camptothecin for our studies.

We cultured HeLa cells in 100 mm tissue culture dishes following, which 10 μM CPT damage was given and cells were harvested after 16 hours. Genomic DNA extraction and precipitation was performed as per the protocol. The isolated DNA was electrophoresed in 2% agarose gel. Since 10 μM CPT induces caspase-3 activation, we expected DNA fragmentation upon the treatment.

Initially, 2% agarose gel was used for resolving the isolated DNA. However, as we observed that the DNA was not being able to resolve even after electrophoresing for 3 hours (Figure 5a), we reduced the percentage of agarose in the gel from 2 to 1.5. As shown in Figure 5b, no fragmentation of DNA (ladder of DNA was not formed) was observed in the treated lane.

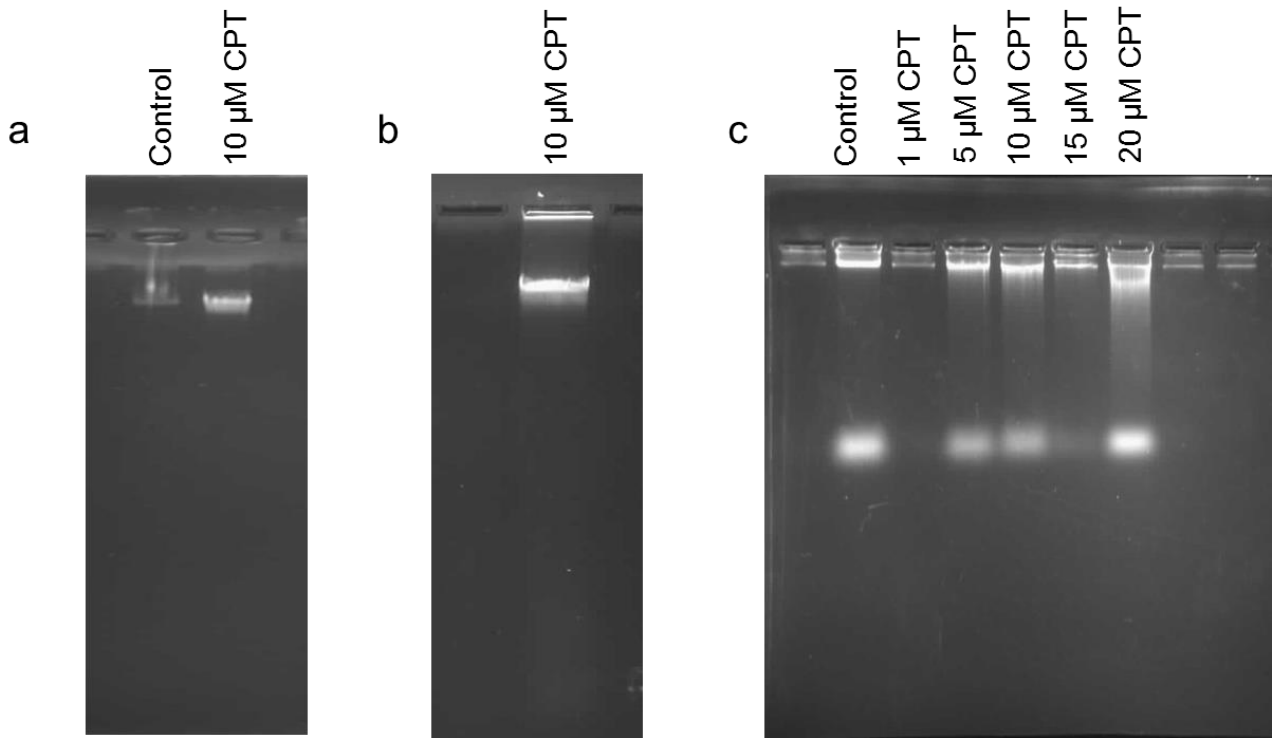

Figure 5: No DNA fragmentation was observed in 10 µM CPT treated HeLa cells for 16 hours. (a) 100 mm dish of HeLa cells either treated with DMSO or with 10 µM CPT treatment for 16 hours were subjected to DNA isolation. Isolated DNA from HeLa cells was electrophoresed for 3 hours in 2% agarose gel. **(b)** DNA isolated from 100 mm dish of HeLa cells treated with 10 µM CPT for 16 hours was electrophoresed for 2 hours in 1.5% agarose gel. **(c)** HeLa cells were treated with different doses ranging from 1, 5, 10, 15 and 20 µM CPT for 16 hours. DNA isolated from these cells was electrophoresed in 1.5% agarose gel for 2 hours.

Hence we decided to perform DNA fragmentation assay over a range of concentrations of CPT to investigate whether apoptosis is induced at this dose regime. A concentration ranging from 1 µM to 20 µM was added to the cells and DNA fragmentation assay was performed (Figure 5c). It was quite surprising that there was no DNA fragmentation throughout the dose range of CPT.

Hence we decided to irradiate HeLa cells with UV (50 J/m^2) and collect cells post damage in a time dependent manner from 2 hours up to 24 hours rather than treating with 10 µM CPT for 16 hours. We required a DNA damage dose as low as possible also because knockdown of Api5 was resulting in cell death and

transfection of mutant Api5 should be able to compensate for the knockdown. Damage dose incubation for longer time point might result in massive cell death, which is not desirable. Since Api5 is an anti-apoptotic protein, we believe that it would function at the borderline of survival and apoptosis. Once apoptosis has kicked in and caspase gets activated, it would be just a cascade of events leading to cell death. Thus we aimed to choose a time point wherein we observe initiation of caspase activation.

A time course experiment was performed to decide on the time point of caspase activation. HeLa cells were seeded in a 6-well dish and irradiated with 50 J/m^2 UV. The time points chosen for study were 2, 6, 8, 12, 18 and 24 hours post damage. We observed massive cell death (floating cells after treatment) in time points 18 and 24 hours post damage. Nearly 80% of cells had undergone apoptosis and were floating. All dead and attached living cells were collected to prepare lysates. 15% SDS-PAGE was carried out where we analyzed the activation of caspase-3 as readout for the activation of apoptosis.

As seen in Figure 6a, we did not observe cleaved caspase-3 band in any of the time points. It was also difficult to distinguish the right sized band due to a lot of non-specific bands in the blot. Unfortunately, the repeated experiment also gave similar results as before. Due to a lot of non-specific bands in caspase-3 blot, we were not able to distinguish the exact band of interest (Figure 6b).

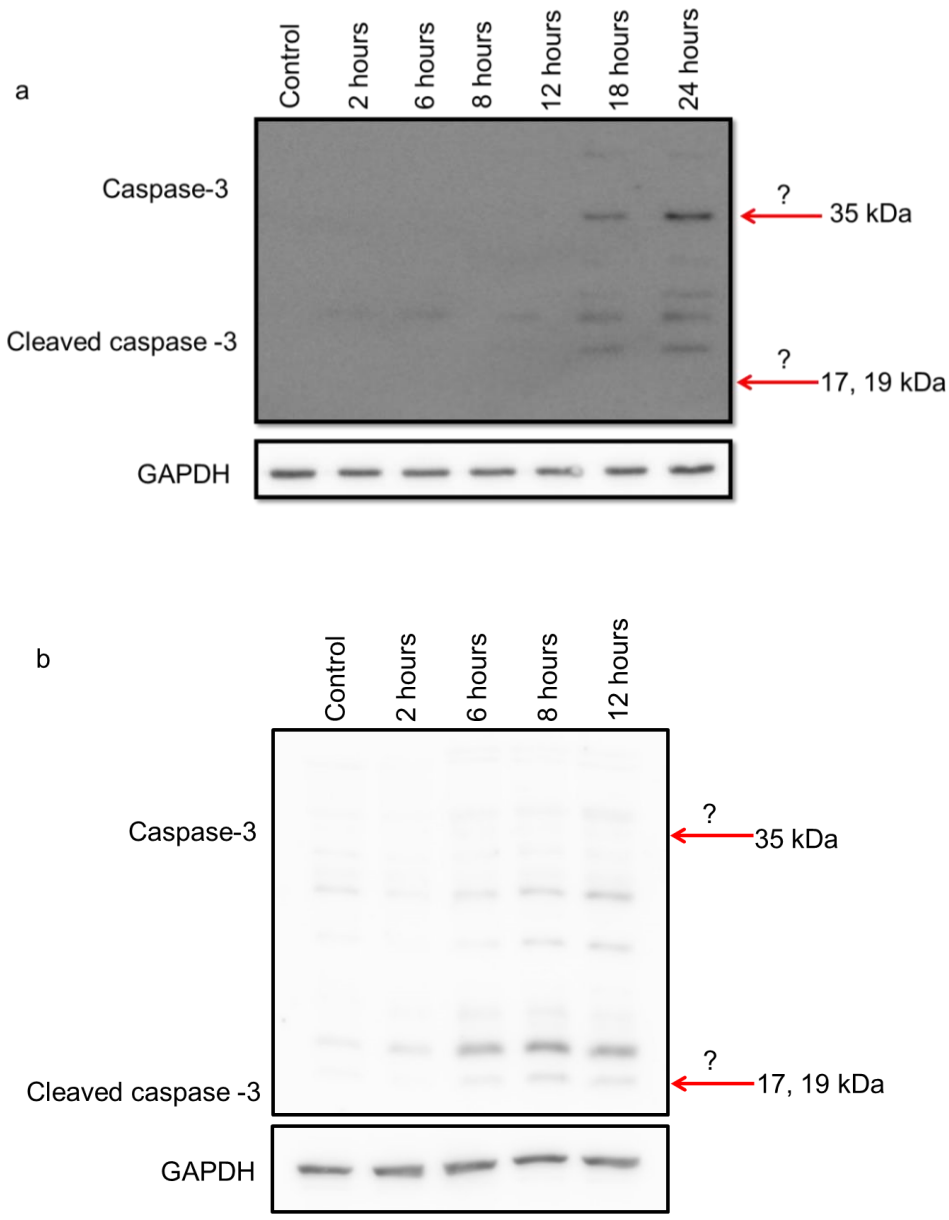

Figure 6: Activated caspase-3 could not be detected in HeLa cells post UV damage. (a) HeLa cells were irradiated with UV (50 J/m^2) and cell lysates were collected at various time points (2, 6, 8, 12, 18 and 24 hours) post UV damage. Apoptosis activation was analyzed by probing for caspase-3 in immunoblotting. **(b)** HeLa cells irradiated with UV (50 J/m^2) were collected and lysed at various time points (2, 6, 8, and 12 hours) post UV damage. Cell lysates were used for the analysis of activation of apoptosis by probing against caspase-3 in western blotting. GAPDH was used as a loading control.

We next decided to check for the activation of initiator caspase, caspase-9 in MCF-7 cells (caspase-3 mutated). Similar time course DNA damage study was carried out in MCF-7 cells and lysates were analysed for the activation of caspase-9 and cleavage of Poly (ADP-ribose) Polymerase 1 (PARP-1).

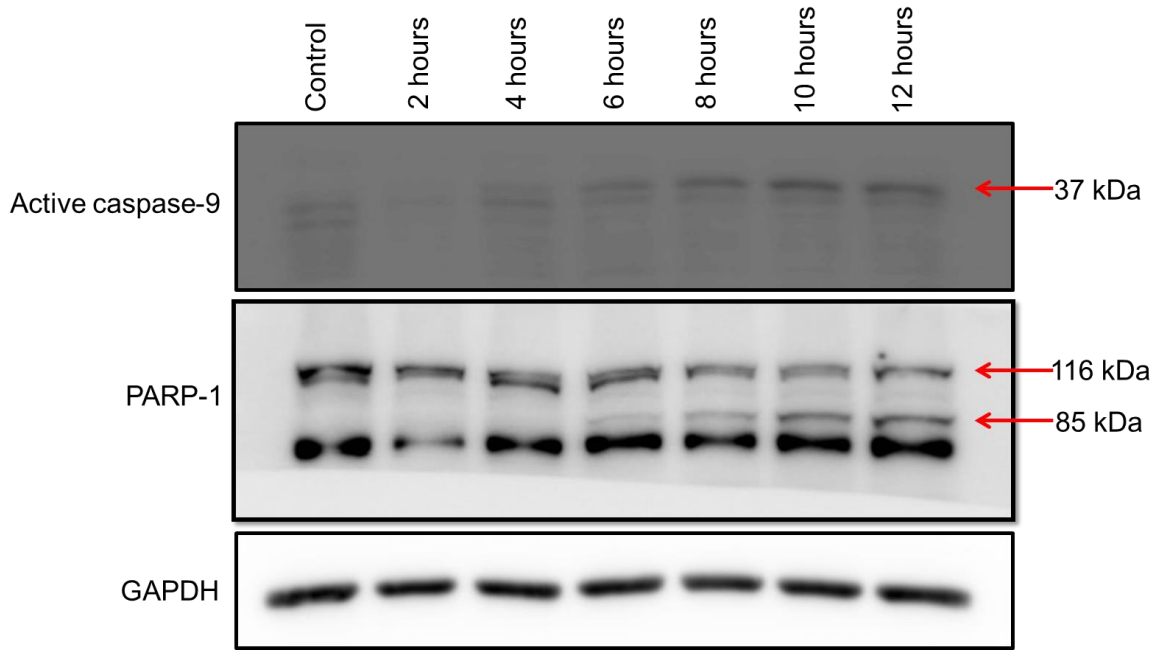

Figure 7: UV damage induced activation of caspase-9 and PARP-1 in MCF-7 cells. MCF-7 cells were irradiated with UV (50 J/m^2) and cell lysates were collected at various time points- 2, 4, 6, 8, 10 and 12 hours post UV damage. Lysates were run on SDS-PAGE and were analyzed for activation of apoptosis by probing against cleaved caspase-9 and PARP-1 antibody, to detect its cleaved product. GAPDH was used as a loading control.

We observed caspase-9 activation and PARP-1 cleavage beginning from 6 hours and persisting till 12 hours post UV damage. No PARP-1 cleavage was observed till 4 hours. The data obtained suggests that apoptosis was induced between 4 and 6 hours of post damage. As we were interested in performing further assays at the boundary between cell survival and apoptosis, under the conditions of the study, we considered 6 hours for further experiments, which will give insights into the role of Api5 acetylation mutants during the initiation of apoptosis and 8 hours, where cells are already in the apoptotic phase.

Nuclear fragmentation

Cells when undergoing apoptosis show distinct nuclear morphology like nuclear chromatin condensation and fragmented nuclei, which are considered as one of the morphologic hallmarks of apoptosis (Saraste and Pulkki, 2000). Also, lamin B, a component of inner nuclear membrane has been reported to undergo cleavage upon activation of apoptotic pathway (Orth et al., 1996; Takahashi et al., 1996). Nuclear morphological change was used as another read out for apoptosis, specifically DNA fragmentation. Towards this end, cells irradiated with UV were fixed at various time points 8, 10 and 12 hours post damage and were stained for lamin B1. Nucleus was counterstained with Hoechst 33342.

Under the conditions of our study, we did not observe nuclear membrane disruption in the UV-irradiated cells. Lamin B1 was clearly localized to the nuclear membrane and did not seem to be disrupted. However, the overall cellular and nuclear morphology of UV-treated cells appeared different as compared to control.

In addition to the above findings, we observed that lamin B1 along with staining the nuclear membrane was found to be localized in the cytoplasm of all the UV-treated cells. As shown in Figure 7, this localization appears to have increased in 12 hours post UV damage even though nucleus seemed intact. Nothing has been reported so far regarding the localization of lamin B1 to the cytoplasm upon activation of apoptosis, which could be an interesting question to explore.

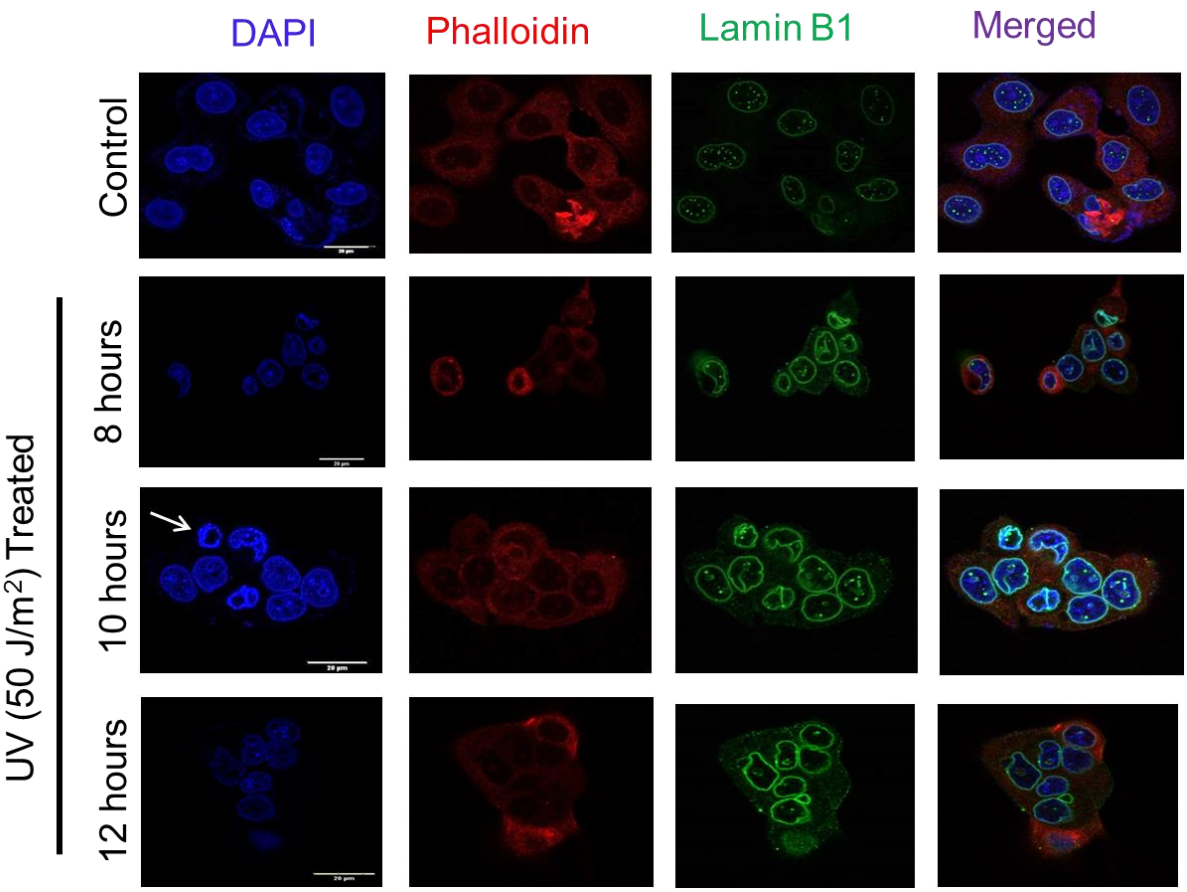

Figure 8: Pre-apoptotic nuclear morphology and cytoplasmic localization of lamin B1 was observed upon UV treatment in MCF-7 cells. MCF-7 cells exposed to UV (50 J/m^2) radiation were fixed at various time points 8, 10 and 12 hours. Cells were then permeabilized, blocked with 10% serum and were stained for lamin B1. Nuclei were counterstained with Hoechst 33342 to observe apoptosis induced nuclear morphological changes, if any. Phalloidin (dye that stains Actin) was used as marker for cell boundary. Images were taken at 63X oil-immersion objective under a Zeiss LSM 710 laser scanning confocal microscope. At least, 50 cells per treatment group were acquired and were analyzed.

Role of Api5 acetylation in its interaction with TopBP1

Previous studies in the lab have shown that Api5 interaction with TopBP1 is independent of acetylation *in vitro* using far western blotting. In the current study, to analyze the interaction of Api5 acetylation mutant protein and TopBP1 *in vivo,*

immunoprecipitation (IP) was performed. Endogenous Api5 was pulled down and checked for its interaction with endogenous TopBP1. After resolving the IP eluent in an 8% SDS-PAGE, Api5 protein was not detected on the PVDF membrane. Repeating the experiment again with a highly confluent 60 mm dish still failed to give desired protein concentration (estimated using protein estimation Kit). We believe that we are losing protein during cell lysis step using RIPA buffer. The procedure has to be repeated with a higher concentration of NP-40 increasing from 0.1% to 0.5%. Once the assay is standardized, we would pull down acetylation mutants and analyze its interaction with TopBP1.

Investigating the degradation pathway of Api5

Reports from Navarro *et al* about the cell cycle regulated levels of Api5 and from Han et al about the stability of Api5 protein prompted us to investigate the degradation pathway of Api5. To begin with, we decided to check whether Api5 is undergoing degradation through ubiquitination pathway. HeLa cells were treated with 5 μM MG-132, a protease inhibitor for 6 hours. Control cells were treated with equivalent volume of DMSO (drug solvent). Cell lysates were collected following treatment, and immunoblotting was performed to check for the enrichment of Api5 protein levels in the treated lane.

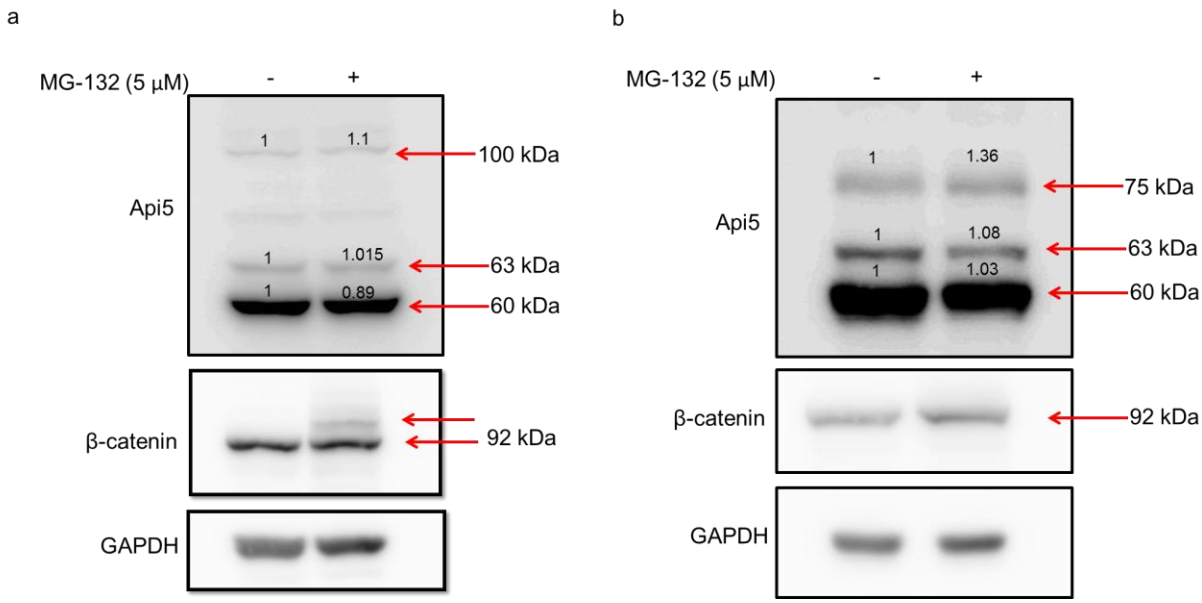

Figure 9: Api5 levels were not altered by MG-132 treatment. (a) HeLa cells treated either with DMSO or with 5 µM MG-132 for 6 hours were collected in sample buffer and were stored at -40°C. Lysates collected were used for immunoblotting, where the blot was probed against Api5 antibody (upper panel) and β-catenin antibody (lower panel). β-catenin is used as positive control. Api5 levels were not altered after MG-132 treatment while β-catenin showed an extra band in the MG-132 treated cells, probably due to the accumulation of ubiquitinated β-catenin. GAPDH was used as a loading control. Data is representative of two independent experiments. **(b)** MCF-7 cells were treated either with DMSO or with 5 µM MG-132 for 6 hours. Following the treatment, cells were collected in 2X laemmli buffer and were stored at -40°C. Lysates were heated at 95°C for 5 minutes and then were loaded on to the polymerized gel. Proteins after transfer on to the PVDF membrane were analyzed for the levels of Api5 (upper panel) and β-catenin (lower panel). β-catenin is used as positive control. Api5 levels were not altered after MG-132 treatment while β-catenin levels were enriched in MG-132 treated cells. GAPDH was used as a loading control. Data is representative of two independent experiments.

We could not find any two-fold enrichment of Api5 after MG-132 treatment in HeLa cells (Figure 8a). We also repeated the experiments in MCF-7 cell line. We observed no significant fold increase upon MG-132 treatment even in MCF-7 cells (Figure 8b).

Discussion

Post translational modifications diversify and modulate the functions of proteins both in a quantitative and qualitative manner. These modifications dynamically regulate proteins in a spatio-temporal manner and can work as molecular switches either activating or inhibiting specific signaling pathways by recruiting proteins in and out of transient preformed protein complexes in response to stress conditions such as DNA damage. Although Api5 acetylation has been shown to sensitize cells in serum starvation induced apoptosis, (Han et al) it is not clear how and why acetylation of Api5 inhibits the anti-apoptotic function of Api5. Here, in the current study we made an attempt to investigate and understand how and why acetylation of Api5 inhibits its function.

Api5 has been shown to interact with Acinus, a protein involved in apoptotic DNA fragmentation (Rigou et al., 2009). This physical interaction of Api5 with Acinus inhibits the caspase-3 mediated Acinus cleavage which otherwise would produce a p17 fragment responsible for DNA fragmentation upon apoptosis induction (Rigou et al., 2009) On the other hand, it has been reported that K251 acetylation negatively regulates anti-apoptotic function of Api5 (Han et al., 2012) We presume that Api5 acetylation could play a negative role in its interaction with Acinus thereby enhancing DNA fragmentation which could result in sensitization of cells to apoptosis.

To address this working hypothesis in the study, our major concern was to choose and standardize suitable assays for apoptosis. As we hypothesize that Api5 acetylation could indirectly affect DNA fragmentation, we tried standardizing DNA fragmentation assay and also monitored nuclear morphological changes induced in cells undergoing apoptosis.

Here, in this study it was observed that HeLa cells treated with 10 µM CPT for 16 hours did not show any DNA fragmentation. This was quite surprising as this concentration could activate apoptosis and induce significant amount of cell death as confirmed using thiazolyl blue tetrazolium bromide (MTT)-based cytotoxicity assay (unpublished data, Lahiri Lab). This could be explained based on the findings from Zare-Mirakabadi and his colleagues (Zare-Mirakabadi et al., 2012). Herein the authors report that CPT can induce both apoptosis and necrosis in HeLa cells. They have shown that concentration as low as 0.1 µg/ml CPT treatment for 48 hours

induces nuclear morphological changes and DNA fragmentation. Any dose of CPT greater than 0.1 µg/ml would induce necrosis in cells. The DNA band we observed in our DNA fragmentation assays is similar to what they had shown for necrosis effect. According to this report, the concentration of CPT used in our experiments could possibly be inducing necrosis and thus does not induce DNA fragmentation.
We further observed that UV (50 J/m^2) exposure caused no prominent change in the integrity of nuclear membrane as evident by intact Lamin B1 staining. However, globally the nuclear morphology of UV treated cells was observed to be different as compared to the control. This change in nuclear morphology has been characterized and reported as pre-apoptotic nuclear morphology (Johnson et al., 2000). Similar nuclear morphology has been observed in MCF-7 cells treated with staurosporine. These are characterized as convoluted nuclei with cavitation and clumps of chromatin connected to inner nuclear envelope (Johnson et al., 2000). We assume that apoptosis has begun and is still at the initial stages even after 12 hours post damage. Also we observed clear cytoplasmic localization of lamin B1 in cells irradiated with UV. This is one of the interesting questions raised in our study, which requires further validation.

Though we did not observe any nuclear fragmentation upon UV treatment within 12 hours post damage, we observed the activation of caspase 9 and PARP-1 cleavage as early as 6 hours post damage. Api5 being an anti-apoptotic protein when knocked down should sensitize cells and induce apoptosis at an earlier time point compared to normal control cells (6 hours for activation of caspase). We investigated the time dependent activation of caspases in UV irradiated Api5 knockdown cells. Following the results from these experiments, rescue experiments using acetylation mutants will be performed and markers of apoptosis will be probed and analyzed to understand the role of acetylation in its anti-apoptotic function. Meanwhile, characterization of interaction of Api5 acetylation mutants with Acinus may strengthen our working hypothesis.

Stable knockdown of Api5 in HeLa cells, suggest that Api5 is vital for cell viability, one of the key findings of the study. Our findings are supported with the published report that Api5 depletion affects cell proliferation in H1299 cells (Garcia-Jove

Navarro et al., 2013) Thus, Api5 could be indispensable for cell survival and a cell-cycle regulated protein.

Degradation pathway of Api5

Api5 is a cell cycle regulated protein, whose levels are high in G1 phase while low in M phase (Garcia-Jove Navarro et al., 2013). Till date there are no reports of how Api5 levels are regulated in a cell cycle dependent manner. Indeed there are no reports pertaining to degradation and turnover of Api5 in the cell. However, Han *et al* proposed the mechanism of turnover of Api5. Here, it was reported that Api5 is immediately acetylated at lysine 251, which resulted in stabilization of the protein. On the contrary, deacetylation of Api5 destabilized the protein and underwent degradation (Han et al., 2012). We predict that Api5 could have a cross regulation between acetylation and ubiquitination at lysine 251 residue. When acetylated, protein is inactive and stable. Upon deacetylation, protein becomes active. But at the same time, it also exposes the K251 site for ubiquitin ligases to act upon. This could be the reason for the Api5 instability. Unraveling Lysine 251 cross talk would be a step ahead in validating the hypothesis.

But interestingly we did not observe any significant enrichment of Api5 upon MG-132 treatment in HeLa as well as in MCF-7 cells. It has been reported that MG-132 (proteasome inhibitor) inhibition blocks apoptosis induced by severe UV damage (Zhang et al., 2011). Since the initial data using MG-132 suggest that Api5 does not undergo proteosomal degradation, the possibility could not be neglected. Api5 being an anti-apoptotic protein can be expressed at its basal level when cells are not threatened by any kind of stress of damage. In such a system, it could be difficult to see subtle changes in protein level upon proteosome inhibitor treatment, MG-132, with a technique such as immunoblotting which depicts population level changes. Hence it is essential to concentrate the level of Api5 protein in these cells. As genotoxic insults such as low doses of UV damage, would threat the very existence of the cells which will activate the DNA repair and anti-apoptotic pathways. We expect a significant increase in Api5 protein levels in such a situation. Therefore, MCF-7 cells will be subjected to UV damage followed by pre-treatment with 5 µM MG-132 for 1 hour in the treatment set whereas control set will be UV-irradiated with

no inhibitor treatment. At a UV dose where damage becomes irreparable, cells would undergo apoptosis. As hypothesized we expect Api5 to be ubiquitinated upon apoptosis signaling. As observed by Zhang *et al (Zhang et al., 2011)*, we expect to see a delay in apoptosis activation in the treated set of cells. Along with it, we may also expect a reduction in the levels of Api5 when treated with UV without pre-treatment with MG-132 and enrichment of the same upon inhibitor treatment if Api5 undergoes degradation through proteasome pathway.

In summary, we generated the necessary acetylation mutants to carry out further experiments and our initial experiments suggest that Api5 is indispensible for cell survival. Also, for the first time, we show the localization of lamin B1 to cytoplasm upon UV treatment. The results obtained in this study are interesting and would be confirmed performing various other experiments.

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