ROLE OF ACETYLATION OF API5 AND ITS INTERACTION WITH TOPBP1

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By

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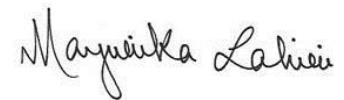Indian Institute of Science Education and Research (IISER)

Pune, India.

Thesis Guide: Dr. Mayurika Lahiri

Thesis Advisor: Dr.Gayathri Pananghat

Certificate

This is to certify that this dissertation entitled *"Role of acetylation of Api5 and its interaction with TopBP1"* towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Marina Victor** at IISER Pune under the supervision of Dr. Mayurika Lahiri, Assistant Professor, IISER Pune Biology Department during the academic year 2014-2015.

Mayurika Lahiri

Assistant Professor

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Declaration

I hereby declare that the matter embodied in the report entitled *"Role of acetylation of Api5 and its interaction with TopBP1"* are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of **Dr. Mayurika Lahiri** and the same has not been submitted elsewhere for any other degree.

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Abstract

The molecules involved in the checkpoint pathways are crucial in implementing different cellular events like cell cycle arrest, DNA repair or apoptosis, depending upon the extent of DNA damage. TopBP1 is a mediator protein in this pathway and is known to inhibit E2F1-mediated apoptosis. Another protein Api5, which is an anti-apoptotic protein, is also shown to inhibit E2F1-mediated apoptosis. Studies performed in the lab have shown an interaction between TopBP1 and Api5. Api5 is acetylated at Lysine 251 and this has been predicted to play a role in its activation and or function. Interestingly, lysine 251 falls in region of Api5, which was shown to be interacting with TopBP1, and this motivated the hypothesis that this acetylation may be playing a role in the Api5-TopBP1 interaction. *In vitro* interaction studies by far western blotting, using Api5 lysine 251 acetylation mimic, deficient and uncharged mutants as prey and TopBP1 as bait provided no difference in the interaction in the absence of acetylation for the wild type and mutants. Preliminary imaging experiment results using similar lysine mutants of Api5 in mVenusC1 mammalian expression constructs suggested that the TopBP1 foci formation upon DNA damage was independent of the acetylation status of Api5. Api5 was initially thought to be essentially a nuclear protein but prior experiments done in the lab showed it to have a cytoplasmic presence as well. Lysine acetylation is also known to be involved in the nuclear-cytoplasmic shuttling of proteins upon certain stimuli, so investigating its role in such possible shuttling of Api5 upon DNA damage may put some light upon a possible mechanism of Api5 function in response to stimuli. Whereas lysine 251 uncharged Api5 mutant was found to be majorly nuclear, wild type Api5, whose lysine 251 residue is capable of undergoing acetylation and de-acetylation, showed a significant cytoplasmic presence, indicating a possible role of the acetylation status of K251 in the subcellular localization of Api5.

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It is a great pleasure to thank my mentor, **Dr. Mayurika Lahiri** for giving me the opportunity to work in her group for the last two years. I do sincerely acknowledge her willingness to share new ideas, enthusiasm to initiate novel projects and determination to drive them to an end. This research experience is a foundation from which I have numerous lessons to carry forward with me and use them in my career as a researcher. I also owe my gratitude to Dr. Gayathri for her helpful suggestions on this project.

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The successful implementation of this project would not have been possible for me without the great scientific and learning environment provided by IISER Pune and the facilities of the Biology Department. I am also highly obliged by INSPIRE providing me stipend throughout my entire duration at IISER, Pune. Above all, I would like to thank my parents and family for their emotional support for last five years.

Introduction

DNA damage and Checkpoint proteins

Cells in our body are constantly subject to various endogenous as well as exogenousgenotoxic stresses which result in DNA damage.These DNA damaging agents can be intrinsic as well as extrinsic. Intrinsic genotoxic agents include products of metabolism like reactive oxygen species (ROS), Nitric oxide (NO), alkylating agents etc (Massague, 2004). Extrinsic DNA damaging agents include ultraviolet radiations (UV) causing T-T dimerization andsingle strand breaks (SSB), ionizing radiations causing double strand breaks (DSB) anda host of various different drugs. If these damages remain unchecked it will result in genomic instability or even cancer. Fortunately, the cell has evolved an elegant genome integrity surveillance mechanism called cell cycle checkpoints that monitor the cellular environment at the G1/S, S, G2/M and M phases (Fig 1A).The signals generated as a result of DNA damage will cause cell cycle to arrest, to allow time for the damage to be repaired. Abnormalities in the activation of checkpoints will lead to genome instability, which will finally result in cancer (Yamane et al., 2002).
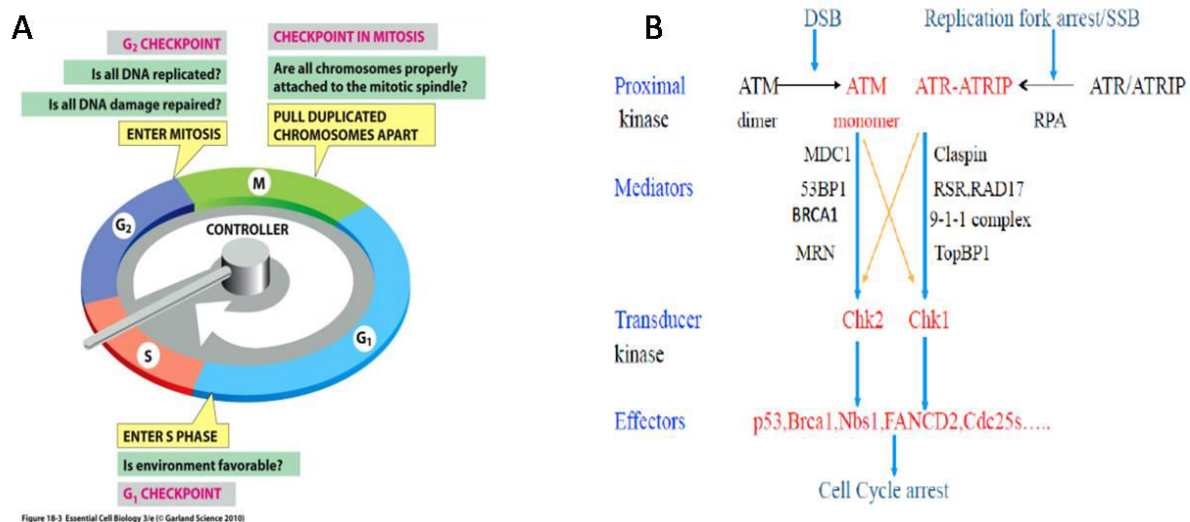

Fig 1: A) Cell cycle checkpoints (Essential cell Biology 3/e). B) The canonical checkpoint signaling pathway and major proteins involved (Abhinav, unpublished)

The checkpoint pathway includes various molecules like initiators, mediators, transducers, and effectors which together trigger the activation of the checkpoint

pathway (Fig 1B). Among these molecules TopBP1 is one of the major mediator proteins which get activated upon single stranded breaks (Wang and Elledge, 2002).

TopBP1: DNA Topoisomerase 2-binding protein 1

The DNA Topoisomerase 2-binding protein 1 or the TopBP1, encoded by TopBP1 gene was shown to interact with the C-terminal domain of the topoisomerase-II beta in a yeast 2-hybrid screen (Wang and Elledge, 2002). TopBP1 shares structural and functional similarities with molecules like Dpb11 (*S. cerevisiae*), Cut5/Rad4 (*S. pombe*), Mus101 (*D. melenogaster*) and Xmus101 (*X. laevis*) *(Araki et al., 1995; Hunt et al., 2013; Morishima et al., 2007; Ogiwara et al., 2006; Parrilla-Castellar and Karnitz, 2003)*. It is a BRCA1 C-terminus (BRCT) domain rich protein that is structurally and functionally conserved throughout all eukaryotic organisms. Human TopBP1 has eight BRCT domains, three BRCT-related regions and one poly-ADP ribose polymerase homologous region and a putative ATR activation domain between BRCT domains VI and VII (Garcia et al., 2005) (Fig 2).

Fig 2: Domain organization of TopBP1.

 In addition to its role as a mediator protein in the ATR-checkpoint pathway, TopBP1 is also involved in chromosome replication and regulation of transcription (Bang et al., 2011; Garcia et al., 2005; Jeon et al., 2011). TopBP1 also has a role as transcriptional activator or repressor. BRCT domain 4 has been shown to be an activator and adjacent BRCT domains 2 and 5 to be repressors of transcription (Jeon et al., 2011). TopBP1 has been shown to activate transcription of HPVE2 transcription factor (Boner et al., 2002) and also repress the transcription of c-Abl (Boner et al., 2002). One of the notable roles of TopBP1 is in its interaction with the E2Fs. TopBP1 interacts with E2F1 through its BRCT domain VI (Liu et al., 2003) and suppresses E2F1-mediated apoptosis during normal cell cycle and DNA damage. This is brought about by the transcriptional repression function of TopBP1. TopBP1 interacts with E2F1 and recruits Brg1/Brm (key components of SWI/SNFchromatin remodelling complex) into a repressive complex at E2F1 responsive promoters, thus inhibiting E2F1 mediated apoptosis (Liu et al., 2004) (Fig 3).
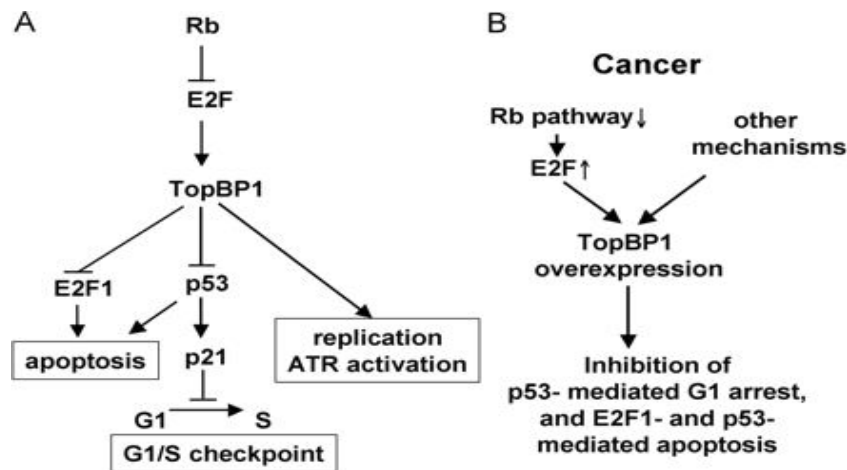
Fig 3: An integrated model for the function of TopBP1 in normal cells and in cancer cells. (A) TopBP1 is induced by E2F during G_1/S transition. (B) TopBP1 is up-regulated through deregulation of the pRb pathway or other mechanisms (Liu et al., 2009).

Api5: Apoptosis Inhibitor 5

Role of programmed cell death (apoptosis) is very crucial in various cellular mechanisms like tissue renewal, repair, development and tumor (Koci et al., 2012). Though there is lot of literature about several proteins that initiate apoptosis; very few reports involve the study of the proteins that can inhibit apoptotic signals. Api5 or Apoptosis Inhibitor 5 also known as AAC-11 (Anti-Apoptotic Clone -11) is a 60kDa nuclear protein. As the name suggests, Api5 mediates anti-apoptotic signaling in tumor cells. Studies have shown that Api5 is up-regulated in some cancers such as non small cell lung cancer, colorectal tumors, B cell chronic lymphoid leukemia as well as cervical cancer (Garcia-Jove Navarro et al., 2013; Gnanasekar et al., 2013; Rigou et al., 2009). Api5 is present in all species, from plants to flies to humans, but is absent in yeast and worms (Morris et al., 2006). It exhibits an all-alpha helical structure made up of 19 α helices and two 3_{10} helices (Han et al., 2012). There are three distinct domains - an domain with LxxLL motif believed to provide stability to the protein, an a typical leucine zipper domain (LZD) predicted to be involved in protein-protein interactions but lacking DNA binding function as well as a nuclear localization signal (NLS) at the C-terminus of the protein (Han et al., 2012) (Fig 4).

Fig 4: Illustration of Api5 protein structure showing domains with LxxLL, LZD and NLS.

In 2006 Morris and Dyson have shown Api5 to function as astrong and specific suppressor of E2F1-mediated apoptosis in *Drosophila* as well as in mammalian cells (Morris et al., 2006). Api5 was shown to be a negative genetic modifier of E2F1 in flies, its inhibition enhancing the E2F1-mediated apoptosis phenotypes and *vice versa*. Over-expression studies showed Api5 to inhibit E2F1-mediated apoptosis inmammalian cells without affecting the transcriptional activation function of E2F1, predicting it to function downstream of E2F1 (Morris et al., 2006).

Api5 and TopBP1

Role of TopBP1 in E2F1-induced apoptosis inhibition has already been shown (Liu *et al.,* 2004) and Api5 has also been shown to inhibit E2F1-induced apoptosis (Morris *et al.,* 2006). In addition to this, previous work done in the lab showed DNA damage-dependent enrichment of Api5 in a full-length GST-TopBP1 pull-down. These observations lead to the characterization of the interaction between TopBP1 and Api5 and investigation of its implications in checkpoint response or apoptosis in response to DNA damage. Furthermore the study done by Abhinav in the lab confirmed the direct biochemical interaction of TopBP1 and Api5 *in vitro* (Fig 5) (Unpublished). His studies also showed that Api5 does not get recruited to sites of DNA damage and does not co-localize with damage-induced TopBP1 nuclear foci.
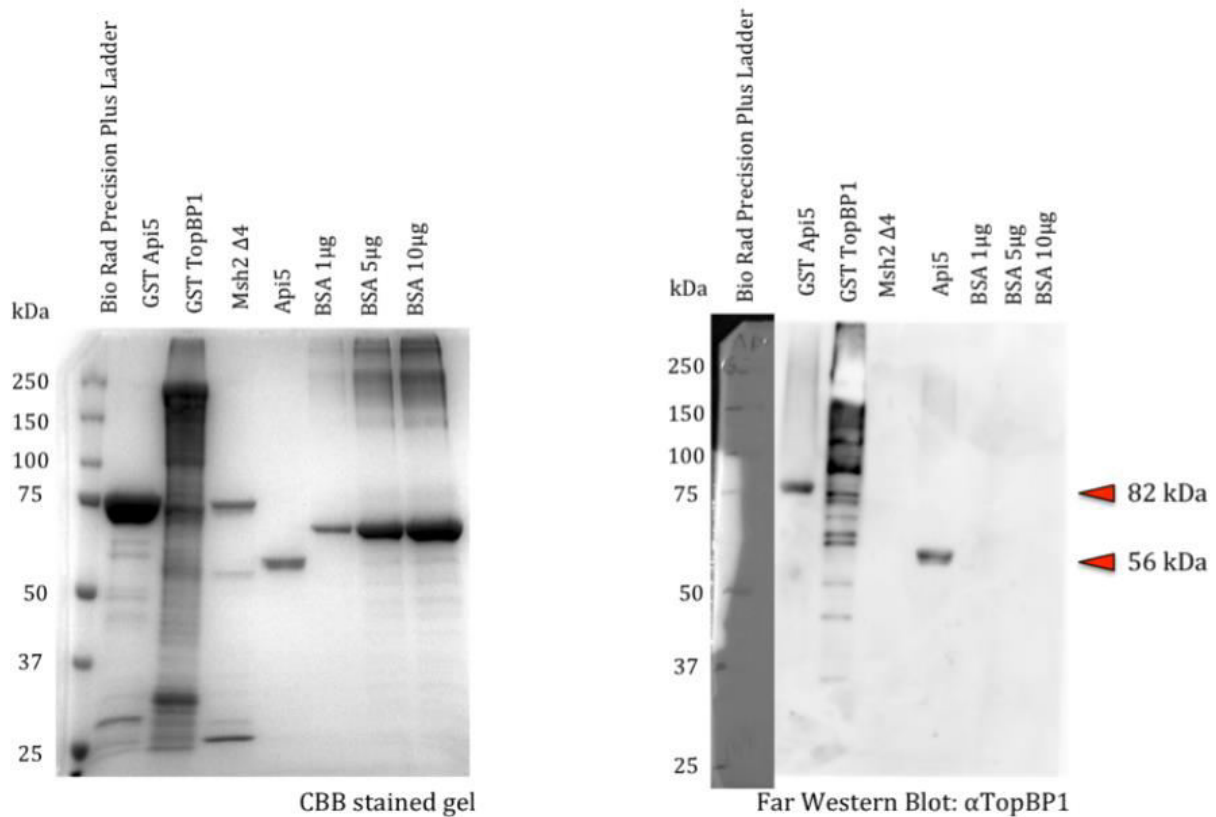
Fig 5: Far western blotting using Api5 as bait protein and GST TopBP1 as "prey"protein and probing using anti TopBP1 antibody to show the interaction betweenthe two *in vitro* (Abhinav, Unpublished).

Lysine Acetylation of cellular proteins

Study on the reversible post- translational modifications of the lysine residues in the cellular proteins and its role in cellular signalling and much other cellular process is emerging beyond the study on its role in chromatin organisation and transcription. More than 250 acetylated proteins are now identified to localise presumably in the cytoplasm following the discovery of tubulin acetylation in the year 1985 (L'Hernault and Rosenbaum, 1985). Now lysine acetylations of cellular proteins have been shown to have role in intracellular trafficking, stress response, metabolism, cytoskeleton dynamics, vesicular fusion, protein folding, protein- protein interaction, cell cycle control, to name a few (Choudhary et al., 2009; Grillon et al., 2012; Mersfelder and Parthun, 2008).

The role of lysine acetylation in cytoplasmic-nuclear shuttling is well known. Most proteins, which shuttle between the nucleus and the cytoplasm, are acetylated by

p300/CBP, including HNF-4, CIITA, PCNA, SRY, cAbl, CtBP2, p53, PAP, and β-catenin, RECQL4 (Naryzhny and Lee, 2004; Shimazu et al., 2007; Thevenet et al., 2004). No general rule for the sub cellular localization of the acetylated proteins has been deciphered until now. Acetylation can enhance the localization in the cytoplasm for some proteins (di Bari et al., 2006; Naryzhny and Lee, 2004) while for others acetylation can favor a nuclear localization (Naryzhny and Lee, 2004; Thevenet et al., 2004).The mechanism by which acetylation influences cellular localization can be mainly two ways:

1. By the modifying an interaction with binding partner which in turn leading to retention in a particular compartment or

2. By an altered interaction with nuclear import/export factors (Sadoul et al., 2011).

Motivation of the project

The post translational modifications of Api5 is not well studied and one among the few post translational modification (PTM) known till date is acetylation at lysine 251 residue, which was identified in a global acetylation-specific mass spectrometry analysis done by Choudhary and his colleagues (Choudhary et al., 2009). Later Han *et al.*, (2012) did further studies on the same and found Lys 251 to be conserved across different species including humans, mice, chicken, salmon and drosophila (Han et al., 2012) (Fig 6). They also showed that acetylation of Api5 can induce apoptosis and alter its stability. Post-translational modifications cannot only govern cell signaling, they also can affect protein interactions. Interaction of Api5 with TopBP1 happens through a region, which contains the K251 residue (Abhinav, unpublished). Hence it may be hypothesized that the acetylation status of Api5 at K251 may be governing its interaction with TopBP1. In addition to that, considering the role of lysine acetylation in cytoplasmic –nuclear shuttling, studying the effect of acetylation in Api5 in its sub cellular localization is also interesting. Considering the data provided by Han and his colleagues, loss of this acetylation can be the trigger to the degradation of Api5 after getting exported to the cytoplasm.
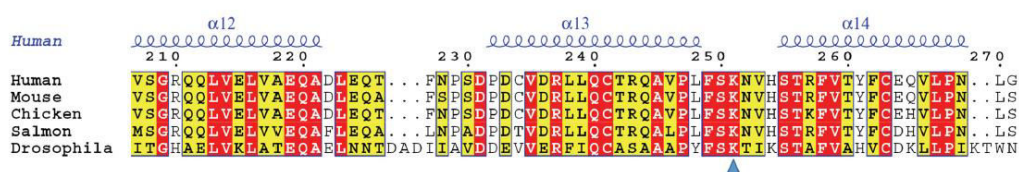
Fig 6: Conservation of Lysine 251 across species (Han et al., 2012)

Objective

1. Elucidate the role of acetylation of lysine 251 in regulating Api5 and TopBP1 interaction.

    I. Construction of 3 Api5K251 mutants (Api5K251R, Api5K251Q, Api5K251A) in full length Api5 tagged with GST, HA and mVenus and confirming its expression.

    II. Study the *in vitro* biochemical interaction of TopBP1 with Api5 acetylation mutants using far western studies.

    III. Study the interaction of TopBP1 with the Api5 acetylation mutants in cell lines using pull down assay.

2. Study the role of Api5 K251 acetylation in context with its cellular localization with and without DNA damage.

Materials and methods

Site directed mutagenesis

The primers for site directed mutagenesis for Api5 K251 mutations were designed and ordered from Sigma-Aldrich.

Api5K251Qfor-1: 5'-CAGTACCCCTCTTCTCTCAGAATGTCCATTCCACAAGGTT-3'

Api5K251Qrev-1: 5'-AACCTTGTGGAATGGACATTCTGAGAGAAGAGGGGTACTG-3'

Api5K251Rfor-1: 5'-CAGTACCCCTCTTCTCTCGAAATGTCCATTCCACAAGGTT-3'

Api5K251Rrev-1: 5'-AACCTTGTGGAATGGACATTTCGAGAGAAGAGGGGTACTG-3'

Api5K251Afor-1: 5'-CAGTACCCCTCTTCTCTGCAAATGTCCATTCCACAAGGTT-3'

Api5K251Arev-1: 5'-AACCTTGTGGAATGGACATTTGCAGAGAAGAGGGGTACTG-3'

Api5K251mutchk-1: 5'-AACAGACCTTCAATCCCTCG-3'.

Pfu Turbo DNA polymerase was obtained from Stratagene, dNTPs from MP Biomedicals and Dpn1 was from New England Biolabs (NEB).

Plasmids

Plasmids used in the study were Api5 mVenusC1 (Kanamycin resistant), GST- Api5 full length (Ampicilin resistant), Api5 HAC NLS (Ampicilin resistant), TopBP1 pGEX 2Tkcs (Ampicilin resistant), pGEX 2Tkcs (Ampicilin resistant), mVenusC1 (Kanamycin resistant), and pCDNA3.1 HAC3 (Ampicilin resistant).

Kits

Plasmid extraction and isolation kit (Mini Prep) was initially provided by Macherey-Nagel (MN) and later from Qiagen. Plasmid extraction and isolation midi prep kits were from Roche and Macherey-Nagel (MN). BCA Assay kit was from Thermo Scientific.

Cell Culture

DMEM, Trypsin and DPBS were obtained from Lonza. Lipofectamin 2000 used for transfection was from Invitrogen and OPTIMEM was from Invitrogen.

Cell Lines

HeLa was from European Collection of Cell Cultures and U2OS was a generous gift from Dr Jomon Joseph (National Center for Cell Sciences, Pune).

Antibodies

Primary antibodies:

Api5 raised in rabbit (1:2500) and Api5 raised in mouse (1:2000) were purchased from Abnova, anti-GFP raised in rabbit (1:2500) was bought from Abcam, anti-HA raised in mouse (1:2000) was from Millipore, anti-TopBP1 raised in rabbit (1:2500 for immunoblotting; 1:500 for immunostaining) was purchased from Bethyl.

Secondary antibodies used in the study were anti-mouse (1:10000) and anti-rabbit (1:10000) from Sigma-Aldrich, and Alexa Fluor 568 conjugated donkey anti-rabbit (1:500) from Invitrogen.

Other chemicals and drugs

Camptothecin, bovine serum albumin (BSA), glutathione reduced and Commassie Brilliant blue was from Sigma - Aldrich. Glutathione agarose beads were bought from Thermo Scientific. Complete mini EDTA free protease inhibitor cocktail tablets were from Roche. Hoechst 333258 for nuclear staining was from Invitrogen.

Methods

Site directed mutagenesis: Primers were reconstituted and 100uM stocks were prepared and stored in -40°C. Site directed mutagenesis was performed for each of the three mutations based on the following reaction mixture (Table 1) and PCR cycles (Table 2). The PCR product was subjected to Dpn1 digestion for 3hr in 37°C. Digested mixture was transformed into DH5α cells and kept in 37°C overnight. Colonies we collected the following day, miniprep was prepared using the Qiagen Plasmid extraction kit and sent for sequencing (1st Base, Singapore).

Table 1: Reaction Mixture for site directed mutagenesis

Plasmid	2µl
10X PFU Buffer	5µl
5'Primer(25µM)	1µl
3'Primer(25µM)	1µl
dNTPs	0.4µl
PFU Turbo	1µl
H$_2$O	39.6µl

Table 2: PCR cycles for site directed mutagenesis

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

Protein expression and purification: GST-tagged constructs were transformed in BL21 cells to get colonies. Single colonies were grown in LB-Amp (20ml LB + 250µg/ml Amp) overnight and secondary culture was made the next day (5ml primary +200ml LB +500ul Amp). When optical density (600nm) of the culture reached 0.6-0.7, 0.4mM IPTG was added and incubated at 25°C for 10hrs. After induction, the cells were harvested at 4000rpm for 15mins at 4°C.1ml of both induced and un-induced fractions were collected, pellets were made and re-suspended in 40ul of 2X Sample Buffer. The harvested cells were re-suspended in 20ml of Extraction buffer (Table 3) and sonicated twice (Amplitude was 50%, time for 2min; Pulse was on for 50s and off for 30s). After spinning down the sonicated cells at 12000rpm for 30min the supernatant was incubated with glutathione-coated agarose beads for 30min at 40°C on a rotator. The solution was then spun down at 600rcf for two minutes and the beads were given three washes with wash buffer 1 (Table 4) and one wash of wash buffer 2 (Table 5). Beads were collected and stored in -80°C. Expression of the protein was qualitatively measured by performing and SDS PAGE.

Table 3:Composition of Extraction Buffer (200ml culture).

200nM Hepes pH 7.8	500µl
0.5M NaCl	2.5ml
0.5mM EDTA	25µl
10% Glycerol	2.5ml
0.5%NP 40	1.25ml
1X Protease Inhibitor	250µl
H_2O	17.975ml

Table 4: Composition of Wash Buffer 1 for GST tagged proteins.

1M Tris pH8	600µl
5M NaCl	900µl
10%Triton X-100	300µl
1mM PMSF	300µl
H_2O	27.9ml

Table 5: Composition of Wash Buffer 2 for GST tagged proteins.

1M Tris pH 7.6	1ml
5M NaCl	200µl
Glycerol	0.5ml
H_2O	8.3ml

Elution of TopBP1: Elution buffer was made using 20mM reduced glutathione (Sigma-Aldrich) in 50mM Tris, pH8. 50ul of GST elution buffer was added to the purified bead bound protein and rotated in wheel for 30mins at room temperature. Then it was spun down for 2min at 800g and supernatant was collected. The samples were mixed with 2X sample buffer and run on an SDS-PAGE.

Thrombin cleavage: In order to prevent the GST dimer formation between the tags of GST-Api5 and GST-TopBP1, the Api5 wild type and mutants were cleaved from

the beads leaving GST tags using Thrombin cleavage buffer (TCB) (Table 6). Equal volumes of the bead bound protein and the TCB were mixed together. Thrombin (1 unit/100µg of protein) was added to the mixture and left overnight in room temperature on a rotating wheel. Then the solution was spun down and supernatant was collected to run on an SDS PAGE.

Table 6: Composition of Thrombin Cleavage Buffer.

Working concentration	Stock
5mM Cacl2	0.1M
150mM NaCl	5M
50mM Tris-Cl pH 7.5	1M
1% Triton X 100	10%

Transfection: To study the expression and localization of mVenus-tagged full length Api5 and the mutants, the constructs were transfected into HeLa cells and U20S. For transfection, required number of cells were seeded into a 12 well/6 well dish and left in incubator (37°C) for 16 hrs to reach confluency. 4µl of Lipofectamine 2000 (Invitrogen) was added to 96µl of OPTIMEM and incubated for 5min. 1µl of DNA was made up to 100µl in OPTIMEM in a different vial. Both the samples are mixed together and incubated for 20minutes. 800µl of OPTIMEM was added to the 200µl mix and added to the respective wells. After 4hr DMEM (Lonza) containing 30% FBS was added to the wells. Lysates were collected after 48hrs.

Immunoblotting: For immunoblot experiments, cell lysates were prepared in Laemmli sample buffer (Tris with SDS, bromophenol blue, dithiotreitol, and glycerol, pH 6.8) and were separated on SDS-PAGE gels at 120V**.**10% gels were used for resolving the proteins while 5% was the stacking gel**.** Resolved proteins were transferred at 250mA for 3 hrs onto a PVDF membrane. The blots were blocked in 5% skimmed milk prepared in TBS-Tween (0.1%) for 1hour at room temperature (RT) and probed with the respective primary antibody overnight at 4^{0}C. For phosho-proteins, Block Ace (ABd Serotec) was used for blocking. Blots were washed thrice with TBS-T (0.1% Tween) and probed with HRP-conjugated suitable secondary antibody (anti mouse or anti-rabbit accordingly) for 1hr at RT. After 3-4 washes with

TBS-T, blots were developed with Immobilon reagent (Millipore). Images were acquired on ImageQuant LAS4000 gel documentation system (GE Healthcare).

Far western: One of the best methods available to study the biochemical interactions of proteins is far western. 1.5µM concentration of thrombin cleaved Api5 and mutants (prey proteins) were loaded on an SDS PAGE. GST and BSA was used as negative controls. Resolved proteins were transferred at 110mA overnight onto a PVDF membrane. The blots were then denatured using AC buffer (Table 7) and re-natured using serial concentrations of the same buffer. Re-natured blots were then blocked in 5% skimmed milk for 1hr at room temperature. 5nM and 10nM bait proteins were mixed with Protein Binding Buffer (Table 8) and the blots were left in this solution overnight at 4^0C. Next day the blots were washed 4 times with TBS-T (0.1% Tween) and immunobloting against anti-TopBP1.

Table 7a.Composition of master mix for AC Buffer

Glycerol	12.5ml
5M NaCl	2.5ml
1M Tris pH7.5	2.5ml
0.5 EDTA	0.25ml
100% Tween 20	0.125ml
1M DDT	0.125ml

Table 7b. Composition of AC Buffer

AC Buffer conc	6M	3M	1M	0.1M	0M
Master Mix	3.6ml	3.6ml	3.6ml	3.6ml	3.6ml
dH$_2$O	2.45ml	12.82ml	18.07ml	10.89ml	21.2ml
Milk	0.5g	0.5g	0.5g	0.5g	0.5g
8MGuanidiumHCl	18.75ml	9.3ml	3.13ml	0.31ml	0ml

Table 8. Composition of Protein binding buffer

5M NaCl	60µl
1M Tris pH 7.6	60µl
0.5M EDTA	3µl
100%Glycerol	300µl
100% Tween 20	3µl
Skimmed milk	60mg
1M DDT	3µl
H_2O	2.57ml

Immunostaining and microscopy: To study the cellular localization of Api5 K251 mutants and its co-localization with TopBP1, U20S cells were transfected with mVenus-tagged mutants. These cells were immunostained and imaged using LSM-710 inverted microscope (Carl Zeiss) at 63X magnification. For immunostaining, acid-treated cover slips were placed in a 6-well plate and kept in UV for 30 minutes until the ethanol dries up. $2x10^5$ cells were seeded on the plate and incubated for 16hr at 37^oC. The cells were then transfected with the required proteins and left in 37^oC for 48hr. 6hr 1µM camptothecin treatment was given for DNA damage. The media was removed and two 1XPBS was given after 48hrs. Cells were fixed using 4% freshly prepared formaldehyde for 20min at room temperature. Fixation was stopped using one Glycine-PBS and two 1XPBS washes. Permeabilization was done using 0.5% Triton X 100 in PBS at 4^oC for 10min. To stop permeablilization three 1X PBS washes were given. 500µl of blocking solution (10% serum in IF buffer) was added to the fixed cells for 1hr. The cells were stained using primary antibody and left for incubation for 1hr at room temperature. Cover slips were then washed for 20 min in IF buffer and two times with PBS. Secondary antibody was added accordingly and left at room temperature for 1hr. PBS washes were given and then nuclei was counterstained with 1X Hoescht 333258 for 10min at RT. One more PBS wash was given and the cover slips were mounted on glass slides after adding 7µl of mounting medium. Sealing was done using nail polish. The slides were then imaged under the confocal microscope and further analysis was performed using ImageJ 1.48(NIH).

Results

The three Api5K251 mutants (Api5K251R, Api5K251Q, and Api5K251A) tagged with GST, HA and mVenus were prepared using site directed mutagenesis.

To study the role of acetylation of Lysine 251, site directed mutagenesis of this site was performed by replacing lysine with arginine (no acetylation), glutamine (acetylation mimic) or alanine (uncharged mutant) in GST-taggedApi5, Api5-mVenus and Api5-HA. Primers were designed and ordered from Sigma-Aldrich. The following mutants were successfully prepared: GST-Api5K251A, GST-Api5K251Q, GST-Api5K251R, HA-Api5K251A, HA-Api5K251Q, HA-Api5K251R, Api5K251A mVenusC1, Api5K251R mVenusC1 and Api5K251Q mVenusC1.

Full length GST-Api5, all the three GST tagged mutants of Api5, GST-TopBP1 and pGEX were expressed in *E. coli* BL21, purified and concentrations were estimated.

To study the role of Lysine 251 acetylation of Api5 in its interaction with TopBP1 all the proteins required to perform far western was expressed in *E. coli* BL21 cells. These proteins were purified according to the protocol (Fig 7). Then the GST Api5 full length and all the mutants were cleaved using thrombin cleavage buffer to avoid the possibility of GST dimer formation with the prey and bait proteins during far western. Concentrations were estimated using BSA standards (Visual estimation) (Fig 8) and BCA assay. GST and GST-TopBP1 was eluted from the beads using GST elution buffer and concentrations were estimated in a similar way.
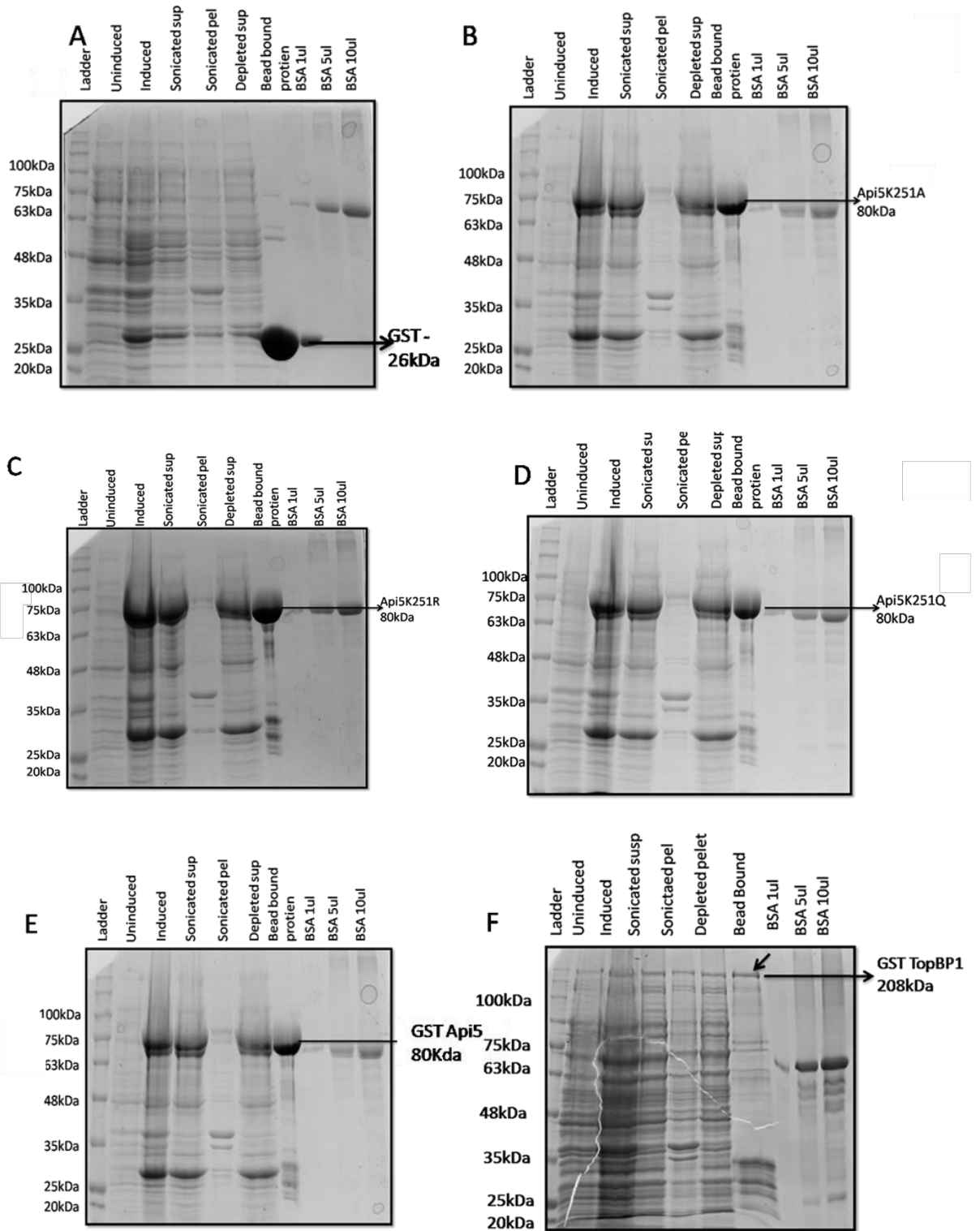
Fig 7: CBB stained SDS-PAGE showing purification profiles of (A) GST, (B) GST Api5K251A,(C) GST Api5K251R,(D) GST Api5K251Q, (E) GST Api5 and (F) GST TopBP1.
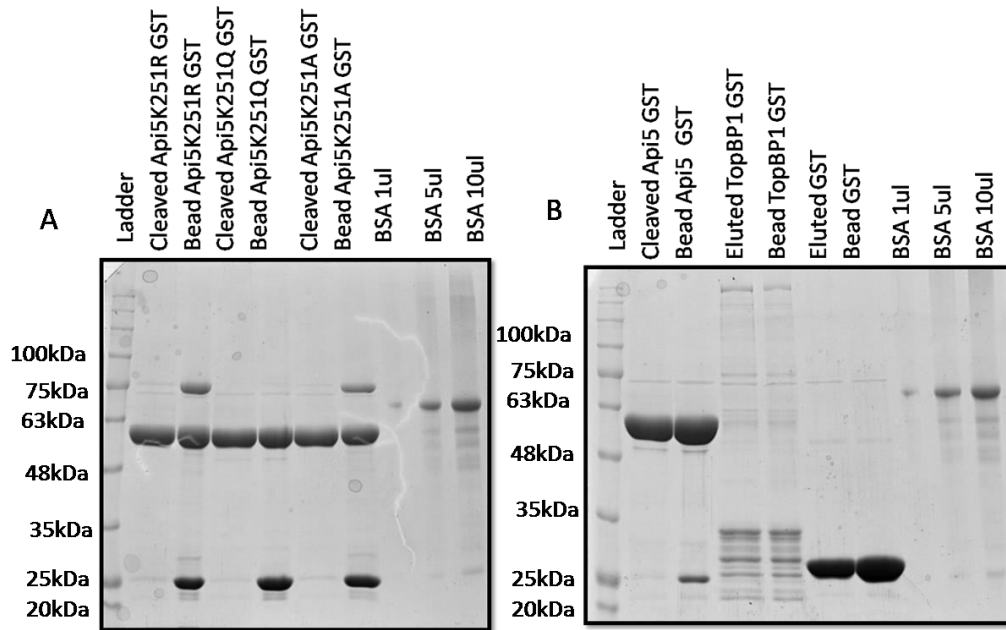
Fig 8: CBB stained SDS-PAGE showing (A) thrombin cleavage of Api5 mutants, (B) thrombin cleavage of wild type Api5 and elution profile of GST-TopBP1 and GST.

Api5 mVenusC1, HA-APi5 and the mutants (Api5K251R mVenusC1, Api5K251Q mVenusC1, Api5K251A mVenusC1, HA-Api5K251R, HA-Api5K251Q, HA-Api5K251A) were transfected into HeLa cells and expression was confirmed.

mVenus constructs were used for the study of cellular localization of the Api5K251 mutants and the HA mutants were made for performing pull down experiments. The mutants were transfected into HeLa cells and lysates were collected 48hr after transfection. A un-transfected well and a well transfected with empty vector were kept as controls. 10μl of each sample was loaded on a 10% SDS PAGE, and then transferred to PVDF membrane. The blot was then probed with anti-GFPand anti-Api5 antibodies to check for expression. On probing with anti-GFP a band was observed at a molecular weight of 85kDa which is supposed to be the Api5-GFP band and the untransfected lane was blank and empty vector band came around 29kDa (Fig 9A). On probing with Api5, the exogenous and endogenous Api5 was visible (Fig 9B). For the HA-tagged Api5 and mutants the blot was probed with anti-rabbit HA and stripped and reprobed with anti-mouse Api5. Since the levels of HA Api5K251R was very low to see whether the loading was proper the blot was again stripped and reprobed with GAPDH. The HA Api5 band was observed at a molecular weight of 60kDa (Fig 10).
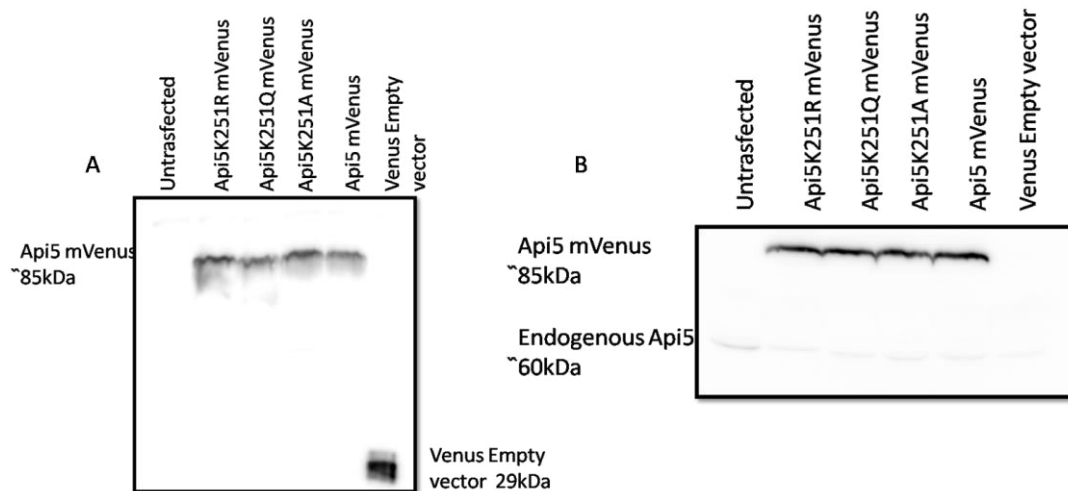
Fig 9: Western blot showing Api5 mVenus mutants expression in HeLa cells (A) probed anti GFP and (B) probed anti Api5.
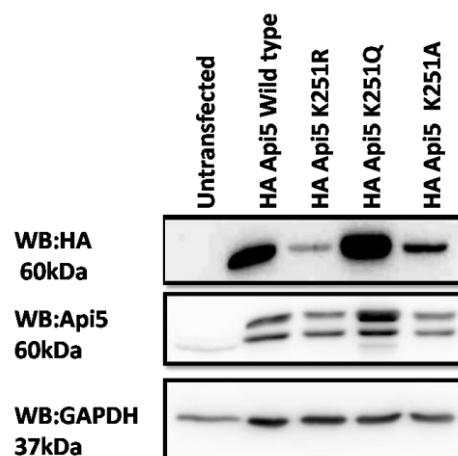

Fig 10: Western blots showing HA Api5 mutants expression in HeLa cells.

Interaction of Api5 with TopBP1 is independent of its K251 acetylation *in vitro*.

Far western blotting was performed to investigate whether Api5K251 acetylation had any effect on its biochemical interaction with TopBP1. Constant molar concentration of thrombin cleaved Api5 and its three mutants along with GST and BSA as negative controls were used as "prey" proteins on the blot. Two different molar concentrations of the TopBP1 bait protein was used to get some estimation about the stochiometry of the interaction. TopBP1 showed an interaction with all the 4 proteins in both the stochiometries, suggesting a strong interaction and also that the Api5K251 actetylation status does not play a role in its biochemical interaction with TopBP1 *in vitro*.The far western analysis was repeated thrice and the bands were quantified

using imageJ. No significant change in interaction was observed between wild type and the different Api5 mutant proteins (Fig 11).
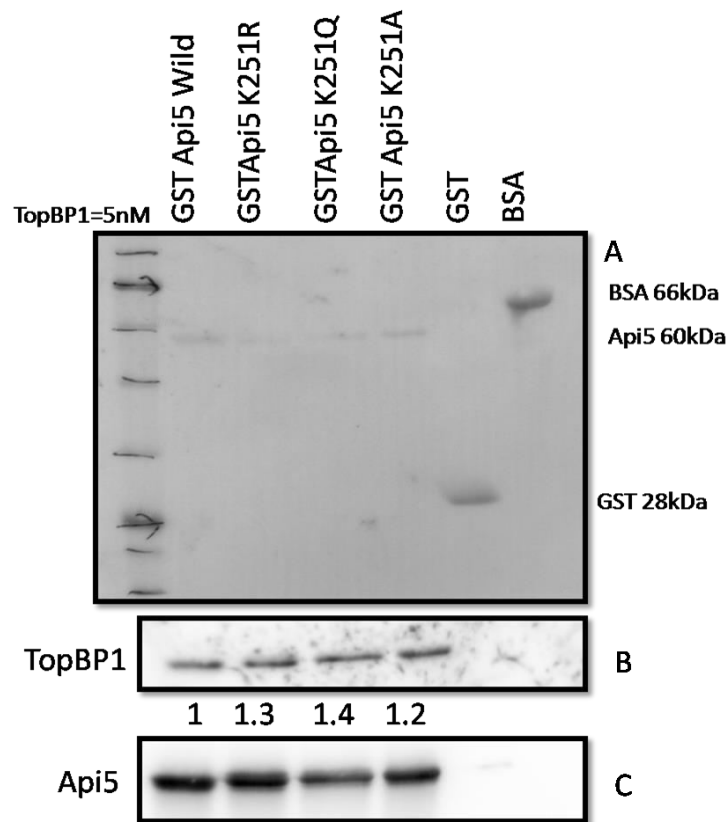

Fig 11: Far western blot showing aninteraction between TopBP1 and Api5 mutants. (A) Ponceau S stained blot to show protein loading. (B) Far western blot probed with TopBP1 and (C) probed with Api5. Quantification is given for the average of threeindependent experiments.

TopBP1 foci formation is independent of Api5K251 acetylation.

TopBP1 foci were observed following camptothecin damage whereas Api5 did not form damage-induced foci (Abhinav, unpublished). Given the hypothesised role of Api5 K251 acetylation on its function as well as interaction with TopBP1, it was interesting to see whether Api5 acetylation status could affect TopBP1 foci formation profile or evenmore, cause Api5 to form foci. Cells transfected with each of the Api5 acetylation mutants were stained for TopBP1 and the number of cells showing TopBP1 foci was counted with or without 1μM Camptothecin treatment for 6 hr (Fig12a). The percentage of cells showing TopBP1 foci increased upon CPT treatment (~70%) in comparison to untreated (~30%) irrespective of the acetylation

status of Api5, suggesting that TopBP1 foci formation is independent of Api5 K251 acetylation (Fig 13). It was further observed that acetylation status of Api5 did not confer upon itself any ability to form damage induced foci and the Api5 nuclear profile of the muatants remained similar as that of the wild type (Fig 12b)
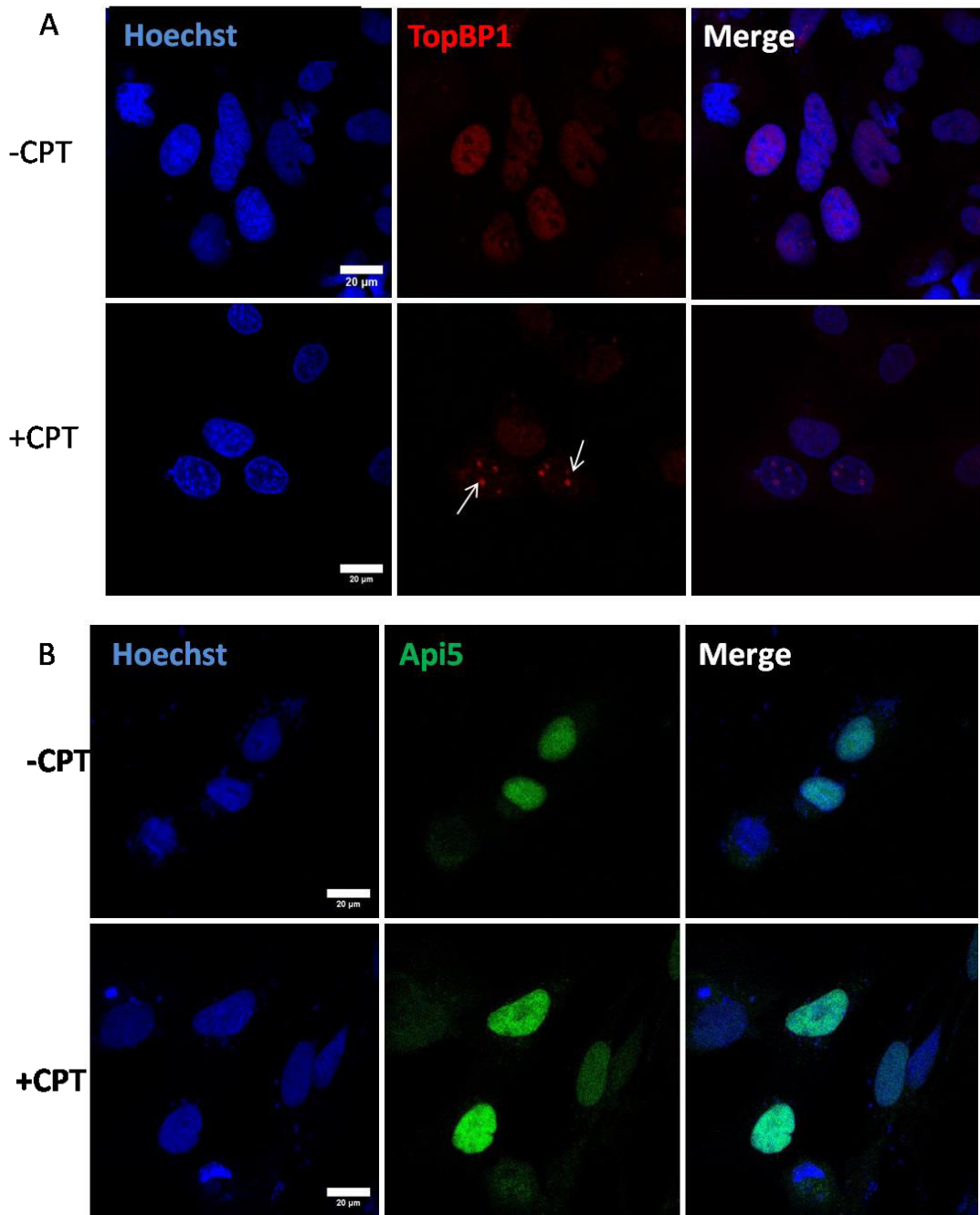

Fig 12: (A) IF staining showing TopBP1 foci with and without CPT treatment. (B) mVenus Api5 showing similar expression profile of Api5 with and without CPT treatment.
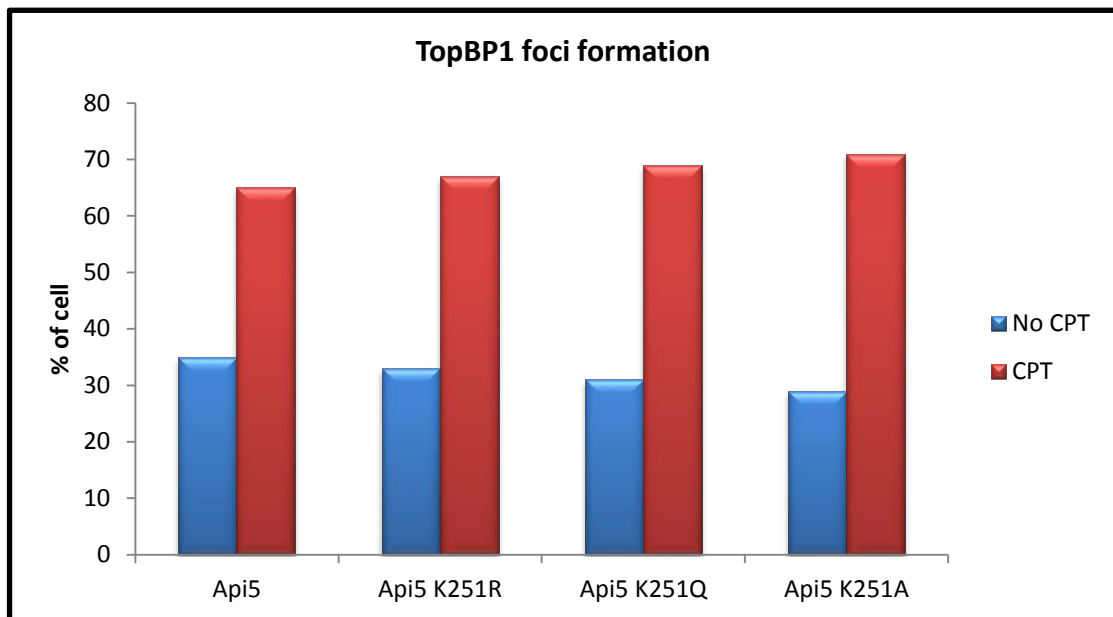
Fig 13: Quantification of cells positive for TopBP1 foci with or without camptothecin treatment (1µM for 6hr) (N=1, n=60).

The uncharged acetylation mutant showed a higher nuclear localization in comparison to wild type cells without DNA damage.

Wild type as well as the mutant Api5 showed two types of subcellular expression profiles, 1) A complete nuclear localization 2) nuclear as well as cytoplasmic distribution (Fig 14).
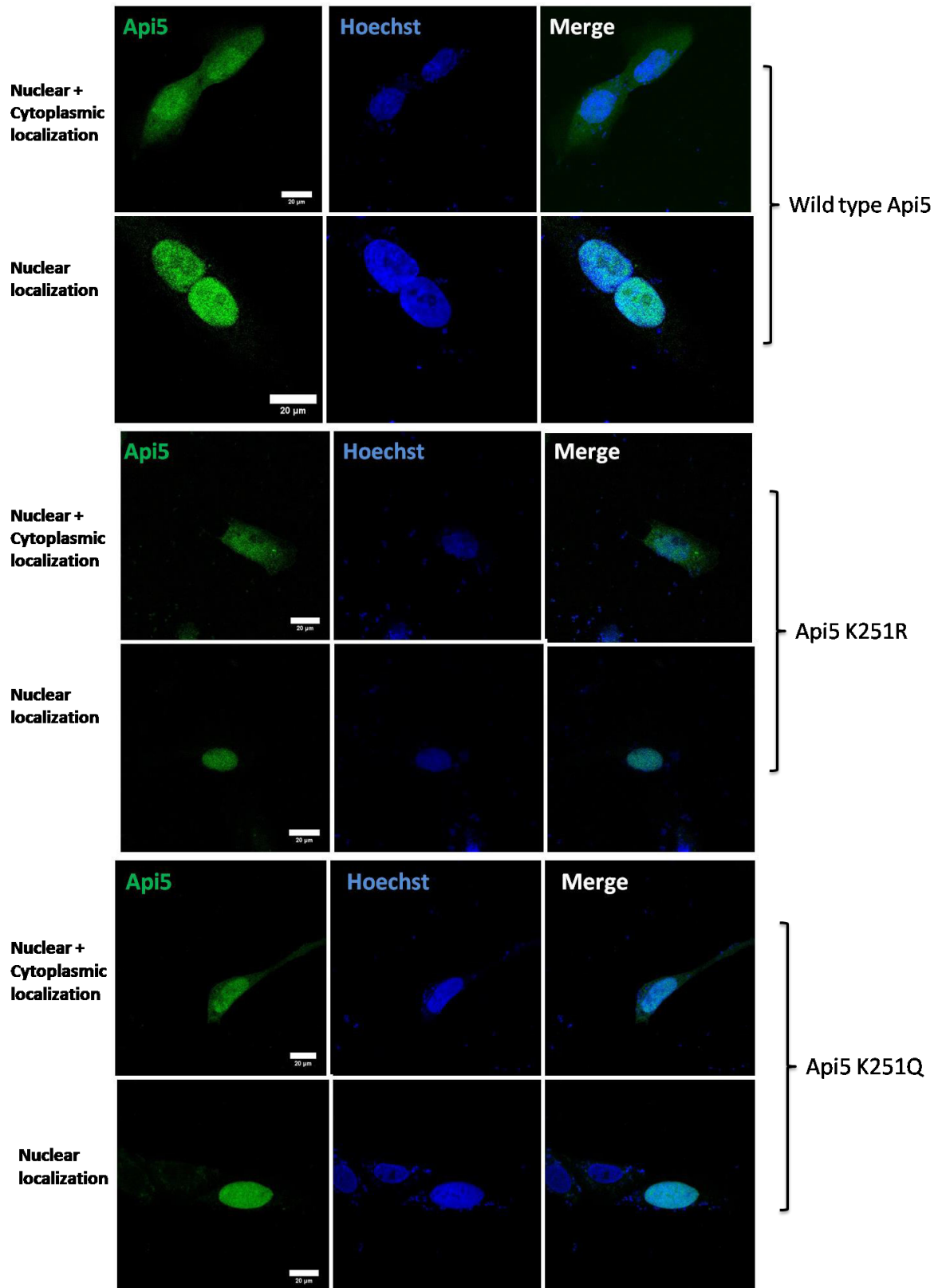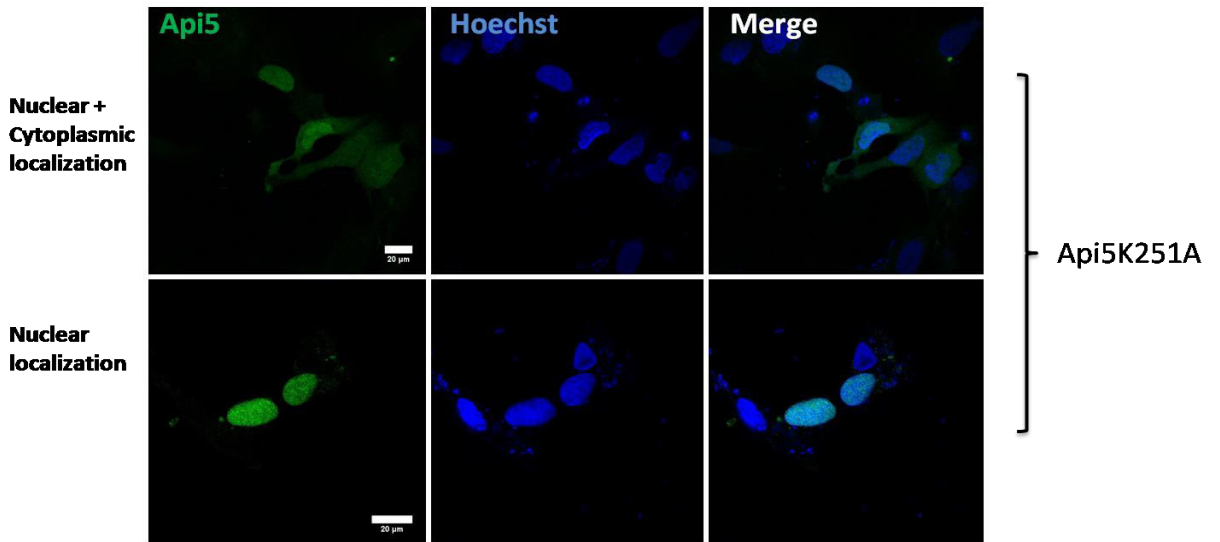
Fig 14: Representative images of Api5 mVenusC1 protein expression showing nuclear and/or cytoplasmic localization. Nucleus is stained using Hoescht 33358.

To investigate whether Api5 subcellular localization was affected by its acetylation status at K251, the above mentioned expression profiles were quantified for each mutant by counting 60 cells. Percentage of cells showing nuclear or cytoplasmic localization was plotted for each of the mutants and the wild type (Fig15). Preliminary data showed a higher fraction (84%) of cells expressing the uncharged mutant (K251A) having a nuclear localization of the protein in comparison to wild type or the acetylation mimic or deficient mutants. complimentarily, the later ones showed a higher cytoplasmic localization than the uncharged mutant.
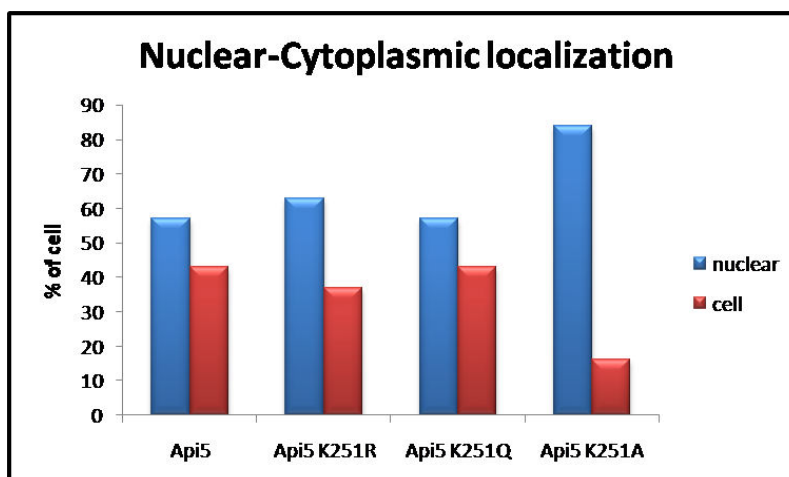

Fig 15: Quantification of fraction of cells showing nuclear (blue) or nuclear +cytoplasmic (red) expression for each of the Api5 K251 mutants (N=1, n=60).

Discussion

Api5 protein has an anti-apoptotic function but its mechanism is poorly understood. It may be undergoing a dynamic regulation in response to different stimuli (like DNA damage) orchestrating its function. These almost instantaneous spatio-temporal regulations are often times brought about by post-translational modifications (PTM). The PTMs can work as molecular switches recruiting or dissociating specific proteins into or out of transient protein complexes and thus activating or inhibiting specific signaling pathways in response to a stress like DNA damage. Api5 interacts with TopBP1 *in vitro* as well as *in vivo* and the *in vivo* interaction is lost upon CPT induced DNA damage (Abhinav, unpublished data). Interaction of the checkpoint protein TopBP1 with anti apoptotic protein Api5 may be one of the novel ways by which checkpoint signaling controls apoptosis signaling in the face of DNA damage, buying time for DNA repair. *In vitro* interaction studies showed that the two proteins can interact biochemically, but the difference in spatio-temporal interaction status between the two proteins upon DNA damage hints towards a possible role of PTM in orchestrating this dynamic regulation of Api5-TopBP1 interaction in response to DNA damage. Since the role of acetylation in governing such regulations of different proteins are well documented and this is the one PTM which has been associated with Api5 function (Han, 2012), it may well be expected to be governing Api5-TopBP1 interaction dynamics *in vivo* in response to DNA damage and thus contributing to the checkpoint-anti-apoptosis signaling which is supposed to be a manifestation of that interaction.

Our *in vitro* interaction studies indicate that there is no effect of the acetylation status of Api5 at lysine 251 residue upon its biochemical interaction with TopBP1. The *in vitro* interaction studies only provide information about the ability of the two proteins to interact with each other accounting for their biochemical and biophysical properties. However, it does not provide any information about the *in vivo* physiological response to DNA damage stimuli, which is critical for the functionality of these two proteins. Hence, *in vivo* interaction studies need to be done with each of the mutants to be able to comment upon the role of acetylation of Api5 in its interaction with TopBP1. A pull down assay after transfecting the HA-tagged Api5 mutants in HeLa cells will help to validate the data and will confirm the results in a more realistic environment.

TopBP1 foci formation is associated with DNA damage and image analysis experiments shows that neither wild type Api5 nor the Api5 mutants are recruited to the sites of DNA damage. This suggests that the interaction of TopBP1 and Api5 may be happening elsewhere in the nucleus and not at sites of DNA damage, irrespective of the K251 acetylation.

Api5 K251 uncharged mutant (which can not change its charge properties owing to its inability to undergo acetylation) showed a significantly lesser cytoplasmic localization in comparison to the wild type which is prone to change its charge properties owing to possibility of its acetylation or de-acetylation. This could be indicative of a role of the acetylation status of Api5 between its nuclear-cytoplasmic shuttling. Since no significant difference was observed in the sub-cellular localization of the protein between wild type, acetylation mimic and deficient mutants, we can not associate a particular acetylation status of the protein with its localization in a particular sub-cellular compartment as of now. Repeating this experiment and looking at the change in sub-cellular localization patterns of the mutants with or without DNA damage may help us associate the acetylation status of Api5 with its nuclear -cytoplasmic shuttling.

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