

Post-translational regulation of Apoptosis inhibitor 5 (Api5) during cell cycle progression and DNA damage-induced apoptosis

A Thesis Submitted in partial fulfilment of the requirements

Of the degree of

Doctor of Philosophy

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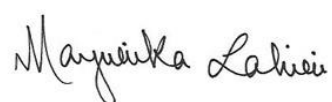
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CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Post-translational regulation of Apoptosis inhibitor 5 (Api5) during cell cycle progression and DNA damage-induced apoptosis**” submitted by **Virender Kumar Sharma** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

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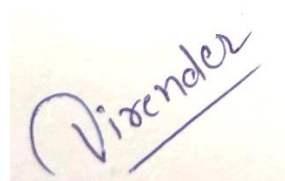
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Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

A handwritten signature in blue ink, reading "Virender", with a horizontal line underneath it.

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Synopsis

Post-translational regulation of Apoptosis inhibitor 5 (Api5) during cell cycle progression and DNA damage-induced apoptosis

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Introduction

The post-translational regulation of proteins is achieved by different post-translational modifications (PTMs). PTMs are covalent modifications that occur at side chains of specific amino acids and control various functional aspects of proteins such as interactions, activity, stability and localisation. PTMs provide far more variation at protein levels than the genome (Prabakaran et al., 2012). PTMs in response to external stimuli work at a much smaller time scale than signals acting on the genome. Some common PTMs are:

1. Phosphorylation: This is the most common PTM and is usually carried out by enzymes called kinases. They play a significant role in several cellular processes like cell survival, cell division, differentiation, and death. In eukaryotes, proteins are usually phosphorylated at either serine, threonine or tyrosine residues.
2. Ubiquitination: Ubiquitin is a 76 amino acid protein that is highly conserved and present in all tissues. Ubiquitin activating enzyme (E1) activates the ubiquitin moiety and transfers it to a complex formed by the ubiquitin-conjugating enzyme (E2) (Pickart, 2001) and ubiquitin ligase (E3) (Drazic et al., 2016). Ubiquitin ligase recognises the target proteins and ubiquitinates them. Later these proteins are transferred towards the proteasome complex for their degradation. Ubiquitination has been classified as mono-ubiquitination and poly-ubiquitination depending upon the number of ubiquitin

moieties attached to target proteins. Poly-ubiquitination of a protein (Yu et al., 2003) ,at specific lysine residues, with the ubiquitin linked via their Lys-48 usually acts as a signal that targets the proteins for proteasomal degradation, thus maintain the stability of proteins.

3. Acetylation is a major PTM where an acetyl group donated from acetyl co-enzyme A is attached to the α -amino acid at the N-terminal or specific lysine residues. During metabolism, acetylation at lysine residues is tightly regulated by lysine acetyltransferases (KAT). Lysine de-acetylases (KDACS) perform acetylation's reverse function by removing the acetyl group from the target proteins. (Drazic et al., 2016).

Apoptosis inhibitor 5 (Api5) is an anti-apoptotic protein and is also known as anti-apoptotic clone 11 (AAC-11) (Tewari et al., 1997), Fibroblast growth factor-2 interacting factor 2 (FIF2) and Migration Inducing Gene 8 (MIG8). Api5 is conserved across species and is expressed ubiquitously in all tissues and at all developmental stages. It was first discovered as a factor that prevented apoptosis that occurred upon growth factor deprivation. Api5 is an elongated all α -helical structure (Han et al., 2012). It has 19 α -helices and two 3_{10} helices. It has also been observed that all these helices interact with neighbouring helices to form antiparallel helix pairs. The N- terminal of Api5 comprises a α -helix from 1 to 11, which shares homology with the HEAT repeat structures of other proteins such as Importin β . C-terminal of Api5 is made up of α -helices 12 - 19 and share homology with ARM-like repeat structures of other proteins, for example, p120 catenin. These repeats provide Api5 a surface for interaction with other proteins and can also act as scaffold for multiprotein complexes. N-terminal of Api5 contains the LxxLL motif, which is an amphipathic α -helix and forms nuclear receptor-co factor interaction regions. This motif is present in the 6th α - helix and is helpful in maintaining the stability of the protein by developing hydrophobic interactions with neighbouring α -helices and helps interact with FGF2. The C-terminal of Api5 has a putative nuclear localisation signal and a leucine zipper domain (LZD). The LZD is present in the α -18 helix between amino acid residues 371- 391. Api5 interacts with acinus, which mediates chromatin fragmentation during apoptosis (Rigou et al., 2009). Api5 prevents dimerisation of caspase 2, a highly conserved initiator and executioner caspase. Api5 binds to the caspase recruitment

domain of caspase 2, preventing its homo-dimerisation, which is crucial for its activation (Imre and Rajalingam, 2017).

The Dyson laboratory has demonstrated Api5 to suppress apoptosis that is induced by dE2F de-regulation in *D. melanogaster*. It neither binds to dE2F nor does it block its transcriptional activity; however, Api5 was observed to act downstream of dE2F (Morris et al., 2006).

Global mass spectrometric analysis of Api5 suggests that it has one conserved acetylation site. This acetylation occurs at lysine 251, which is present in the hinge region between α -13 and α -14 (Han et al., 2012). Api5 acetylation at lysine 251 is conserved from protists to mammals (Han, Kim et al., 2012). The de-acetylated form of Api5 is not stable and undergoes post-translational degradation. However, the mechanism of degradation and the enzymes involved in the process of acetylation and de-acetylation of Api5 is not yet known.

Api5 acts as an immune escape gene in tumors. Api5 upregulates pErk levels in the cells, which in turn phosphorylates BIM, causing it to be degraded by the proteasomal pathway (Noh et al., 2014). BIM is a pro-apoptotic protein that induces sensitivity to antigen tumor specific T-cells. Upregulated Api5 levels promote the proliferation of cancer cells. High Api5 expression was significantly associated with low survival of patients, particularly in ER-positive sub-population in which Api5 was also shown to induce cell migration (Basset et al., 2017). Api5 overexpression promotes invasion and metastasis in cancer cells. Api5 levels have also been associated with chemo-resistance of tumors cells while reduced levels of Api5 make these cells susceptible to apoptosis (Rigou et al., 2009).

Objectives

1. To identify the enzyme(s) involved in K251 acetylation of Api5
2. To investigate the mechanism and regulators engaged in post-translational degradation of Api5
3. To understand the mechanism of regulation of Api5 during cell cycle progression
4. To study the regulation of Api5 upon DNA damage-induced apoptosis

Results

1. p300 histone acetyltransferase acetylates while HDAC1 de-acetylates Api5 at K251

p300/CBP is a histone acetyltransferase and regulates gene expression by targeting histone and non-histone proteins (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). p300/CBP function as transcriptional co-activators and scaffold for various factors involved in proliferation, differentiation and apoptosis (Chan and La Thangue, 2001; Giles et al., 1998; Giordano and Avantaggiati, 1999; Goodman and Smolik, 2000). Histone acetyltransferase activity peaks at G1/S. Phosphorylation by cyclin E-cdk2 stimulates its activity. p300 also controls E2F movement (Martinez-Balbas et al., 2000), whose binding is stabilised by its phosphorylation by cyclin E-cdk2.

Histone de-acetylases, de-acetylates lysine residues on histones and non-histones proteins by removing the acetyl group attached to their amino acid residues. They function in various cellular processes, such as cell cycle regulation, DNA repair, apoptosis, proliferation and differentiation (Hubbert et al., 2002; Inoue and Fujimoto, 1969; Luo et al., 2000; Marmorstein and Zhou, 2014). The overexpression of class 1 HDACs (which includes HDAC1) is seen in multiple cancers. A regulator of G1/S transition, pRb, indirectly recruits HDAC1 and HDAC2 and inhibits p21 transcription which can arrest the cell cycle.

We found that p300 histone acetyltransferase is the enzyme that leads to the acetylation of Api5 and HDAC1 is the histone de-acetylase involved in the de-acetylation of Api5. p300 and HDAC1 physically interact and regulate Api5 protein levels. The acetylation of Api5 at lysine 251 by p300 and de-acetylation by HDAC1 was also shown to control the localisation of Api5. Acetylated Api5 stabilised in the nucleus, while upon de-acetylation, Api5 moved to the cytoplasm for its degradation. We propose a model where inhibition of activation of Akt and reduction in Api5 protein levels upon inhibition of AMPK confirms that p300-mediated stability of Api5 is achieved through the AMPK-Akt pathway.

2. Api5 undergoes post-translational degradation by ubiquitin-proteasomal pathway by FBXW2, FBXO3 and FBXO6 E3 ubiquitin ligase

To identify the pathway involved in the degradation of Api5, both lysosomal and proteasomal pathways were inhibited using small chemical inhibitors, and Api5 levels were analysed. Accumulation of Api5 upon inhibition of proteasomal pathway suggests that Api5 undergoes post-translational degradation through the proteasomal pathway. Further studies proved that Api5 underwent ubiquitination, another post-translational modification. This ubiquitination makes Api5 a target for proteasomal pathway-mediated degradation. E3 ligases are the key enzymes that provide specificity to the substrate proteins for their ubiquitination. E3 ligase is an enzyme that has multiple protein subunits. FBOX is the main component of the E3 ligase enzyme which harbours the domain that can bind to target proteins and perform ubiquitination. Our studies revealed that FBXW2, FBXO3 and FBXO6 SCF E3 ubiquitin ligases were found to reduce the Api5 protein levels significantly. To attain their molecular function, FBOX proteins must interact with substrate proteins. Interaction of Api5 with FBXW2, FBXO3 and FBXO6 proved that These select FBOX proteins degrade api5 by the proteasomal pathway.

3. De-acetylated Api5 undergoes ubiquitination and proteasomal mediated degradation in the G2/M phase of the cell cycle through Aurora Kinase B activity

During cell division, a cell must replicate its DNA and duplicate other cellular contents, segregate them equally and then divide to give rise to two daughter cells. The time between two successive divisions is called the cell cycle. There are four cell cycle phases in eukaryotes: first growth (G1) phase. This is when the cell senses its external and internal environmental conditions like space and nutrients/ energy sources. If the conditions are favourable, the cell commits to divide, implying it has crossed the first checkpoint/restriction point. The second phase is the synthesis (S) phase. This is the central event of the cell cycle where the DNA is duplicated. For successful division, the replication should be accurate and should occur only once per cycle. The cell cycle control system oversees the latter. The third phase of the cell cycle is the second-growth (G2) phase. It follows the S phase when all the other organelles like Golgi, mitochondria and ribosomes are duplicated. This also serves as a checkpoint that ensures the DNA has been replicated correctly.

The last phase of the cell cycle is the mitosis (M) phase. In this phase, the duplicated DNA is compacted to chromosomes, the copies are then segregated into two nuclei, and cytokinesis gives rise to two daughter cells. There are multiple proteins whose levels fluctuate during the different cell cycle phases, and Api5 is one of them. It has been reported that Api5 protein levels fluctuate during the cell cycle (Garcia et al., 2013). Api5 peaks during the G1 phase, while in the G2/M phase, Api5 is at its lowest levels. Api5 alters G1/S transition by regulating gene expression by the known transcription factor, E2F1 (Garcia et al., 2013). Henceforth it was decided to identify the mechanism of regulation of Api5 during the course of the cell cycle.

Our results confirm that Api5 undergoes post-translational degradation during the G2/M phase of the cell cycle. Api5 undergoes poly-ubiquitination in the G2/M phase and thus undergoes post-translational degradation. FBXW2 is the E3 ubiquitin ligase that facilitates the post-translational degradation of Api5 through the proteasomal pathway in the G2/M phase of the cell cycle. This cell cycle phase-dependent degradation is also regulated by Aurora kinase B. Aurora kinase B is a serine/threonine kinase that is part of the chromosomal passenger complex responsible for correcting wrong spindle attachment to the chromosome. Its RNA levels and kinase activity are low at G1/S, gradually increasing and reaching a peak 8-9 hours after the G1/S transition. Our results conclude that Api5 gets phosphorylated by the Aurora kinase B in the G2/M phase before undergoing ubiquitination by FBXW2, which eventually leads to proteasomal pathway-mediated degradation of Api5. This degradation of Api5 is mandatory for the regular cycle and functioning of cells, while the non-degradative form of Api5 imbalances cyclins' levels of cyclins, thus hampers the cell cycle pattern.

4. Proteasomal pathway-mediated degradation of Api5 upon DNA damage requires ATR kinase activity

Apoptosis is a cell death method in which cells undergo different genetic and morphological changes to accomplish their death. It is also called programmed cell death. The anti-apoptotic role of Api5 upon has been investigated in multiple contexts like serum starvation, viral infection, and DNA damage. However, the regulation of Api5 during DNA damage-induced apoptosis had not yet been elucidated. We observed that following DNA damage, Api5 inhibits apoptosis until a

certain threshold. But when the cell finally decides to undergo apoptosis, Api5 levels decrease. This decrease in Api5 levels is due to Api5 undergoing proteasomal degradation upon DNA damage. It was observed FBXW2 regulate Api5 levels upon DNA damage. ATR is the major kinase that senses the damage and initiates the signalling cascade to repair damaged DNA and apoptosis, depending upon the extent of DNA damage. Our experiments observed that Api5 interacts with ATR upon DNA damage and gets phosphorylated at serine 138. This serine site is crucial for maintaining the stability of Api5 as the site-directed mutant (S138A) does not undergo post-translational degradation. It has also been observed serine 138 also regulate the process of apoptosis upon DNA damage. Previous results confirmed the link between the K251 acetylation by p300 and the stability of Api5. It has also been established that Api5 is de-acetylated before undergoing degradation. Therefore, we analysed the stability of all the three Api5 mutants of K251 following DNA damage. It was observed that only the de-acetylated form of Api5 underwent post-translational degradation by the proteasomal pathway upon DNA damage, while the acetylated protein remained stable. This stable form of Api5 also regulated DNA damage-induced apoptosis differentially. Taken together, we conclude that Api5 undergoes post-translational degradation upon DNA damage through the proteasomal pathway. p300, HDAC1, ATR and FBXW2 are the primary regulators of Api5 upon DNA damage-induced apoptosis.

Summary

Our study reports that Api5 undergoes post-translational degradation by the ubiquitin-proteasomal pathway. Cellular levels of Api5 is maintained by p300-mediated acetylation of Api5 at lysine 251, while de-acetylation by HDAC1 leads to reduction of Api5 levels. It has been shown that levels and activity of p300 increase in the G1 phase of the cell cycle while the activity of HDAC1 is at its peak during the G2/M phase of the cell cycle. In the G1 phase, p300 regulates the stability of many proteins like E2F1 and p53. Both E2F1 and p53 are known to regulate the pattern and profile of the cell cycle. Studies from Garcia and group and our lab concluded that levels of Api5 are at their peak in the G1 phase of the cell cycle and minimum in the G2/M phase. This indicates that Api5 might play a significant role in the regulation of the cell cycle.

Reduced protein expression of Api5 upon induction of DNA induced apoptosis also confirms its role in the regulation of apoptosis. Its basal physiological levels help in preventing apoptosis upon DNA damage. It might be possible that during mild DNA damage (below the required threshold for initiation of apoptosis), Api5 might be regulating the levels of DNA repair proteins so that the cell cycle can continue. It might also be possible that the basal levels of Api5 are not permitting cells to sense the damage.

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Abbreviations

Api5	Apoptosis inhibitor 5
AAC11	Anti-apoptotic clone 11
AKB	Aurora kinase B
ATR	ATM and Rad3 related
BRCA1	Breast cancer 1
CDK	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CPT	Camptothecin
DMEM	Dulbecco's modified eagle's medium
dNTP	deoxy-nucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DSB	Double strand break
ETP	Etoposide
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FIF	FGF2 interacting factor
G1	Growth 1 phase
G2	Growth 2 phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
LZD	Leucine zipper domain
M	Mitosis phase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PIZSA	Protein Interaction Z Score Assessment
PTM	Post-translational modification
RFP	Red fluorescence protein
RPA	Replication protein A
S	Synthesis phase
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	small interfering RNA
SSB	Single strand break
TBS-T	Tris buffered saline-Tween-20

Chapter 1

Introduction

1.1 DNA damage response

The environment in which a eukaryotic cell resides contains numerous DNA damaging agents that can attack its genome. The damaging agents can be either endogenous or exogenous (De Bont and van Larebeke, 2004). One of the major endogenous agents is reactive oxygen species (ROS) which include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen that can oxidize the DNA (De Bont and van Larebeke, 2004). Lipid peroxidation produces aldehydes that form exocyclic adducts and thus hampers Watson-Crick base pairing. Exogenous agents like UV radiation, IR rays, tobacco smoke and adulterants/pollutants in food or alkylating agents also attack the DNA. The kind of DNA damage caused by different agents can be different like UV induces single-strand DNA break, IR causes double-strand DNA break and some alkylating agents cause single base change. Single strand DNA breaks are recognised and coated by the activated RPA while the double-strand breaks are identified by γ H2AX (Kuo and Yang, 2008; Zou et al., 2006). These different types of DNA breaks lead to the activation of different sensor proteins like single-stranded breaks leads to the activation of ATR and double DNA strand breaks are sensed by the ATM (Abraham, 2001). ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) along with DNA-PK and mTOR comprise the main members of the phosphatidylinositol 3-OH kinase-like kinases family. Their functioning upon DNA damage also varies. ATR phosphorylates and activates Chk1 while ATM phosphorylates Chk2 (Abraham, 2001). These activated checkpoint proteins mediate DNA damage response through p53. It has also been reported that ATM and ATR can also directly activate p53 by phosphorylating it (Banin et al., 1998; Nakagawa et al., 1999; Tibbetts et al., 1999). These signalling cascades can also initiate DNA repair pathways.

The cell recruits specific repair proteins based on the kind of damage that occurred. Mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), Homologous Recombination repair (HRR) and non-homologous end joining (NHEJ) are the major DNA repair pathways in eukaryotes (Bernstein et al., 2002). The key players in most of these pathways have dual functions in damage repair and pro-apoptosis signalling. Any defect in these repair pathways leads to the accumulation of damage which causes genomic instability. This irreparable damage induces apoptotic signalling leading to the up-regulation of proteins involved in apoptosis (Figure 1.1).

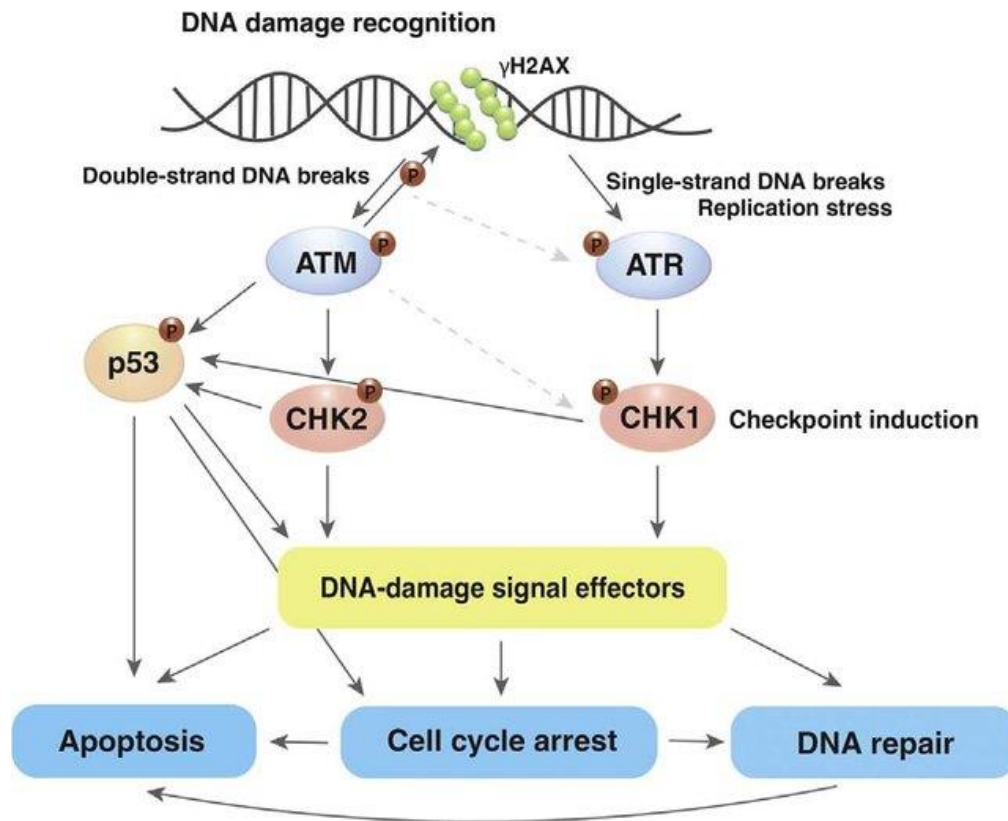


Figure 1.1: Schematic representation depicting DNA damage response

Reproduced from (Tsuiko et al., 2019)

1.2 Apoptosis

Apoptosis is a programmed and specialised form of cell death. Apoptosis happens normally to maintain homeostasis of the tissues where excessive and unnecessary cells undergo cell death with special morphological characteristic features like cell shrinkage, pyknosis, chromatin condensation, and apoptotic body formation (Bortner et al., 1995) (Bratton et al., 1997) (Zhang and Xu, 2000). This process has been observed to occur during normal embryonic development, aging, immune system, and in chemotherapies targeting cancers. It is necessary for removing excess and damaged cells from the body, maintaining the size of tissues, and shaping organs during development. Several signals and pathways that include many molecular players are involved in the events that ultimately lead to apoptosis (Elmore, 2007). The cell survives within a balance of endogenous as well as extracellular survival signals and death signals that are constantly regulated. An override of this regulation and tipping of the balance towards more growth signals or less death signals leads to uncontrolled cell growth and this is one of the hallmarks of cancer. Apoptosis takes place through two main pathways like intrinsic pathway and extrinsic pathway

(Elmore, 2007). However these two pathways are not mutually exclusive, one pathway player can also initiate the other kind of apoptotic pathway. In the intrinsic pathway or mitochondria-dependent pathway, the cell kills itself upon sensing some stress while in the extrinsic or ligand-dependent pathway cell senses some death signal from the nearby cells before undergoing cell death. Both the apoptotic pathways induce cell death with the help of special enzymes known as caspases. Caspases are a special kind of proteases that degrade proteins. Both the pathways include activation of caspases as a key step to complete the process of apoptosis. Initiation of apoptosis is a very well regulated and crucial step because if the cell starts to undergo apoptosis the final is cell death (Figure 1.2).

1.2.1 Intrinsic pathway of cell death

In the intrinsic pathway of cell death, cells start intracellular apoptotic signalling after perceiving some stress. This stress can be through various ways like the binding of the steroid hormones to the nuclear receptors, temperature, upon infection with certain viruses, during hypoxia, and upon dysregulation osmoregulation by the variable concentration of calcium and other ions intracellularly (Fulda and Debatin, 2006). These kinds of stresses release various apoptotic signalling molecules from the cells. These signalling molecules are capable enough to induce the initiation of apoptosis. During the initiation of apoptosis by intrinsic pathway mitochondria is targeted. There are several ways by which mitochondria can be affected through apoptotic molecules (Rosenblatt et al., 2001). Apoptotic molecules can cause pore formation through the membrane and can disrupt the permeability of the mitochondria and cause the leakage of apoptotic effector molecules. There are some other methods also through which mitochondria can also get affected for example nitric oxide, a gaseous form of molecule that can disrupt the membrane potential of the mitochondria and lead to apoptosis. After targeting the mitochondria, cytochrome c a regulatory protein is released from mitochondria (Ferri and Kroemer, 2001). This release of cytochrome c is achieved through the function of Bax and Bak on the mitochondrial membrane. Bax and Bak can form both homo and heterodimers and get inserted into the outer membrane of mitochondria (Wolff et al., 2008). Once the cytochrome is released its binds to the Apaf-1 and ATP which then binds to procaspase-9 to form a complex called Apoptosome, which in turn cleaves and activates caspase-3, an executioner caspase (Bratton and Salvesen, 2010).

Mitochondria also release some other regulators of apoptosis, like SMACs. This release is achieved after the disruption of membrane permeability. These SMACs bind to the different inhibitors of Apoptosis and inactivate them. IAPs are known to inhibit the activity of a group of cysteine proteases called caspases. Thus the mitochondria and their released products are the major factors involved in the regulation of apoptosis (Silke and Meier, 2013).

1.2.2 Extrinsic pathway of cell death

There are two main methods through which the extrinsic pathway of apoptosis can initiate, TNF induced and Fas-Fas ligand-mediated. TNF- α is the main component of Apoptosis regulation through the extrinsic pathway. During the process of inflammation activated macrophages secrete this cytokine. This binds to its receptor TNFR1 and TNFR2. This binding of the cytokine to its receptors leads to the activation of caspases. This activation of caspases is achieved by intermediate and transient membrane protein complexes named TNF receptor-associated death domain (TRADD) and Fas-associated death domain. Signalling through TNFR1 induces apoptosis by transcribing the genes involved in the regulation of apoptosis. It has also been reported that TNF α also includes DR4 and DR5. These receptor proteins upon binding with protein TRAIL mediate apoptosis (Kasper et al., 2010).

Fas receptor is a transmembrane protein. This receptor upon receiving the apoptotic signals binds to its ligand called Fas ligand. This interaction of the fas receptor with its ligand leads to the formation of a death-inducing signalling complex. This complex consists of FADD, caspase 8 and caspase-10 (Cai et al., 2019). Thus this complex helps in the initiation of the extrinsic pathway of apoptosis by increasing pro-apoptotic molecules from mitochondria and enhancing activation of caspase 8 (Powley et al., 2016).

Caspases are the members of large family proteases. Caspases are known to regulate all types of cell death-like necrosis, necroptosis, and apoptosis. Thus caspases help in maintaining the normal homeostasis of tissues. They are named caspases because of their specific activity of cutting at cysteine and aspartic acid residues. According to a report in 2009, there are 12 caspases known in humans and 10 in mice. The role of these caspases in the regulation of apoptosis is very well known (Powley et al., 2016). Activation of caspases leads to the degradation of

cellular products with minimal surrounding side effects. Caspases are also tightly regulated inside the cells because less activation or absence of caspases can lead to tumor progression and higher activity can also lead to certain diseases like Alzheimer's disease (Thawkar and Kaur, 2019). The apoptotic caspases have been divided into two main categories depending upon their role in apoptosis.

1. Initiator Caspases (Caspase 2, Caspase 8, Caspase 9, Caspase 10)
2. Executioner Caspases (Caspase 3, Caspase 6 and Caspase 7)

Once the initiation of apoptosis occurs, it leads to the activation of initiator caspases and activated caspases leads to the activation of executioner caspases which ultimately regulate the whole process of apoptosis (Powley et al., 2016).

Some well-known pro-apoptotic proteins that induce cell death include Bax, Bim, Apaf-1, tumor necrosis factor (TNF) family proteins, and caspases (Fulda and Debatin, 2006). Anti-apoptotic proteins like Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and inhibitors of apoptosis (IAPs) and growth factors work against these pro-apoptotic inhibitors to inhibit the process of apoptosis (Miyashita et al., 1995).

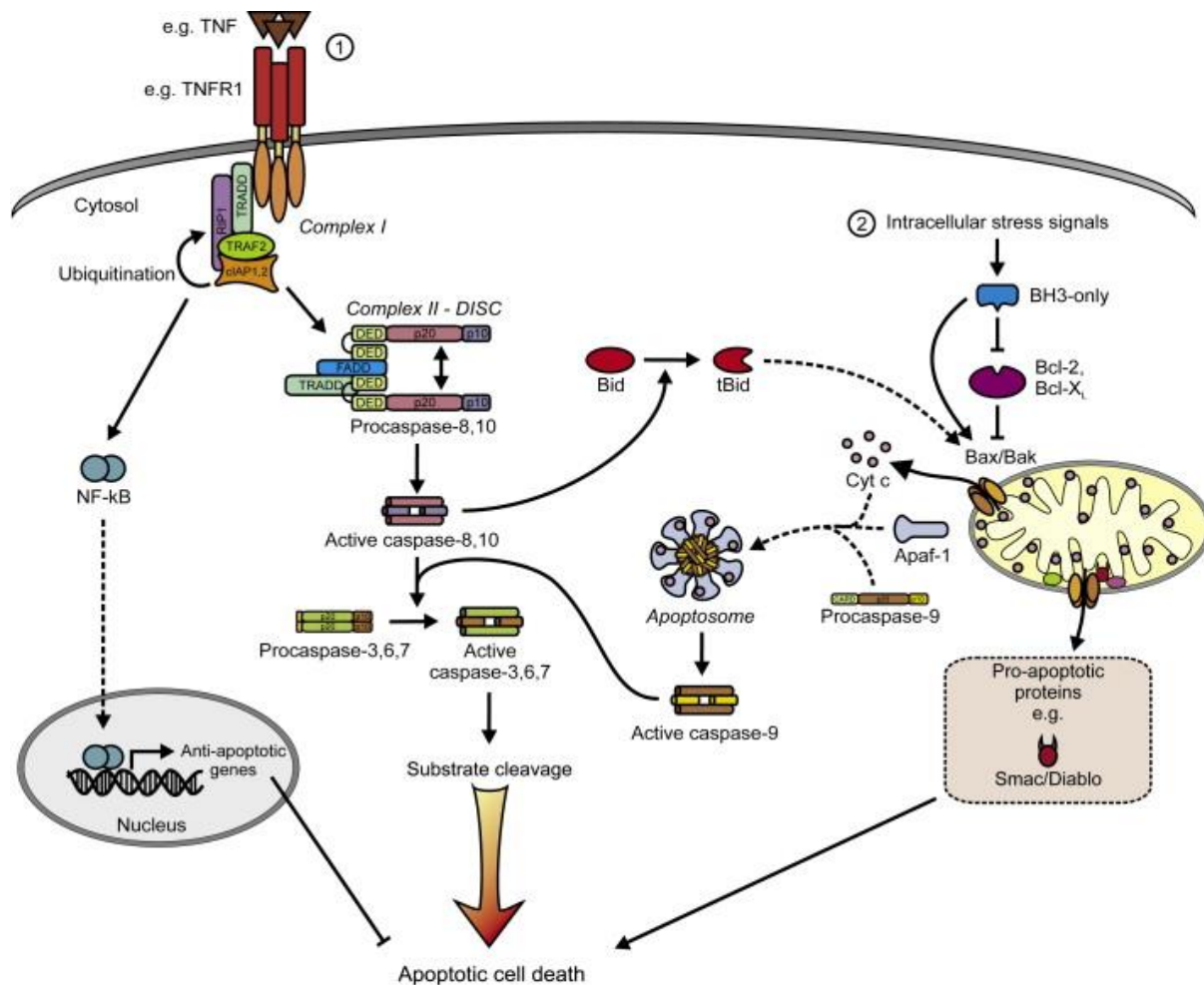


Figure 1.2: Schematic representation of major apoptotic pathways

Reproduced from (Duprez et al., 2009)

1.3 Inhibitor of Apoptosis Protein (IAP)

Inhibitor of Apoptosis (IAP) proteins are a family of proteins involved in cell death regulation by inhibiting apoptosis and regulation of the cell cycle. The presence of one to three baculoviral IAP repeats (BIR) domain is the characteristic feature of the members of this family. It was first identified by Miller and Crook in 1993 as a baculovirus gene with a zinc finger-like motif that inhibited apoptosis (Crook et al., 1993). Further studies from the same group suggested the presence of a Cys/His sequence motif in the peptide. Cellular IAPs are identified as components of the TNFR2 complex (Birnbaum et al., 1994).

c-IAP1, c-IAP2, XIAP, survivin, NAIP, ILP-2, ML-IAP, and Apollon are the major 8 classes of IAPs in humans. Some of them control apoptosis directly while others regulate cell survival by cell cycle control or inflammation. XIAP binds to Caspase

through its BIR domain and prevents its dimerization and activation by blocking the active site of Caspase (de Almagro and Vucic, 2012).

IAPs have a C-terminal RING domain apart from its BIR domain which provides an E3 ubiquitin ligase activity to the proteins. Dimerization of the RING domain triggers the ubiquitin ligase activity. Homo-dimerization or hetero-dimerization of the RING domain of IAP can lead to auto-ubiquitination or trans-ubiquitination of its binding partners and thus leads to their degradation.

1.3.1 Survivin: Major IAP

Survivin, a unique member of IAP, has a direct role in the inactivation of Caspase and regulation of cell cycle (Sah et al., 2006). The telomeric region of chromosome 17 encodes for the survivin gene. It has a single BIR, an amphipathic C-terminal helix but lacks the RING domain. Cancer cells that showed an up-regulation in survivin were poor in prognosis as they had acquired drug resistance. Meanwhile, normal differentiated cells didn't express survivin. This differential expression pattern makes it a good target in cancer treatment.

Survivin can be detected in biological fluids of cancer patients so has been now used as a prognosis marker to understand the different stages of carcinogenesis.

Disruption of the surviving mediated anti-apoptotic pathway increased apoptosis and decreased tumor growth. Administration of drugs against survivin along with other chemotherapeutic agents increased the efficiency of cancer treatment and opened a promising field for new efficient drug discovery. Cancer cells acquire immortality by inhibiting apoptosis proteins. So inhibiting apoptosis inhibitors had shown to be an efficient way by which we can treat cancer (LaCasse et al., 1998). Many different drugs against apoptosis inhibitors have been shown to increase the chemotherapeutic efficiency, specificity, and avoided drug resistance provides better chances of survival.

So studies to identify new apoptosis inhibitors and their drug targets will be more helpful in the field of cancer treatment. Also, the identification and characterization of apoptosis inhibitor proteins that have interacting partners in other major cellular pathways like DNA damage repair pathways will provide new insights into the field. Api5 is one such apoptosis inhibitor that has been shown to interact with a major mediator protein in the repair pathway.

1.4 Apoptosis inhibitor 5 (Api5)

Apoptosis inhibitor 5 (Api5) is also known as anti-apoptotic clone 11, AAC-11, Fibroblast growth factor 2 interacting factor, FIF2 and migration inducing gene 8, MIG8 (Morris et al., 2006b; Tewari et al., 1997; Van den Berghe et al., 2000). Api5 was initially discovered as a nuclear protein, which was responsible for the inhibition of apoptosis under serum starvation conditions in murine fibroblast (BALBc3T3) cells (Tewari et al., 1997). These cells were transfected with murine cDNA library cDNA constructs. It was observed that cells transfected with AAC-11 clones were able to survive for around 40 days in serum-free media while cells transfected with other constructs showed apoptosis after 10 days in serum-free media (Tewari et al., 1997). Cells transfected with AAC-11 showed higher survival than cells transfected with the known anti-apoptotic gene bcl2 (Figure 1.3). This observation established Api5 as an anti-apoptotic protein.

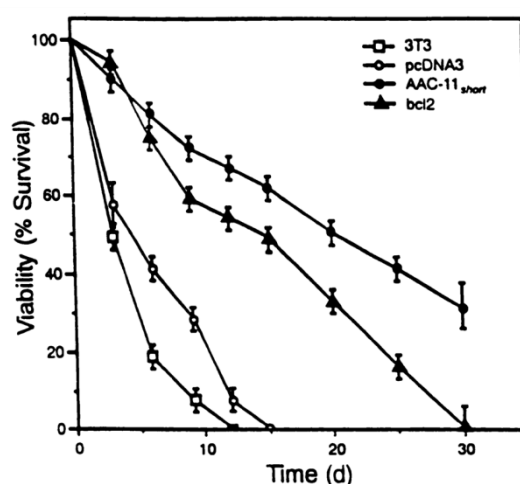


Figure 1.3: Over-expression of AAC11 inhibits apoptosis in serum-starved murine fibroblast cells (Adapted from Tewari et al., 1997)

Api5 is a right-handed superhelix that is composed of all helical repeats (Figure 1.4). It has 19 α -helices and two 3_{10} helices (Han et al., 2012). It has also been observed that all these helices interact with neighbouring helices to form an antiparallel helix pair. N- terminal of Api5 comprises of α -helix from 1 to 11 which shares homology with the HEAT repeat structures of other proteins such as Importin β . C- terminal of Api5 is made up of alpha-helices from 12- 19 and share homology with ARM like repeat structures of other proteins, for example, p120 catenin (Han et al., 2012).

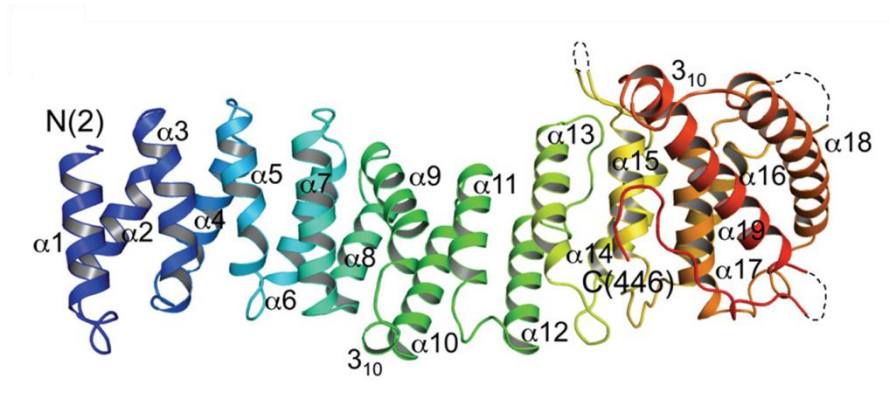


Figure 1.4: Structural representation of Api5 (reproduced from Hans et al., 2012)

N-terminal of Api5 contains LxxLL motif which is an amphipathic alpha-helix and forms nuclear receptor-co factor interaction regions (Figure 1.5). This motif is present in the sixth alpha-helix and helps maintain the stability of the protein by forming hydrophobic interactions with neighboring alpha-helices and helps interact with FGF2. C-terminal of Api5 has nuclear localisation signal and a Leucine Zipper domain (LZD). Leucine Zipper Domain is present in the alpha -18 helix between 371-391 amino acid residues. It interacts with Acinus, a protein that mediates fragmentation of chromatin during apoptosis. However, this LZD does not bind with DNA (Han et al., 2012).

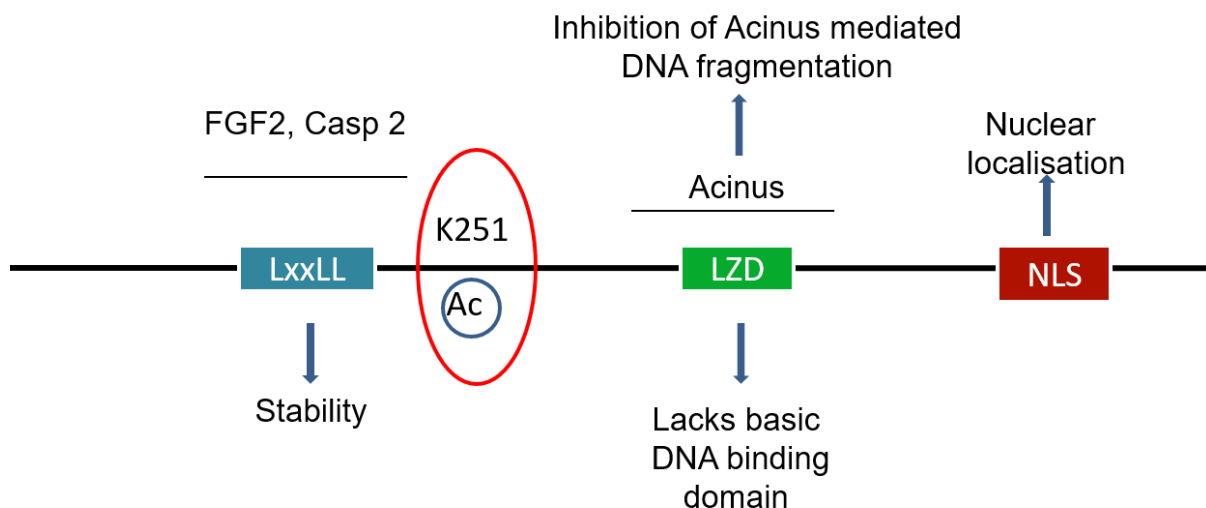


Figure 1.5: Characteristic domain representation of Api5

Global mass analysis of Api5 suggests that it has one conserved lysine site at 251. One post-translational modification has also been reported to be conserved at K251.

This lysine 251 acetylation is present in the hinge region between alpha 13-alpha 14 (Han et al., 2012). K251 acetylation is the only known post-translational modification for Api5.

It has been proven that Api5 inhibits the dE2F1 mediated apoptosis in Drosophila cells. dE2F1 is a well-known pro-apoptotic gene responsible for apoptosis in Drosophila cells. In Drosophila embryonic cells, SL2, RNAi mediated depletion of Api5 was found to be responsible for higher apoptotic cell death as compared to the dE2F1 overexpressed cells. A similar result has also been observed in human osteosarcoma cell lines, Saos-2, where ectopic expression of Api5 decreased E2F1 mediated apoptosis in E2F1 over-expressed cells without affecting its transcriptional activity (Morris et al., 2006a).

According to the study of Rigou et al., AAC-11 binds to the Acinus which is a nuclear protein and plays a significant role in chromatin condensation during apoptosis. Upon acute DNA damage, the acinus is cleaved by caspase 3 and an active fragment of acinus is translocated to the nucleus. Inside the nucleus fragment of the acinus initiates the heterochromatisation and fragmentation of DNA that eventually leads to the initiation of apoptosis. Upon ectopic expression, AAC-11 binds to acinus and does not allow its degradation by caspase 3, in turn inhibiting DNA fragmentation. This eventually inhibits the further apoptotic signalling pathway. Hence, inhibition of cleavage of Acinus by Api5 reduces apoptosis in cervical cancer, HeLa cells after chemically induced DNA damage (Rigou et al., 2009).

Further studies carried out in melanoma cell line, 526 melanoma cells, has shown that Api5 modulates the FGF2 and FGFR1 signalling pathway. This signalling activates ERK. This activated ERK causes the phosphorylation of Bim, a pro-apoptotic protein. Phosphorylated Bim is the target for proteasomal degradation. Thus ubiquitin-mediated degradation of Bim is a way by which Api5 inhibits apoptosis in HeLa and 565mel cells (Noh et al., 2014).

In addition, Mayank and group showed that Api5 inhibits the transcription of the APAF1 gene. APAF1 is the main component of the apoptosome complex and is transcribed by the transcriptional activity of E2F1. During the viral infection, nucleoprotein, a viral protein binds and sequesters Api5. This binding of nucleoprotein to Api5 has been reported to enhance the E2F1 binding to its target

promoters, in this case, APAF1. This leads to the formation of an apoptosome complex that regulates programmed cell death of cells. However, upon overexpression Api5 sequesters the nucleoprotein which in turn diminishes the binding of E2F1 to the APAF1 promoter. Thus Api5 does not allow the apoptosome formation in turn inhibiting the apoptosis (Mayank et al., 2015). One recent study suggests that Api5 physically interacts with Caspase 2, which acts as both initiators as well as executioner caspase and does not allow it to get activated. Upon α toxin-mediated stress caspase 2 first forms homodimers and in late stages this homodimer undergoes proteolytic cleavage. These overall processes cause the activation of caspase 2. Upon ectopic expression Api5 forms heterodimers with caspase 2 and does not allow its activation, thus inhibiting the apoptosis (Imre and Rajalingam, 2017). These are known anti-apoptotic functions of Api5 that are generally initiated upon induction of some kind of stress such as DNA damage.

1.5 Cell cycle

Api5 has also been reported to be involved in the regulation of E2F1, a transcriptional activator of G1/S cell cycle transition genes, by enhancing the binding affinity of E2F1 to its target promoters. Knockdown of Api5 arrests H1299 cells, non-small cell lung carcinoma cells, at G1 phase. This proves that apart from regulating apoptosis, Api5 also has a role in maintaining normal cell cycle progression (Garcia-Jove Navarro et al., 2013)

Further study revealed that the protein levels of Api5 are regulated in a cell cycle-dependent manner (Garcia-Jove Navarro et al., 2013). To prove the cell cycle-based fluctuation in protein expressions of Api5, H1299 non-small cell lung carcinoma cells were synchronised in the G1-S boundary using the double thymidine method. These were then released in thymidine-free media to obtain the cells in the G1, S, and G2-M stages of the cell cycle. It was observed that the levels of Api5 were higher in the G1 phase and were stabilised during the G1-S transitions. The levels of Api5 were observed to decrease as the cells proceeded further to the G2 and G2-M phases of the cell cycle (Navarro et al., 2013). This degradation of Api5 was at the post-translational level as the *Api5 transcript* levels were unaltered in the G2-M phase of the cell cycle. This suggests that Api5 undergoes degradation during cell cycle progression (Figure 1.6).

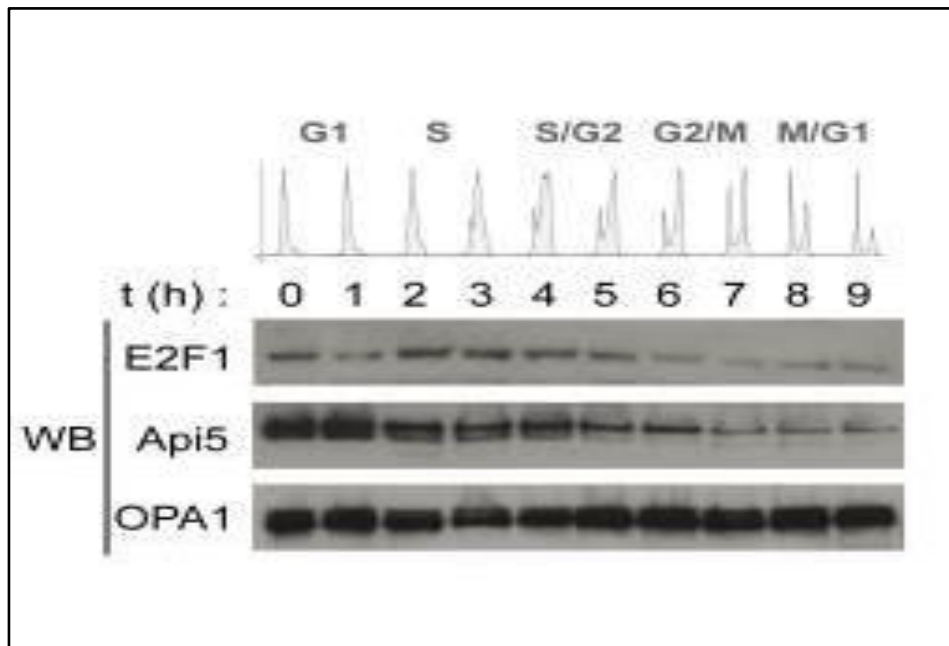


Figure 1.6: Api5 levels fluctuate across the cell cycle

Western blot analysis depicting the posttranslational regulation of Api5 in H1299 cells (reproduced from Garcia et al., 2012)

Cell division is a process in which duplicates of its contents, segregates them equally, and then divides to give rise to two daughter cells. The time interval between two successive divisions constitutes a cell cycle. The completion of a cell cycle requires the functioning of various proteins that will guide cells to certain sequences to achieve two daughter cells. Central to all Cyclin-dependent kinases Cdks and cyclins are the crucial regulators of cell division. These sequential events have been categorised as the different phases of the cell cycle. In each phase, the cell has various ways to control this cycle such as checkpoints at different stages, a series of switches, and many external and internal cues. All this makes up the cell cycle control system and it is essential in controlling the number of cells. There are four phases of the cell cycle in eukaryotes:

First Growth (G1) phase: This is a preparatory phase of cell division where cells sense for different external and internal clues. For external clues cells sense for space, availability of nutrients, and growth factor for survival of two daughter cells. For the internal clues, cells sense the proteins and other regulators required for the DNA synthesis and follow up processes like transcription and translation. This is the time for the cell to carefully study its external and internal environment and if the

conditions are favourable, the cell commits to enter the next phase of cell division, which implies that the cell has crossed the first checkpoint/restriction point that is known as G1-S restriction point (Hume et al., 2020).

Synthesis (S) phase: This is the central event of the cell cycle where the DNA is duplicated where a cell completes the G1 phase and prepares itself for replication of DNA. During the S phase of the cell cycle, certain enzymes like DNA polymerase, topoisomerase, gyrase, and ligases become active. For successful division, the replication should be accurate and should occur only once per cycle since the replication more than once during the S phase cell can lead to genomic instability that eventually can be lethal for the cell. The latter is overseen by the cell cycle control system where certain regulatory proteins like MCMs check for the firing of multiple origins of replications. These proteins interact with multiple ori sequences of DNA and inhibit their firing ensuring that DNA replication takes place through only a single origin of replication. This is the mechanism by which a cell keeps control of ploidy levels of its genetic material (Takeda and Dutta, 2005).

Second growth (G2) phase: It follows the S phase and this is the time where all the other organelles like mitochondria, Golgi apparatus, and ER are duplicated for their proper segregation in two daughter cells. This also serves as a checkpoint that ensures that the DNA has been replicated properly. This is also a kind of preparatory phase of cell division in which a cell senses the faulty free replication of its DNA and initiates the signalling cascade to activate the regulators of cell division so that cell can be divided in the next phase of the cell cycle (Liskay, 1977).

Mitosis (M) phase: This is the shortest and very complex stage of the cell cycle. Mitosis is divided into multiple sub-stages that are prophase, metaphase, anaphase, and telophase. In the prophase, the duplicated DNA starts condensing to form a compact structure that is chromosomes. These chromosomes for their proper segregation align at the centrally aligned plate during the metaphase. During the metaphase chromosomes are positioned to the metaphase plate by microtubules. These microtubules during the anaphase start depolymerising and thus chromosomes are migrated to the respective poles hence the segregation of chromosomes is achieved. For metaphase to anaphase transition, APC/C, a ubiquitin ligase, marks a bunch of proteins such as securin that hold sister

chromatids together for degradation, thus leading to the progression of the cell cycle. Cyclins have certain sequences in them so that they can be proteolytically degraded once they have completed their function and carried the cell through the particular phase of the cell cycle (Glutzer et al., 1991). Cdk activation also depends on the phosphorylation present on them. There are inhibitory and activating phosphorylation sites on Cdk. This provides another layer of regulation (Solomon and Kaldis, 1998). During the telophase, the two sets of chromosomes are positioned at the two ends of a single midway dividing cell. Later the whole divided (cytokinesis) to give rise to two daughter cells (Figure 1.7).

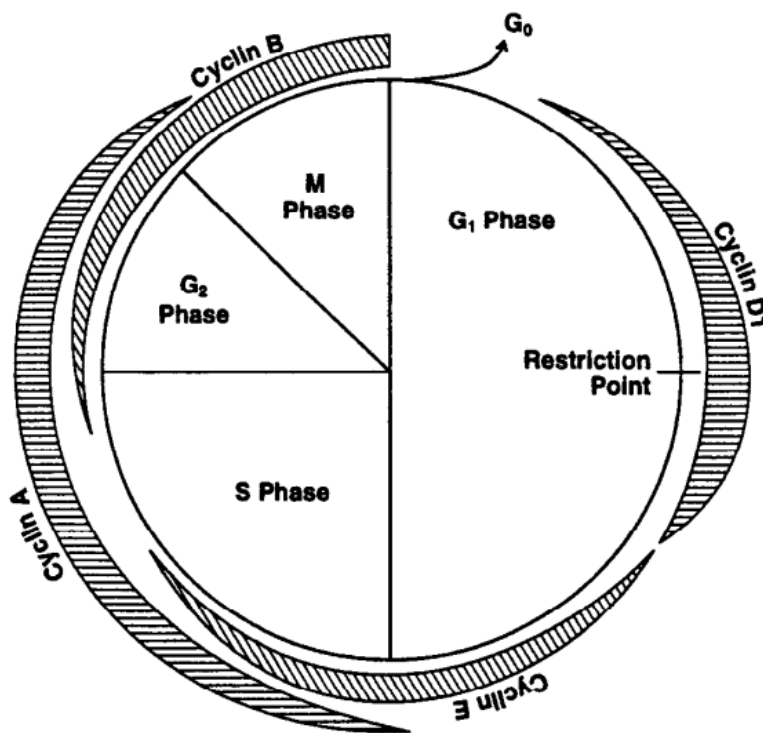


Figure 1.7: Schematic representation of cell cycle and cyclins involved in each phase

Adapted from (Schafer, 1998)

The cell must follow these four phases in an appropriate sequence for proper cell division. The cell cycle control system is like a cue that ensures that each phase is completed before the initiation of the next one. It is also a security system that order of phenomenon required in the completion of a cell division. One of the major executors of the cell cycle control system is cyclin-dependent kinases (Cdks), which are serine/threonine kinases. The protein levels of Cdks are constant during the cell

cycle (Malumbres and Barbacid, 2009). Activation of Cdks requires the functioning of a family of proteins called cyclins (Koepp et al., 1999). The protein levels of cyclin vary during the cell cycle like cyclin D is synthesised and maintained as long as there is growth factor signalling. Cyclin D activates Cdk4 and CDK6 and ensures the entry of the cells in the G1 phase of the cell cycle. For the transition from G1 to S, Cyclin E combines with Cdk2. After which Cyclin A forms a complex with Cdk2 for S phase entry. In G2 and entry into M phase, cyclin A combines with Cdk1, and for the rest of the M phase, Cdk1 associates with Cyclin B1 (Vermeulen et al., 2003). SCF and the anaphase-promoting complex (APC/C) are the important E3 ubiquitin ligases in cells that target various proteins for degradation, which eventually leads to successful cell division.

To make sure all the essential steps have been accomplished in the previous stage before the cell cycle proceeds further, there are checkpoints. Since the intact and pure DNA should accurately be transferred to the newly formed daughter cells, any defects in DNA activate checkpoints at G1/S and G2/M, via activation of ATM/ATR, which in turn activates Chk1 and Chk2 respectively, that eventually leads to the activation of p53. Activated p53 facilitates the activation of p21, a Cyclin-Cdk inhibitor, thus halting the cell cycle till the damage is repaired (Rodier et al., 2007). Unfavourable environmental conditions like lack of nutrient availability and space can also activate these two checkpoints. Incomplete replication can activate the G2 checkpoint where cells scan for the accurate synthesis and replication of entire DNA. Any defects in the synthesis or replication of DNA can cause the cell to be arrested at the G2 phase of the cell cycle. Another important event that ensures that DNA content gets equally distributed among the daughter is metaphase to anaphase transition, hence there is a checkpoint here too, which ensures that all the chromosomes are attached in a bipolar fashion before anaphase begins (Tanaka et al., 2002).

As mentioned earlier, protein levels of Api5 fluctuate during the cell cycle (Garcia-Jove Navarro et al., 2013). It has also been reported that Api5 knockdown induces a significant decrease in cyclin D (G1 cyclin), cyclin E1 (G1/S cyclin), cyclin A (S phase cyclin), and Cdk mRNA levels, which is the same effect observed upon knockdown of E2F1. This suggests that both Api5 and E2F1 could possibly be the regulators of

the same pathway as Api5 has been shown to facilitate the E2F1 recruitment to E2F1's target promoters.

Although the literature suggests about the post-translational degradation of Api5 during the G2-M phase of the cell cycle, however, it does not mention anything about the molecular mechanism behind the degradation of Api5. Taken all these together we aimed to find out the pathway and regulators of Api5 degradation in the G2-M phase of the cell cycle.

There is another study by Han and group that revealed that acetylation at K251 of Api5 is a crucial regulator of its stability (Han et al., 2012). To establish the relation between stability and K251 acetylation of Api5, Flag-tagged site-directed mutants of K251 Api5 such as K251A acetylation deficient mimic, K251R charge mimic mutant and K251Q a constitutive acetylated mimic mutant along with wild type were ectopically expressed in HeLa cells and their stability was analysed in the presence of a protein synthesis inhibitor, cycloheximide. It was observed that levels of wild type, as well as K251R Api5, were decreasing upon treatment with cycloheximide while K251A levels were not stable even in the absence of cycloheximide (Figure 1.5A). Interestingly, K251Q, a constitutive acetylated mimic mutant, levels were not reduced upon treatment with cycloheximide. This suggested that K251 acetylation plays a significant role in maintaining the stability of Api5. The role of K251 acetylation on maintaining the stability of Api5 was further confirmed by the experiment where the Flag-tagged wild type construct was expressed and its stability was analysed upon treatment with cycloheximide, SAHA, a deacetylase inhibitor, and both. Reduced levels of wild-type Api5 upon treatment with cycloheximide were rescued upon treatment with deacetylase inhibitor (Figure 1.8). This suggests that acetylation, a post-translational modification at K251 of Api5, regulates its stability while the de-acetylated form of Api5 undergoes post-translational degradation. Acetylation and deacetylation are amongst the known post-translational modifications of the proteins.

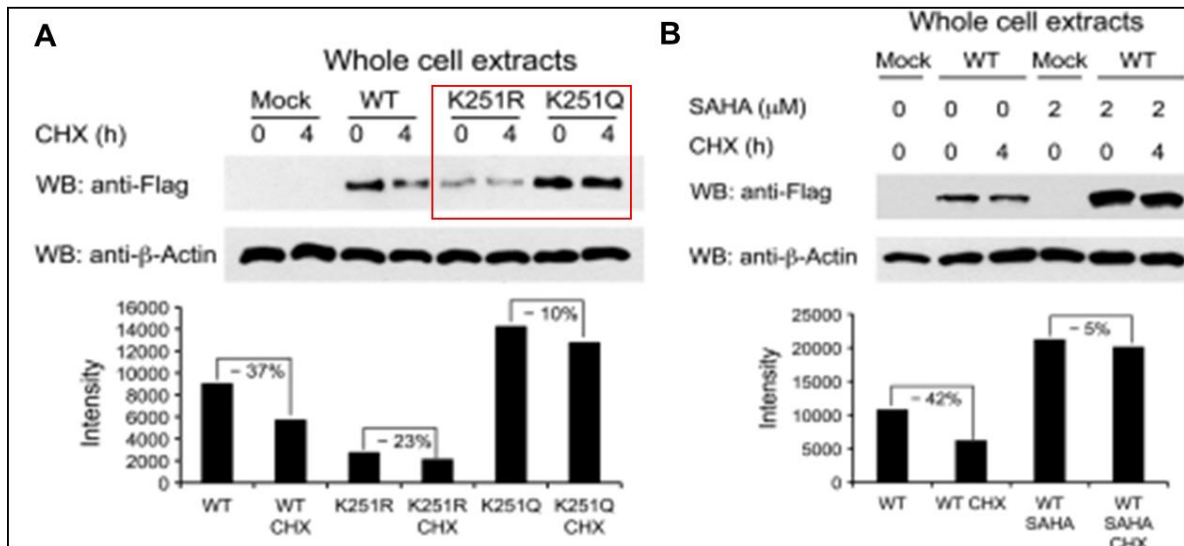


Figure 1.8: Acetylation of Api5 at K251 regulate its stability

Western blot analysis depicting the A and B) role of Lysine 251 acetylation in the stability of Api5 in HeLa cells (reproduced from Hans et al., 2010).

1.6 Post-translational modifications

Post-translational modifications, PTMs are the decorations of the proteins and performed after their synthesis. During the process of PTMs different types of moieties like small peptides or functional groups like acetyl and phosphates are attached to the surface of proteins. Various types of PTMs have been discovered for different proteins. Phosphorylation, acetylation, ubiquitination, sumoylation, methylation are the most studied types of post-translational modifications. Hydroxylation, tyrosination, glutamylation, and glycosylation PTMs are also known to regulate different physiological aspects of the cells (Vu et al., 2018).

1.6.1 Sumoylation

Sumoylation is a reversible posttranslational modification. During this modification, a small ubiquitin-like modifier is covalently attached to the substrate proteins. This process of sumoylation is similar to the ubiquitination with some differences like attached sumo moiety is low in molecular weight protein and unlike ubiquitination, sumoylation can also stabilise the proteins. This PTM has been reported to be involved in various cellular processes such as nuclear-cytoplasmic transport, transcriptional regulation, apoptosis, and protein stability, a response from stress and progression through the cell cycle (Eifler and Vertegaal, 2015; Yang et al., 2017; Yau et al., 2020).

1.6.2 Methylation

This is a post-translational modification in which a small chemical group, methyl, is added to the protein while removal of methylation occurs during the demethylation. Methylation of proteins is also involved in various cellular processes but the most studied role of methylation is in transcriptional regulation (Traube and Carell, 2017). Histones are the proteins that get methylated frequently depending upon the requirement of expression or silencing of genes (Hashimoto et al., 2010). Methylation of histones 3 at various lysine residues helps in the silencing of genes (Hashimoto et al., 2010). Methyltransferases are the enzymes involved in methylation while demethylases are the enzymes that help in removing the methyl group from the substrate proteins.

1.6.3 Phosphorylation

It is one of the most essential and crucial types of a post-translational modification. It is a reversible decoration regulated by the two key enzymes. Kinases are the enzymes that recognise the substrate proteins and add the phosphate group to the nascent proteins while the phosphatases are the enzymes that recognise the phosphorylated substrate proteins and remove the phosphate groups after receiving the context-based signal. AMPK, Akt, PI3K, GSK3 β , and mTORCs are the kinases involved in normal physiological responses like cellular proliferation, cell division, and growth. Multiple cascades of signalling are regulated by these kinases (Pradere et al., 2016; Singh et al., 2017). ATM and ATR are the main kinases that get activated upon DNA damage. They phosphorylate downstream players like p53, Chk1, and Chk2 to initiate cell cycle arrest and DNA damage repair pathways (Abraham, 2001; Moiseeva et al., 2019). Phosphatases are the enzymes involved in removing the phosphate group from substrate proteins. Alkaline phosphatases and acid phosphatases are the main forms of phosphatases that regulate the normal physiological processes of cells. p53 is a protein that has its significant role in almost all cellular aspects and undergoes various kinds of post-translational modifications depending upon its fate and function. p53 is phosphorylated by ATM and ATR after sensing the DNA damage and this phosphorylated form of p53 is functional in the repair of damaged DNA and arresting of the cell cycle. p53 also enables the cell to sense the degree and intensity of the damage. If the damage intensity is more than the threshold of repair then phosphorylated p53 also helps in making cells undergo

apoptosis, a programmed cell death. De phosphorylation of p53 is carried out by protein serine/threonine kinase 1 which makes a direct impact on the functions of activated p53.

CDKs, polo-like kinases, and aurora family kinases are the main kinases involved in cell cycle progression. Aurora Kinases, Aurora Kinase A, B, and C are a family of serine-threonine kinases. Out of these three kinases, Aurora kinase B has been studied extensively. Aurora Kinase B is part of the passenger complex, which is a set of proteins that have a unique localization pattern throughout the G2-M phase. The passenger complex proteins are nuclear in the G2 phase, associate throughout the chromatin in prophase, by prometaphase, they are localized to the inner centromere region, at anaphase they are found at the central spindle, and then gathers at the midbody during cytokinesis (Vagnarelli and Earnshaw, 2004). Aurora Kinase B localization requires the activity of Survivin and INCEP (Adams et al., 2001). Aurora Kinase B forms a complex with INCEP, that increases the turnover of Kinetochore connection to the microtubules from the spindle pole bodies until they are bi-oriented, that is the two kinetochores on one chromosome are attached to microtubules from different poles. In yeast, it has been shown that the absence of Aurora Kinase B activity, led to the persistent connection of kinetochore-spindle pole bodies throughout G1. The turnover will continue to occur till bi-orientation is achieved, by which stage the tension will pull apart the sister chromatin, exposing the Aurora Kinase B- INCEP complex to phosphatases. This tension is relaxed during anaphase as separase acts on cohesion, so by this time, the complex must no longer be present at the kinetochore at this stage (Tanaka et al., 2002). When inactive Aurora kinase B was overexpressed, centromeres moved in one direction and there were defects in the velocity of the chromosome, and also the spindles tended to detach from the kinetochore. Aurora Kinase B is involved in the interaction of kinetochore and motor proteins (Murata-Hori and Wang, 2002).

1.6.4 Acetylation

It is a kind of posttranslational modification in which an acetyl group is attached to the substrate proteins while in deacetylation the acetyl group is removed from the substrate proteins. Like phosphorylation, acetylation also helps in various physiological events of cells. Several acetyltransferases carry out the function of acetylating target proteins while many deacetylases remove the acetyl groups from

the substrate proteins. CBP/p300, GCN5/PCAF, and TIP60/MYST1/2/3/4 are major acetyltransferases involved in the acetylation of most of the cellular proteins. Histone acetyltransferase acetylates the ϵ -amino acid of lysine in histones as well as non-histone proteins. HATs are classified into approximately 5 families. p300 and CBP form a family of their own. They were independently discovered as binding factors of E1A adenovirus protein. These enzymes contain one bromodomain, three zinc finger regions, HAT domain, and more than two domains that interact with a multitude of transcription factors, and act as scaffolding factors (Roth et al., 2001).

p300 catalytic activity uses the Theorell-Chance reaction also known as hit and run mechanism which has an additional half-reaction followed by the elimination half-reaction. This is an unusual mechanism for HATs (Liu et al., 2008; Zhang et al., 2014). The structure of HAT can be divided into two, the central catalytic core that is conserved across HATs and the region flanking the core, which is very different from other HAT, that is required to interact with various substrates (Marmorstein, 2001).

p300/CBP participates in a wide variety of functions ranging from cell proliferation to apoptosis depending upon the context. p300 histone acetyltransferase initially was identified as a transcriptional activator that performs its function by acetylating histones in eukaryotic cells. Later it was discovered that p300 also acetylates non-histone proteins like E2F1, p53, p73, Rb, E2F, myb, myoD, HMG(I)Y, GATA1, and α -importin (Bannister et al., 2000; Boyes et al., 1998; Chan and Ng, 2001; Costanzo et al., 2002; Gu and Roeder, 1997; Martinez-Balbas et al., 2000; Marzio et al., 2000; Polesskaya et al., 2001; Sakaguchi et al., 1998; Yamamoto et al., 2000). These proteins upon acetylation become more stable.

It can suppress cellular growth by acetylating p53 (Goodman and Smolik, 2000). In response to DNA damage, H3K56 gets acetylated by p300/CBP, which then assembles and forms foci at sites of DNA repair (Das et al., 2009). Inhibitor studies against p300/CBP HAT activity confirmed that its inhibition reduced the proliferation growth of various cancer cells and also sensitized cells to DNA damage-induced apoptosis (Yan et al., 2013). The HAT activity of p300/CBP is regulated in a cell cycle-dependent fashion, with the highest activity at G1-S transition. Inhibition of p300/CBP using functional blocking antibodies showed that cells were stuck at the G1-S boundary and were taking significantly higher duration to enter the S phase.

The transcriptional activity of E2F, which is important in G1-S transition, is also affected upon inhibition of p300. p300 has been shown to acetylate pRb, the protein that controls the cell cycle proceeding from G1 to S phase. This acetylation leads to another layer of control on the cell cycle. The acetylation hinders phosphorylation of pRb, hence causing the cell to remain in the G1 phase. This acetylation of Rb is dependent upon the scaffolding function of adenovirus protein E1A that provides proximity to p300 and Rb together in a complex (Chan et al., 2001). p300 plays an important role in development stages also as it has been reported that mice embryos with p300 knockout could not survive past 11.5 days. When both p300 and CBP were both in heterozygous conditions, it led to death much earlier, implying that they have some redundant function in development and survival (Yao et al., 1998). p300 also plays a role in differentiation. In the early stages of keratinocyte and myoblast differentiation, p21 activation is mediated by p300 independent of p53 (Missero et al., 1995). In muscle cells, the knockdown of p300/CBP resulted in inflammation and loss of adhesion, which are characteristics of dystrophies. p300 also functions as coactivators of transcriptional factors such as MyoD (Fauquier et al., 2018).

Humans have 18 Histone deacetylase enzymes, which are classified into four classes. HDAC1 is a class I HDACs. Class I HDAC requires Zn^{2+} for their enzymatic activity. HDAC1 carries out its catalytic function, zinc-dependent hydrolysis of the acetamide bond between the acetyl and lysine of the substrates (Seto and Yoshida, 2014).

Most studies on HDAC1 were performed in a context of deacetylation of histones at lysine residues mostly in the tail region, but they also have a plethora of non-histone substrates as well. In many instances, p300 and HDAC1 have antagonist functions on a substrate (Seto and Yoshida, 2014). Histone deacetylases, HDACs, are the major deacetylases involved in the deacetylation of histone as well as non-histone proteins. HDAC de-acetylates proteins involved in almost all the cellular events like cell cycle, replication and proliferation, and apoptosis. There are 4 classes of HDACs known so far in mammalian cells. E2F1, p53, and Api4 proteins get de-acetylated by these classes of HDACs. Their biological functionality, as well as stability of these proteins, is regulated upon deacetylation by HDACs. For example, E2F1 stability is

regulated by HDAC1 mediated deacetylation (Luo et al., 2000; Martinez-Balbas et al., 2000)

HDAC1 phosphorylation at ser421 and 423 is essential for its deacetylase activity as well as recruitment to various repressor complexes. Upon de-phosphorylation, it is released from the Rb complex. Sumoylation enhances the enzymatic activity of HDAC1 while ubiquitination by E3 ligases makes it susceptible to proteasome pathway mediated degradation. They have also been reported to get acetylated that impairs their deacetylase activity (Di Marcotullio et al., 2011). HDAC1 performs a diversity of functions. Inhibition of HDAC1 downregulates various DNA repair proteins such as Ku86, RAD51, and BRCA1 (Rosato et al., 2008). HDACs play multiple roles in the immune system (Shakespeare et al., 2011). HDAC 1 inhibits skeletal muscle differentiation via inhibition of MyoD transcriptional activity (Mal et al., 2001). HDAC1 forms a complex with mSIN3 that is recruited to Rb, a tumour suppressor, and a major G1 restriction protein. This complex suppresses the transcription of many cell cycle genes in the G1 phase of the cell cycle (Lai et al., 2001). Knockout of HDAC1 is accompanied by p21 upregulation, leading to cell cycle arrest (Lakshmaiah et al., 2014; Zupkovitz et al., 2010).

HDAC1 and HDAC2 are very similar, and if any of them are absent, the other compensates for it. When both are knocked out, there is a marked increase in histone acetylation throughout the cell cycle that regulates cell viability. The double knockout has also been shown to decrease the levels of pluripotency factors such as Oct4 (Jamaladdin et al., 2014). HDAC1 and HDAC2 double deletion lead to senescence-like G1 cell arrest and upregulation of p21 (Wilting et al., 2010). HDAC1 can be considered as a pro-apoptotic factor as its upregulation enhances TGF- β induced apoptosis.

1.6.5 Ubiquitination

Proteasomal degradation of cellular proteins is mostly involved poly-ubiquitination of cellular proteins. Poly-ubiquitination is the process of conjugation of ubiquitin (Ub) to the substrate protein. The nature of Ub-Ub conjugation determines the fate of poly-ubiquitinated proteins. For instance, lysine-48 (K-48) linked poly-ubiquitination of proteins are marked for proteasomal degradation. Ubiquitination is a post-translational modification wherein ubiquitin is attached to the target protein (Yu et al.,

2003) (Figure 5). Ubiquitination is a multi-step enzymatic process. First, ubiquitin is activated by the E1 activating enzyme. Activated ubiquitin then is transferred to the E2 conjugating enzyme and finally, it will be transferred to the targeted substrate proteins via E3 ubiquitin ligases (Pickart, 2001). The E3 enzyme is the most heterogeneous type of enzyme in the proteasome pathway (Liu and Nussinov, 2009).

There are more than 600 known E3 enzymes, which have been classified in four different groups (HECT type, RING Finger type, U-box type, and PHD finger type) based on the presence of characteristic domains and depending on the mechanism of ubiquitin transfer to the substrate (Kuroda et al., 2002). Ubiquitination is involved in cell cycle progression, apoptosis, and stability of proteins. Ubiquitination is the marker for proteasomal pathway-mediated degradation of proteins. Cyclins are the proteins that undergo ubiquitination depending upon the phase of cell cycles. Cyclin E is the protein that undergoes ubiquitin-mediated degradation during the progression of G1-S transition of cells, cyclin A undergoes ubiquitination after the S phase of the cell cycle, cyclin B undergoes ubiquitination in the G2-M phase of the cell cycle.

1.6.5.1 RING-finger E3 ubiquitin ligases

RING-finger E3 ubiquitin ligases are the most abundant ubiquitin ligases and are characterised by the presence of a zinc-binding domain called RING (**R**eally **I**nteresting **N**ew **G**enes). RING-finger E3 ubiquitin ligases mediate a direct transfer of ubiquitin from E2 to substrate proteins. RING-finger E3 ubiquitin ligases are further classified in SCF (S- phase kinase-associated protein 1 Skp1, Cullin and F-box proteins, described in details below), Cullin RING complex (Joazeiro and Weissman, 2000), and anaphase-promoting complex (APCs) or cyclosomes which are responsible for the cell cycle progression (Sudakin et al., 1995).

1.6.5.2 SCF E3 ubiquitin ligase

SCF E3s belong to the group of RING E3s. SCF E3s utilise a special kind of interchangeable F-box substrate recognising element (Joazeiro and Weissman, 2000). The human genome encodes 69 genes coding for F-box proteins. Each member of the F-box protein family harbours conserved 40 – 50 amino acids. F-box motif mostly at the N-terminal domain. F-box proteins are further categorised into

three families (detailed below) based on the presence of substrate interacting domains present in the C-terminal domain (Figure 1.9).

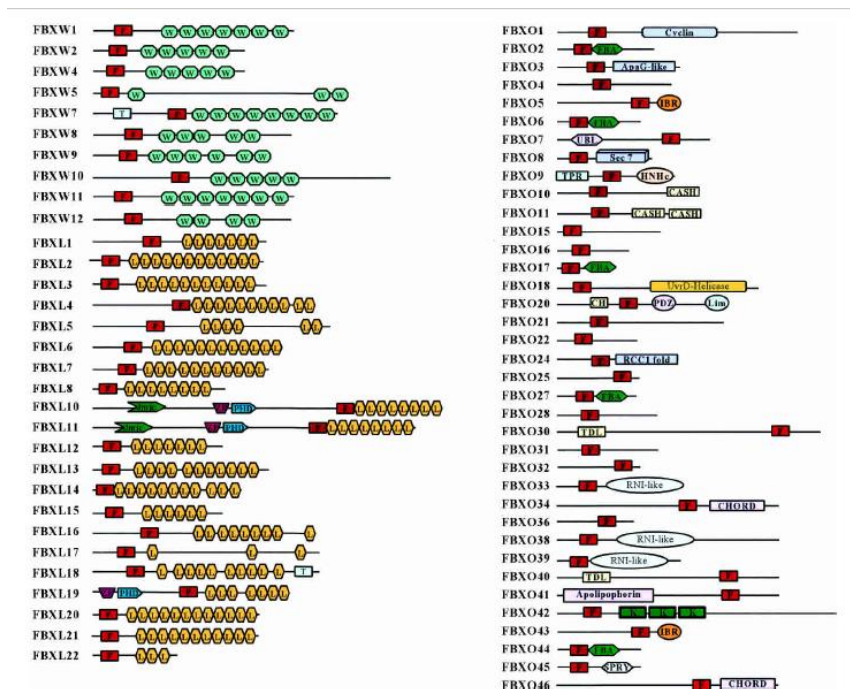


Figure 1.9: Classification of different F-box proteins into three families.

(Adapted from Jin et al., 2004)

FBXW:

F-box proteins having WD40 (Tryptophan and Glutamic acid repeats) repeat domain as a substrate interaction domain, are coined as FBXW family proteins. This series has 12 member proteins. These 12 proteins are named FBXW1 to FBXW12 by the order of their discovery (Jin et al., 2004).

FBXL:

This class of F-box proteins has characteristic Leucine-rich repeats as substrate interaction domain. Twenty-one F-box proteins belong to this group and are named based on their order of discovery (Jin et al., 2004). However, the function of the majority of these F-box proteins remains elusive.

FBXO:

These F-box proteins do not have any specific substrate interaction domain. Few of the F-box proteins have other domains which probably play a role in substrate recognition (Jin et al., 2004). Like other families, these proteins have also been

named on the order of their discovery (Kipreos and Pagano, 2000). However, the function of the majority of these F-box proteins remains elusive.

After getting insights from the literature about Api5 and its function during DNA damage-induced apoptosis as well as in cell cycle we aimed to understand the Post-translational regulation of Apoptosis inhibitor 5 (Api5) during cell cycle progression and DNA damage-induced apoptosis. This aim was further divided into five objectives as follows:

1.7 Objectives

1. To identify the enzyme(s) involved in K251 acetylation of Api5

Acetyltransferases are the enzymes that perform the acetylation of proteins. Acetylation of target proteins by these enzymes is always followed by de-acetylation by deacetylase enzymes. Share and contribution by these two enzymes, acetyltransferases and deacetylases are context-specific. Acetylation of Api5 leads to its stability while de-acetylation causes degradation of Api5. To find out the acetyltransferase and deacetylase involved in the regulation of acetylation of Api5, specific chemical inhibitors of these classes of enzymes will be used. Api5 level will be analysed upon inhibition of these enzymes.

2. To investigate post-translational degradation of Api5

Cellular proteins are regulated by either proteasomal pathway or lysosomal pathway. To find out the pathway through which Api5 is regulated, specific chemical inhibitors of these two pathways, proteasomal and lysosomal, will be used. Api5 level will be analysed upon inhibition of each pathway. This will help determine which pathway is involved in the regulation of Api5. Ubiquitination is a marker for protein undergoing degradation by the proteasome system. Api5 ubiquitination will also be checked.

3. To identify the molecular players involved in the degradation of Api5

Ubiquitination is a multi-enzymatic process. E3 ligases are the rate-limiting enzymes that direct the ubiquitinated product to the proteasomal system. Screening of E3 ligases involved in the regulation of Api5 will be done. Candidate E3 ligase will be selected. Interaction studies of Api5 and selected E3 ligase will be performed at exogenous as well as endogenous levels. shRNA mediated knockdown of selected

candidates will be done and the level of Api5 will be analysed at both mRNA as well as protein levels. Ubiquitination of Api5 will also be checked in the presence as well as in the absence of selected E3 ligase. To check whether selected E3 is regulating the Api5 during particular cell cycle phases, Api5 levels in each phase of the cell cycle will be analysed in the presence or absence of the E3 ligase.

4. To understand the mechanism of regulation of Api5 during cell cycle progression

According to the study of Garcia et al., Api5 levels, as well as stability, varies during the cell cycle progression. However, mRNA levels of Api5 remain unchanged during cell cycle progression. The goal of this objective is to find the pathway of degradation as well as molecular players involved in its cell cycle phase-specific degradation.

5. To study the regulation of Api5 upon DNA damage-induced apoptosis

Api5 is an anti-apoptotic protein whose ectopic expression inhibits DNA damage-induced apoptosis. It has also been proven that Api5 over-expression provides chemoresistance to different cancerous cells. All these studies show the role of Api5 upon ectopic expression. There are no studies that give an idea about the role or what happens to Api5 upon DNA damage-induced apoptosis. To prove this cells will be exposed to different DNA damage causing chemicals like camptothecin, etoposide, and radiation, UV. The levels of Api5 will be analysed by using western blotting.

Chapter 2

Materials and Methods

2.1 Chemicals

MG-132 (Z-Leu-Leu-Leu-al - C2211), p300 inhibitor (382113), Cycloheximide (C1988), Chloroquine (C6628), camptothecin (C9911), Etoposide (E1383), Thymidine (T1895) CDK-1 inhibitor (RO-3306- SML0569), Nocodazole (M1404), Aurora kinase B inhibitor (AZD1152-HQPA- SML0268), Protease inhibitor cocktail (P2714), dimethyl sulfoxide (DMSO- D8418) and propidium iodide (P4170) were purchased from Sigma-Aldrich; romidepsin was from Selleck Chemicals (USA) and the selective ATR kinase ATR inhibitor (VE 821) was purchased from Axon Medchem, USA. Lipofectamine 2000 (H668) was purchased from Invitrogen. Protein A-Sepharose® CL-4B (GE17-0780-01) beads were purchased from GE.

2.2 Antibodies

GFP (ab290), p300 (ab10485), ATR (ab2905), RPA T21 (ab61065), Caspase 9 (ab2324), Caspase 2 (ab182657), PARP1 (ab191217), Aurora kinase B (ab2254), Cyclin B1 (ab32053), Cyclin D (ab226977), Lamin A (ab26300), Lamin A/C (Jol2-ab40567), Lamin B1 (ab16048), p21 (ab109520) and Rad 51 (ab63801) antibodies were obtained from Abcam. HDAC1 (sc-8410), Chk1 (G4, sc-8408), Ubiquitin (sc-8017), Cyclin A (sc-271682) were purchased from SantaCruz while Api5 (PAB7951) was from Abnova. Chk1 S345 (2348), Chk2 T68 (2661), Chk2 (3440), p53 S15 (9284), p53 S37 (9289), p53 S46 (2521), PCNA (2586) and c-Myc (9402) were from Cell Signalling while p53 (247A) and BRCA1 (000A) were from Bethyl Laboratories and GAPDH (G9545) and Tubulin (T6199) were from Sigma-Aldrich. Alexa fluor 488 goat anti-rabbit (A11034), Alexa fluor 568 goat anti-rabbit (A-11011), Alexa fluor 568 goat anti-mouse (A-11004), Phalloidin 568 (A12380) and Phalloidin 633 (A22284) were purchased from Invitrogen.

2.3 Plasmids

CSII-EF-MCS plasmid was a gift from Dr. Sourav Banerjee (NBRC, Manesar, India). pCAG-HIVgp and pCMV-VSV-G-RSV-Rev plasmids were purchased from RIKEN BioResource Centre. pLKO.1 eGFP was a kind gift from Dr. Sorab Dalal, ACTREC. Lentiviral pGIPZ shFBXW2 plasmids were a kind gift from Prof. Michael R. Green, University of Massachusetts Medical School, USA. psPAX2 and pMD2.G were obtained from Didier Trono (Addgene, Watertown, MA, USA 12260, 12259). Human

cDNA encoding FBOX genes were procured from Origene (Rockville, MA, USA). mVenusC1 was gifted by Dr. Jennifer Lippincott-Schwartz, NIH, USA.

2.4 Cloning

siRNA-resistant mVenus C1 wild-type Api5 and K251 mutants were available in the lab. These siRNA-resistant wild type and K251 mutants of Api5 were cloned into mCherry-CSII-EF-MCS vector for preparing lentiviral plasmids. siRNA resistant wild type Api5 and K251 mutant genes from Api5-mVenus C1 plasmid were PCR amplified using primers with XbaI and NotI restriction site overhangs (Table 2.1) with 30 cycle PCR cycle as follows 95°C for 60 sec, 60°C for 1 min, 72°C for 3 mins sec and final extension for 5 min. PCR products were purified using a PCR purification kit (Qiagen) and digested using XbaI (New England Biolabs -NEB) and NotI HF (NEB) for 4 hours (Table 2.2). mCherry-CSII-EF-MCS vector was also digested using the same restriction enzymes at 37°C for 3 hours and calf intestinal phosphatase (CIP; NEB) treatment for 1 hr was performed. Digested plasmids were gel extracted after resolving on agarose gel using gel extraction kit (Sigma). siRNA-resistant wild type and K251 mutants of Api5 were ligated into mCherry-CSII-EF-MCS vector with an insert to vector ratio of 75 ng:25 ng using T4 DNA ligase (Takara) at 16°C overnight (Table 2.3). The ligation mixture was used to transform DH5α competent cells and incubated overnight at 37°C. Plasmid DNA was isolated from the obtained colonies and screened colonies by PCR to check for the insert.

Table 2.1: PCR amplification of siRNA resistant wild type and K251 Api5 from Api5-mVenus C1

DNA Template	3 µl
Forward Primer	3 µl
Reverse Primer	3 µl
10 mM dNTPs	12.5 µl
10X Pfu buffer	10 µl
DMSO	5 µl
dH ₂ O	6.5 µl
Pfu polymerase	2 µl

Table 2.2 Digestion of PCR product and CSII-EF-MCS-mCherry

DNA Template	5 µg
10 X Cut smart buffer	5 µl
Not 1 HF	5 µl
Xba 1	5 µl
dH ₂ O	To make up the reaction to 50 µl

Table 2.3 Ligation reaction of siRNA resistant wild type and K251 Api5 into mCherry tagged CS-II-EF-MCS digested vector

Insert	75 ng
Vector	25 ng
10X T4 Ligase buffer	1 µl
T4 DNA Ligase	1 µl
dH ₂ O	To make up the reaction to 10 µl

2.5 Site-directed mutagenesis to generate S138A Api5 mutant.

PCR reaction was set up with siRNA resistant mVenus C1 Api5 wild type as a template with Api5 S138A-forward and reverse primer to generate S138A mutants of Api5 (Table 2.4). The PCR cycle was as follows: After initial denaturation at 95°C for 2 minutes, a 20 cycle reaction was set for 95°C for 30 secs, 55°C for 1 min, 68°C for 20 mins and final extension for 5 min. The PCR product was DpnI digested at 37°C for 3 hrs and transformed into DH5α cells. Colonies obtained were screened by sequencing.

Table 2.4: PCR amplification of siRNA-resistant S138A mutant of Api5

DNA Template	500 ng
Forward Primer (25 µM)	1 µl
Reverse Primer (25 µM)	1 µl
10 mM dNTPs	4 µl
10X Pfu turbo buffer	5 µl

dH ₂ O	To make up the reaction to 50 μ l
Pfu turbo polymerase	1.5 μ l

2.6 Cell culture and drug treatments

MCF7 cells were obtained from the European Collection of Cell Cultures (ECACC). HEK293T and HeLa cells were a gift from Dr. Jomon Joseph (NCCS, Pune) and Dr. Sorab Dalal respectively. The cells were maintained in 100 mm dishes (Eppendorf or Corning) and were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Lonza) containing 10% heat-inactivated FBS (Invitrogen) and 100 units/mL penicillin-streptomycin (Invitrogen) and incubated at 37°C humidified 5% CO₂ incubators (Eppendorf or Thermo Scientific).

7×10^5 cells were seeded on 35mm dishes 16 hrs before treatment. p300i, MG132 and cycloheximide treatments were for 4hrs, Chloroquine for 8 hrs, HDACi, ATRi and UV treatments were for 12 hrs, nocodazole and camptothecin for 16 hrs and Etoposide for 24 hrs with concentrations mentioned except for time-dependent experiments. For double thymidine treatment, 5×10^5 cells were seeded and treated with 2mM thymidine for 18 hrs. Cells were released into thymidine free media for 9 hrs, and Cells were again treated with 2mM thymidine for 17 hrs for obtaining S phase arrest

2.7 Plasmid Transfection

For transfections, 6×10^5 cells were seeded in 35mm dishes and incubated at 37°C overnight. siRNA duplexes targeting Api5 and LacZ were purchased from Dharmacon (Thermo Scientific). Sense sequences of the siRNA are: Api5, 5'-GACCUAGAACAGACCUUCAUU-3', LacZ, 5'-CGUACGCGGAAUA CUUCGAdTdT-3'. 2 μ g of plasmid or siRNA was mixed with 200 μ l of OPTIMEM for 5 minutes. Meanwhile, 8 μ l of Lipofectamine 2000 is incubated with 196 μ l of OPTIMEM. 200 μ l of lipofectamine-OPTIMUM mixture was added to the plasmid/siRNA-OPTIMUM mixture and incubated at room temperature for 20 minutes. The cells were preconditioned in 1ml optimum meanwhile. After 20 minutes, 600 μ l of OPTIMEM was added to the mixture and it was added to cells. 30% FBS containing DMEM was added to the cells 4hrs post-transfection. 1ml fresh media was added to the cells 24 hrs after transfection. The cells were collected 48 hrs post-transfection for

immunoblotting or FACS staining. In knockdown and rescue experiments, Api5 K251 mutants were transfected 24 hrs post knockdown of Api5.

2.8 Generation of stable cell lines

MCF7 cells stably expressing mCherry-tagged Api5 were generated using lentiviral-mediated transduction. 7.5×10^5 HEK293T cells were seeded on a 35mm dish and transfected with 1 μ g of mCherry-CSII-EF-MCS or mCherry-CSII-EF-MCS-Api5 plasmid along with 1 μ g of pCAG-HIVgp and 0.5 μ g pCMV-VSV-G-RSV-Rev packaging plasmids using Lipofectamine 2000. 1 ml DMEM containing 30% FBS was added to the cells 24 hours post-transfection. 5×10^5 MCF7 cells were seeded on a 35 mm dish for transduction. Viral supernatant was collected 48 hours post-transfection and filtered through a 0.45 μ m filter to get rid of the cell debris. Filtered viral supernatant containing media along with 1 ml fresh media was added to the MCF7 cells. 4 μ g polybrene was added to the cells to increase the transduction efficiency. Cells were replenished with fresh medium 48 hours post-transduction. Transduced MCF7 cells were passaged and used for further experiments.

MCF7 cells stably expressing pLKO1.eGFP and shApi5.eGFP were generated using lentiviral transduction. 7.5×10^5 HEK293T cells were seeded on a 35mm dish and transfected with 1 μ g of pLKO1.eGFP or shApi5-eGFP plasmid along with 1 μ g of psPAX2 and 0.5 μ g pMD2 packaging plasmids using Lipofectamine 2000. 1 ml DMEM containing 30% FBS was added to the cells 24 hours post-transfection. 5×10^5 MCF-7 cells were seeded on a 35 mm dish for transduction. Viral supernatant was collected 48 hours post-transfection and filtered through a 0.45 μ m filter to get rid of the cell debris. Filtered viral supernatant containing media along with 1 ml fresh media was added to the MCF-7 cells. 4 μ g polybrene was added to the cells to increase the transduction efficiency. Cells were replenished with fresh medium 48 hours post-transduction. Transduced MCF-7 cells were passaged and sorted using BD Aria Fusion sorter. Lentiviral particles containing mCherry tagged siRNA resistant Api5 wild type and K251 mutants were generated similar to mCherry-tagged Api5. shApi5 cells after sorting were transduced with lentiviral particles containing mCherry tagged siRNA resistant Api5 wild type and K251 mutants and sorted for mCherry fluorescence.

HeLa cells expressing shFBXW2 were generated similar to shApi5 stable cell line preparation. The non-specific and FBXW2 shRNA sequences are as follows:

shNS: 5'-TCTCGCTTGGGCGAGAGTAAG-3', shFBXW2-

1: AATGACGAGGTTTCAAAGG, shFBXW2-2: TTCATAGGCAACCAATTAG,

shFBXW2-3: TGATTTCTCTCCCAATTGG

2.9 Immunoblotting and Immunoprecipitation

MCF7 or mCherry-tagged Api5 over-expressing MCF7 stable cells with or without treatments were lysed in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na₃VO₄ and protease inhibitors. Cell lysates were incubated with 2 µg of Api5, Myc, GFP, RFP, mCherry, Aurora kinase B, and ATR specific antibodies antibody and incubate overnight in 4⁰ with rotations. Protein A-Sepharose beads after washing with IP buffer are incubated with cell lysate-antibody complex overnight at 4⁰ with rotations. This mixture is washed twice with IP buffer and the protein complex was eluted by adding 2X sample buffer and heating at 96⁰ C for 5 minutes. Eluted proteins or cell lysates are loaded on SDS-PAGE and transferred to PVDF membrane to perform western blotting. The membrane was blocked in 5% skimmed milk or blockase for 1 hr and incubated with primary antibody for a specific time. After washing with TBS-T, the membrane was incubated with HRP conjugated secondary antibody (1:10000), prepared in skimmed milk for 1hr. The membrane is developed in ImageQuant LAS400 (GE Healthcare) using immobilon Western Detection Reagent kit (Millipore) Densitometry analysis of images was done using ImageJ software

2.10 Immunofluorescence Assay

5X 10⁵ cells were seeded on coverslips placed in 35mm dishes. After treatment or transfection, cells were fixed in 4% paraformaldehyde for 20 minutes in dark conditions and permeabilised using 0.5% TritonX for 10 minutes after PBS washes. After PBS-Glycine wash, cells were blocked using 10% FBS for 1 hr and incubated overnight with Api5, Tubulin, pRPA 32 T21, BRCA1, Rad51, Lamin A, Lamin B1, or GFP antibodies at 4⁰C at humid conditions. Cells were incubated with secondary antibodies conjugated with fluorophore Alexa Flour (Invitrogen) for 1 hr at room temperature along with phalloidin after 3 washes with IF buffer. Cells were washed with IF buffer once and PBS twice after secondary. 0.5 µg/ml of Hoechst 33258 in

PBS was added to cells and incubated for not more than 5 minutes and immediately washed with 1X PBS for 10 minutes. Excess PBS was removed and mounting reagent was added to mount the cells. Later cells were imaged under 63X oil immersion objective of Leica sp8 microscope (Leica, Germany). Corrected total cell fluorescence (CTCF) was calculated to measure the intensity of Api5 fluorescence in nucleus and cytoplasm of the cells. Briefly, the nuclear and cytoplasmic boundary was marked manually and measure the area, integrated density and mean grey value of Api5 fluorescence. Also measure the mean intensity of background to calculate corrected fluorescence. CTCF was calculated with the formula

CFTC= Integrated density- (Area of selected area X Mean fluorescence of background readings). CTCF of at least 200 cells were calculated per experiment set.

2.11 Semi-quantitative PCR

MCF7 cells with or without treatment were collected in TRIzol (Invitrogen 15596018) and RNA was extracted using the isopropanol and chloroform method. cDNA was prepared using M-MuLV Reverse Transcriptase (NEB) and oligo-dT (Sigma). Semi-quantitative PCR was performed using API5 specific forward (5'-CGAGTGGCAGATATACTAACGC-3') and reverse (5'-TCCTCTCCTTGAAGTATTTGGC-3') primers. GAPDH was used as endogenous control and was amplified using forward (5'-ACCACAGTCCATGCCATCAC-3') and reverse (5'-TCCACACCCTGTTGCTGTA-3') primers. The following PCR cycle was used for the amplification: 95°C for 60 sec, 58°C for 45 sec, 72°C for 60 sec and final extension for 3 min.

2.12 MTT assay

For the MTT assay, 1×10^4 cells were seeded in each well of a 96 well plate. 16 hours post seeding, cells were treated with different concentrations of camptothecin and etoposide and incubated for 24 hours at 37°C. After removing the drug containing medium, 0.5 mg/ml thiazolyl blue tetrazolium (MTT) containing DMEM was added to the cells and incubated at 37°C after covering with aluminum foil. 4 hours post MTT addition, MTT-DMEM medium mixture was aspirated from the wells and 100 µl of DMSO was added to dissolve the purple MTT-formazan crystals. The

absorbance was recorded at 570nm using the multimode Varioskan Flash plate reader (Thermo Scientific, USA).

2.13 Flow cytometry

MCF7 stable cells or after transfection were trypsinised using 1X trypsin and collected in media. After pelleting, the cells were washed with cold PBS twice to remove excess media. The cells were then fixed in 70% ethanol overnight at 4°C and stained with 1 mg/ml propidium iodide in the presence of RNase A (20 mg/ml) at 37°C for 1 hr. Cell cycle profile was analysed on the BD Accuri™ C6 flow cytometer (BD Biosciences).

2.14 Cell proliferation assay

6 x 10⁵ MCF7 cells were seeded in 35 mm dishes and incubated at 37°C overnight. mVenus-tagged WT and K251 mutants of Api5 plasmids were transfected as mentioned before. 48 hrs post-transfection, cells were trypsinised and pelleted down by centrifugation at 1000 rpm for 5 mins. The cell pellet was suspended in 1X PBS. 1:10 dilution of the suspended cell pellet was prepared in 1X PBS and the cell count was measured using a 60 µm sensor connected to Sceptar™ hand-held automated cell counter (Millipore, Sigma-Aldrich).

2.15 Colony formation assay

1 ml of MCF7 stable cell suspension containing 1000 cells was seeded on a 35 mm dish. Cells were replenished with fresh media after 4 days of seeding. After 7 days of seeding, cells were washed with 1X PBS twice and fixed with 100% chilled methanol at 4°C for 15 minutes. Cells were stained with 0.5% of crystal violet stain for 15 minutes with shaking at room temperature. After removing the stain, cells were washed with distilled water and images were taken after drying the plates. The number of colonies was manually counted

2.16 BrdU incorporation assay

6 x 10⁵ pLKO1 of shApi5 stable cells were seeded in a 35 mm dish and incubated for 16 hrs. 10 µl of Ready to Use BrdU labelling solution (Sigma) was added to 1 mL complete DMEM and added to cells. After 6 hrs, cells were fixed and immunofluorescence assay was performed as mentioned before using BrdU (Abcam) specific antibody.

2.17 Statistical analysis

Results were analyzed from a minimum of three independent experiments and plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Student's t-test and Mann Whitney U test were used to test the significance in difference of Api5 levels between two samples and One-way ANOVA, Dunnett's multiple comparisons or Tukey's multiple comparisons test for more than two samples. p-value > 0.05 was considered non-significant. *, ** and *** correspond to $p < 0.05$, $p < 0.001$, and $p < 0.001$ respectively.

Chapter 3

Interplay between p300 and HDAC1 regulate acetylation and stability of Api5 to regulate its localisation

3.1. Background

Cells rely upon the protein-protein interactions to achieve biological functions like DNA replication, transcription, translation, cell division regulation, and cell signalling responses (Phizicky and Fields, 1995; Shoemaker and Panchenko, 2007; Westermarck et al., 2013). These interactions are dependent on the context or the signal received for a specific biological function. These interactions can be of both type's i.e. transient interaction and permanent interaction. Attachment of sumo and ubiquitin-protein moieties to the substrate proteins through the covalent bonds is an example of permanent protein-protein interaction. Interactions of kinases to their substrate proteins to phosphorylate them is a kind of transient type of protein-protein interaction. Transient interactions are achieved by the ionic, hydrogen, Vander Waal, and hydrophobic forces of attractions (Acuner Ozbabacan et al., 2011).

Protein-protein interactions can further be categorised like homo and hetero types of interaction depending upon the similarities between the interacting subunits (Shoemaker and Panchenko, 2007). Interaction of one protein subunit with itself to form a dimer is a kind of homo protein-protein interaction while the interaction of one protein subunit with another different type of subunit is an example of a hetero type of protein-protein interactions.

These protein-protein interactions can also be direct or indirect. For example binding of cyclins to cyclin-dependent kinases is an example of direct interactions (Beach et al., 1982) whereas binding of cohesin and condensin proteins to the anaphase-promoting complex is an example of the indirect type of interaction (Jones and Sgouros, 2001). Apart from providing various molecular functions to proteins inside cells these interactions also regulate the stability of several proteins (Tada, 2007). Stable and unstable forms of proteins are required to attain certain biological functions. Generally, stable forms of proteins perform constitutive signalling that gets discontinued after achieving the required biological response. This can either be achieved by degrading or making the signalling protein unstable (Siu et al., 2012). The stability and function of proteins are also coupled with their proper localisation inside the cell (O'Keefe et al., 2003). Different cellular compartments like the nucleus, mitochondria, endoplasmic reticulum, Golgi complex, lysosomes, and plasma membrane not only provide a favourable micro-environment for proteins but also help in maintaining their stability (Ohkuma and Poole, 1978).

These compartments are categorised into two main pools. The nuclear pool comprises its nuclear space and chromatin while all the other compartments collectively comprise the cytoplasmic pool.

The cytoplasmic pool is enriched with heterogenous biomacromolecules. In these compartments, proteins become active and functional. For example lysozymes, a class of hydrolases that are secreted by lysosomes are stable only inside the lysosome or the membrane-bound organelle-like endosomes. Several other examples emphasise the crucial role of niche on the stability and functions of proteins. For example enzymes of oxidative phosphorylation and ATP synthesis are destined to the mitochondrial membrane for their biological functions (Chaban et al., 2014). Transmembrane proteins and receptors also migrate to their respective locations to achieve their physiological function (Kesten et al., 2018).

Most of the proteins which are synthesised by ribosomes are destined to the different locations inside the cell depending upon the requirement of this protein to attain specific biological functions. Switch between the two localisation compartments also aid in a different signalling cascade for a protein. For example, β -catenin is synthesised by the ribosomes and transported to the nucleus to carry out its functions. Inside the cytoplasm, β -catenin remains inactive as an outcome of its interaction with other proteins. Upon receiving the signal these interacting partners get detached. Thus the protein is allowed to transport inside the nucleus. Inside the nucleus, it attains its active and functional confirmation. Once the function is achieved inside the nucleus, β -catenin is transported back to the cytoplasm for its degradation (Krieghoff et al., 2006).

It has been reported in the literature that Api5 is a nuclear protein (Van den Berghe et al., 2000) that is stabilised inside the nucleus upon acetylation at lysine 251 (Han et al., 2012). However, the enzyme that leads to its acetylation and stability inside the nucleus has not been discovered yet. Moreover, the physiological relevance of deacetylation is yet to be elucidated. This chapter reveals the K251 acetylating and de-acetylating enzymes of Api5 and also unveils the compartmentalisation switch of Api5 upon acetylation and de-acetylation at K251 by these respective enzymes.

3.2 Results

3.2.1 *in silico* analysis predicts p300 histone acetyltransferase to acetylate Api5 at lysine 251

It has been reported in previous studies that Api5 undergoes acetylation at lysine 251, however, the enzyme that is required for the acetylation function has not yet been identified. Therefore, to identify the enzyme involved in the acetylation of Api5 we performed *in silico* analysis using the ASEB web server tool (van Zundert et al., 2016; Wang et al., 2012) to predict the site as well as the enzyme which may be responsible for acetylation of Api5 at lysine 251.

It was observed that amongst CBP/p300, GCN5/PCAF, and TIP60/MYST1/2/3/4, CBP/ p300 had a lower p-value suggesting a higher possibility of acetylating Api5 at lysine 251 (Figure 3.1A). Active site residues of p300 histone acetyltransferase were obtained from the literature (Dancy and Cole, 2015). Docking was performed between p300 and Api5 using the HADDOCK webserver (Dhawanjewar et al., 2020). The output was analyzed using Protein Interaction Z Score Assessment (PIZSA) (Roy et al., 2019) and the best scoring model was chosen as shown in figure (1B-D). Interestingly the acetylating domain of p300 showed interaction with three amino acid residues of Api5 including lysine 251 (Figure 3.1C-D).

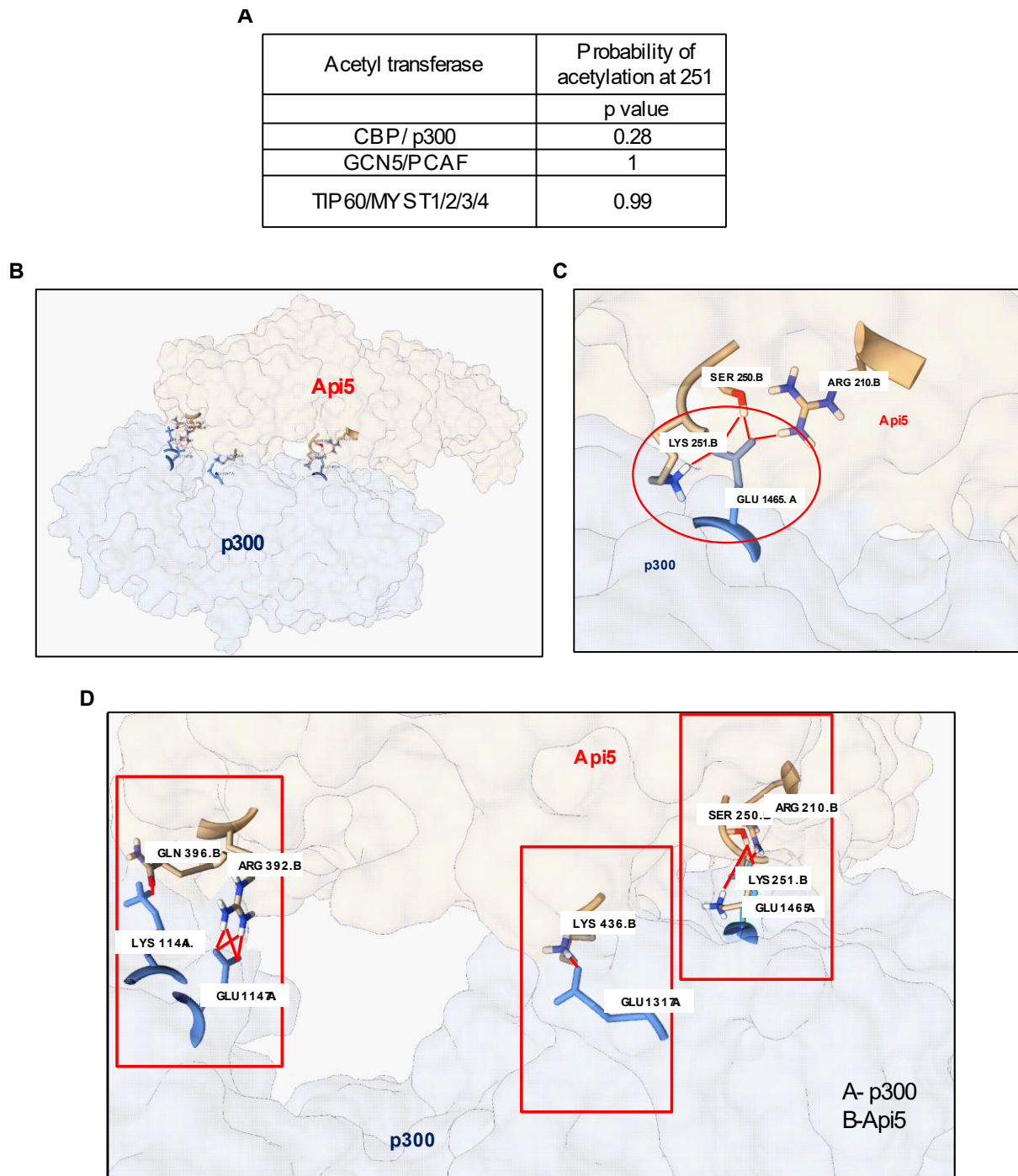


Figure 3.1: Api5 interacts with p300 in silico

A) Various acetyltransferases and their probability of acetylating lysine 251 of Api5 represented as p-value using ASEB server, where lower p-value corresponds to a higher probability. B) The lowest-energy docking position of Api5 and p300 was analysed using HADDOCK software, and output was analysed using PIZSA represented as a cartoon diagram. C) Interaction between Lysine 251 of Api5 with p300. D) Various amino acid interactions between Api5 and p300.

3.2.2. p300 interacts with and regulates Api5

To date, it has been reported that Api5 undergoes only post-translational modification; acetylation at lysine 251 (Han et al., 2012). This K251 acetylation is responsible for maintaining the stability of Api5 as de-acetylated Api5 undergoes post-translational degradation. Acetyltransferases are the enzymes that perform the acetylation of proteins. Acetylation of target proteins by these enzymes is always followed by de-acetylation by de-acetylase enzymes. These two enzymes, acetyltransferases and de-acetylase, function in a context-specific manner. *In silico* analysis predicted that p300 histone acetyltransferase might be the enzyme involved in the acetylation of Api5. p300 histone acetyltransferase is the enzyme that was initially discovered to acetylate histone proteins. Histone H3 is acetylated and stabilised by p300 mediated acetylation. Apart from histones, there are other nuclear proteins like E2F1, p53, and Api4 that are also known to be acetylated and stabilised by p300 histone acetyltransferase. After considering these studies it was hypothesised that p300 might be involved in acetylation and ultimately the stability of Api5. To prove that p300 acetyltransferase is the enzyme responsible for acetylation, a small molecule chemical inhibitor of p300 was used to block the acetyltransferase activity of p300 in MCF7 cells, and protein levels of Api5 were analysed using immunoblotting. It was observed that protein levels of Api5 were reduced in a similar manner to acetylated p53 and E2F1 upon inhibition of p300 (Figure 3.2A-B). Acetylated p53 and E2F1 were used as a positive control for the functional inhibition of p300 as it had been shown that p300 regulates both p53 and E2F1 at the post-translational level through acetylation (Figure 3.2A-D). Henceforth it was demonstrated that p300 might be regulating Api5 stability.

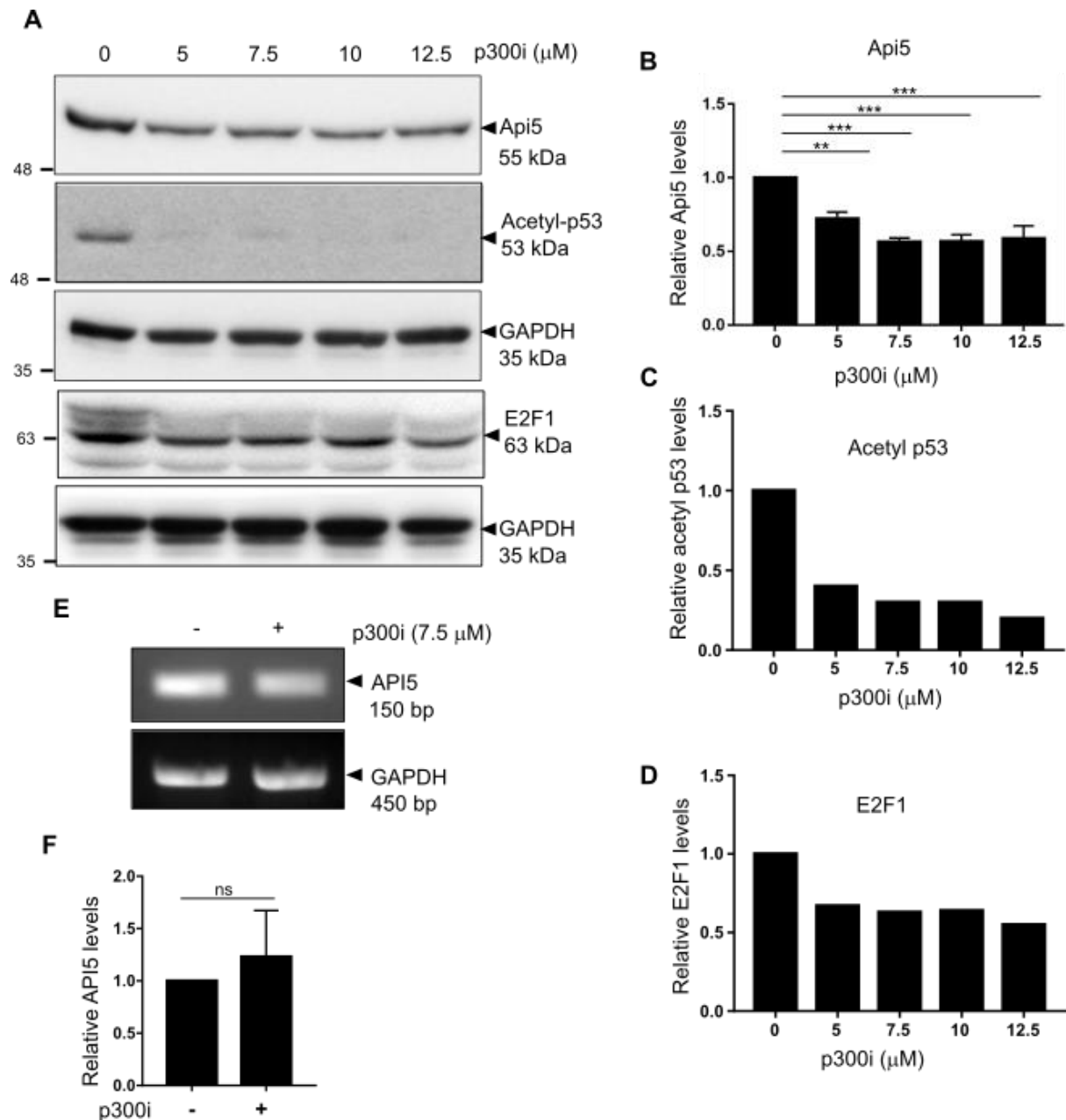


Figure 3.2. p300 regulates Api5

A) MCF7 cells treated with varying concentrations of p300 inhibitor for 4 hrs were lysed, and Api5 levels were analyzed using western blotting. B) Quantification showing the fold change in Api5 protein levels after normalization with GAPDH. A – D) Acetyl p53 and E2F1 were used as a positive control for p300 inhibition. E) API5 transcript levels upon p300 inhibition were analyzed using semi-quantitative PCR. F) Quantification of transcript levels after normalizing to GAPDH. Data is representative of three independent experiments and statistical analysis for (B) was performed using One way ANOVA while for (F) was performed using T-test; ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$

Reduction in protein levels of Api5 upon inhibition of p300 could also be an outcome of a decrease in transcription of *API5* gene. To rule out this possibility, cells were treated with p300 inhibitor for 4 hrs, and transcript levels of Api5 were analysed. It was observed that *API5* transcript levels were not affected upon inhibition of p300 histone acetyltransferase (Figure 3.2E-F), thus demonstrating that p300 might be regulating Api5 at the post-translational level.

In order to carry out its function of acetylating Api5, p300 should essentially interact with Api5. Thus to find whether p300 interacts with Api5, MCF7 cells stably expressing mCherry-tagged Api5 were generated using lentiviral-mediated transduction. These cells were lysed, and immunoprecipitation was carried out using GFP-specific antibody. The presence of p300 immunoblotting confirmed that p300 interacts with Api5 (Figure 3.3A). This interaction was further corroborated by reverse immunoprecipitation using p300 antibody, and the presence of Api5 was confirmed using GFP specific antibody (Figure 3.3B). These results conclusively demonstrated p300 to interact with Api5 and thus regulate Api5 protein expression without affecting the transcript levels.

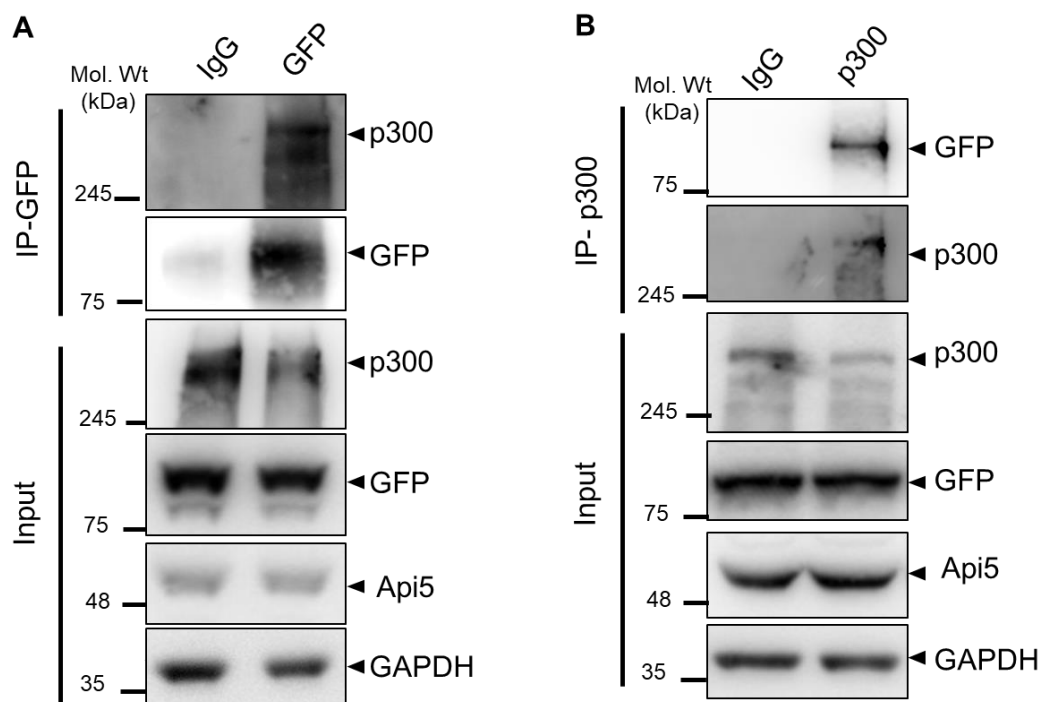


Figure 3.3. p300 interacts with Api5

MCF7 cells stably expressing mCherry-Api5 were lysed, and immunoprecipitation was carried out using A) GFP or B) p300 specific antibodies.

3.2.2 HDAC1 interacts with and regulates Api5

From the previous results, we discovered p300 to be the histone acetyltransferase to regulate Api5 protein levels. Next, we were interested in identifying the de-acetylase enzyme which might be involved in regulating the stability of Api5. The study by Han and group (Han et al., 2012) also suggested that histone deacetylase can also be responsible for the deacetylation of Api5. From the literature, it was found out that class1 HDACs, particularly HDAC1 and HDAC2 enzymes, are involved in the deacetylation of proteins regulating cell cycle and apoptosis. Literature survey suggested that HDAC1, a member of class 1 HDACs, activity is coupled with p300 histone acetyltransferase. It was hypothesised that HDAC1 is the enzyme involved in the deacetylation of Api5. Interestingly, HDAC1 has also been reported to be a part of the Api5 interactome (Bong et al., 2020).

HDAC1 is one of the members of the large family of histone de-acetylases. The de-acetylation activity of HDAC1, a class 1 HDAC protein, is coupled with the acetylation activity of p300. Interestingly, HDAC1 has also been reported to be a part of the Api5 interactome (Bong et al., 2020).

To investigate whether HDAC1 regulates Api5, cells were treated with different concentrations of romidepsin, an HDAC class 1 inhibitor (HDACi), and Api5 protein levels were analyzed. Api5 protein levels increased upon treatment with HDACi similar to p21, which is known to be destabilised upon deacetylation by HDAC1, suggesting that HDAC1 may be involved in regulating the stability of Api5 (Figure 3.4A-C). The accumulation of Api5 upon inhibition of HDAC1 could also be the result of increased transcription of Api5. To rule out this possibility, Api5 transcript levels were analyzed upon HDAC1 inhibition. It was observed that Api5 transcript levels remain unaltered upon romidepsin treatment, as shown in Figure 3.4 D-E. These results suggest HDAC1 play a role in regulating the stability of Api5 at the post-translational level.

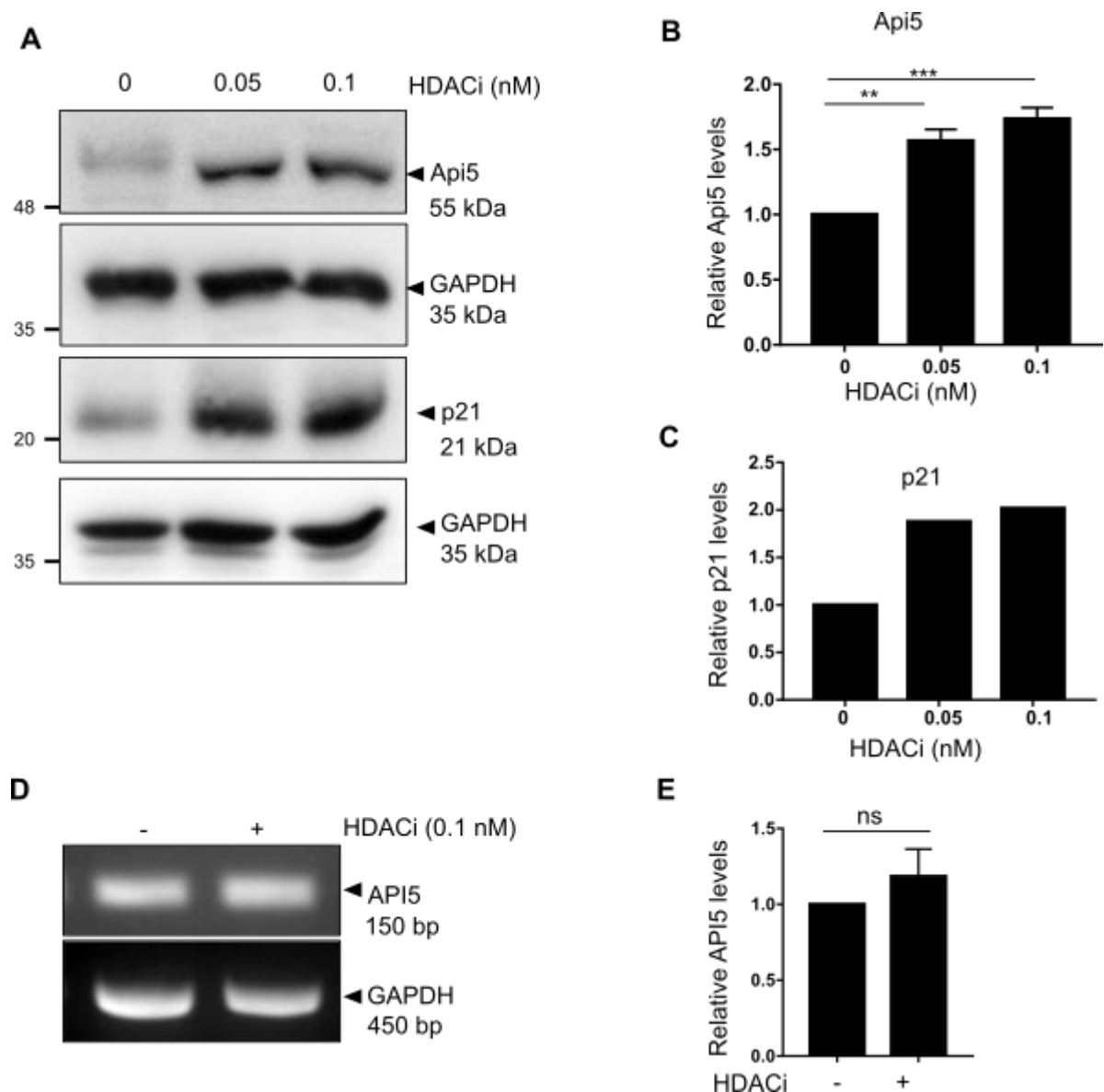


Figure 3.4: HDAC1 interacts and regulates Api5

A) MCF7 cells treated with varying concentrations of the HDAC1 inhibitor, romidepsin for 16 hrs were lysed, and Api5 levels were analyzed using western blotting. B) Quantification showing the fold change in Api5 protein levels after normalization with GAPDH. A and C) p21 levels were analyzed to confirm the HDAC1 inhibition. D) API5 transcript levels upon romidepsin treatment was analyzed using semi-quantitative PCR and E) quantified after normalising with GAPDH. Data is representative of three independent experiments and Statistical analysis for (B) was performed using One way ANOVA while for (E) was performed using T-test; ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$

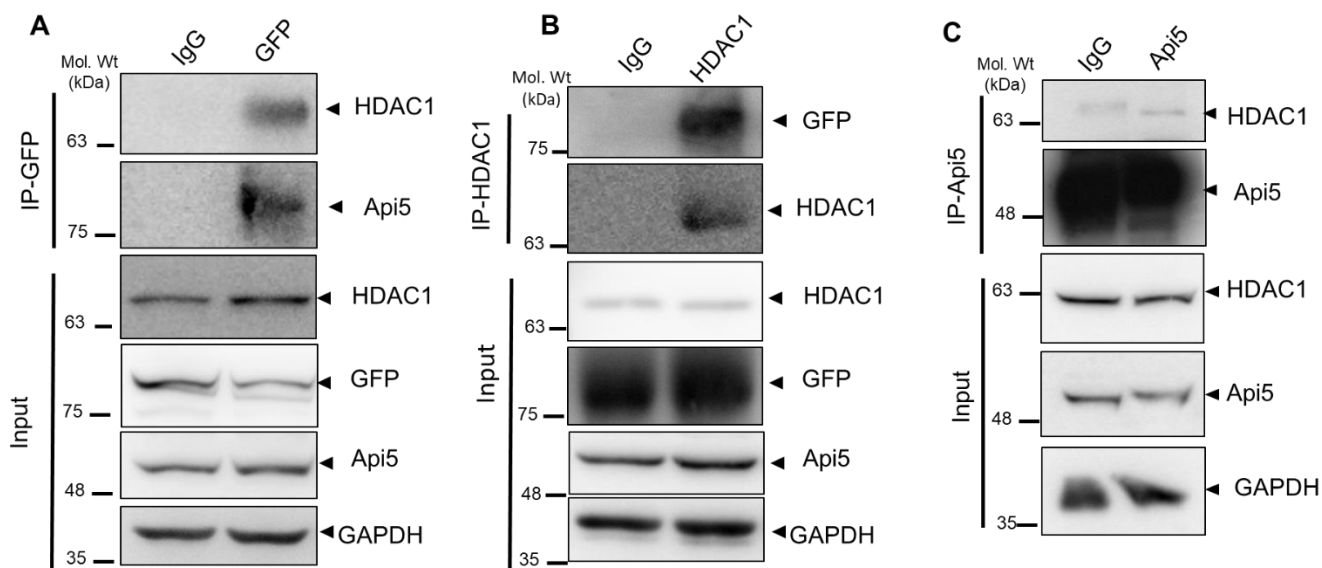


Figure 3.5: HDAC1 interacts and regulates Api5

MCF7 cells stably expressing mCherry-Api5 were lysed, and immunoprecipitation was carried out using A) GFP or B) HDAC1 specific antibodies. C) MCF7 cells were lysed, and endogenous Api5 was pulldown using Api5 specific antibody

In order to decipher whether HDAC1 interacted with Api5 to bring about the regulation, immunoprecipitation studies were conducted. To demonstrate the interaction of Api5 with HDAC1, whole-cell lysates of mCherry-Api5 overexpressing stable cells were immune-precipitated using GFP-specific antibodies (Figure 3.5 A). The presence of HDAC1 in the GFP pull-down lysates confirmed the interaction between HDAC1 and Api5. This interaction was further corroborated by reverse immunoprecipitation, and pull-down was performed using HDAC1 specific antibody. Api5 was found to be present in the HDAC1 pull-down lysates (Figure 3.5B). To prove the interaction of Api5 and HDAC1 at endogenous level, MCF7 cells were lysed, and lysates were immunoprecipitated using Api5 specific antibody. The presence of HDAC1 in the pulldown of Api5 confirmed the interaction between Api5 and HDAC1 (Figure 3.5C). These studies thus validate that HDAC1 interacts with and regulate Api5 at the protein level.

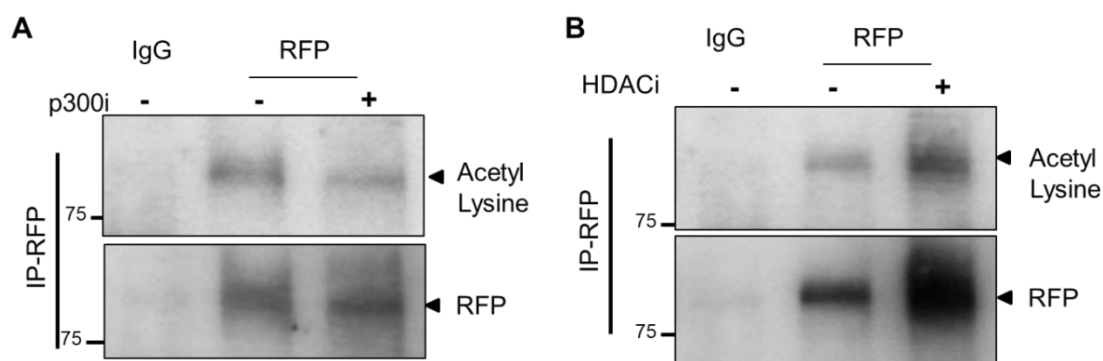


Figure 3.6: p300 and HDAC1 acetylates and deacetylates Api5

Api5 over-expressing MCF7 cells treated with A) 7.5 μM of p300i for 4 hrs or B) 0.1 nM of HDACi romidepsin for 16 hrs were lysed, and immunoprecipitation was performed using RFP specific antibody. Western blotting analysis was performed to analyse the acetylation status of Api5 using acetyl lysine-specific antibodies.

3.2.3 p300 and HDAC1 acetylates- deacetylates Api5

Previous experiments suggested p300 histone acetyltransferase and HDAC1 as the acetylating and de-acetylating enzymes, respectively, that regulate the levels of Api5. It was hypothesized that these two enzymes might also regulate the dynamics of acetylation and de-acetylation of Api5. Therefore, the acetylation status of Api5 was investigated upon inhibition of both p300 and HDAC1.

To investigate whether Api5 is acetylated by p300, mCherry-Api5 overexpressing stable cells were treated with and without p300i, and immunoprecipitation studies were performed. Acetylated Api5 was detected using L-Acetyl lysine-specific antibody. Api5 acetylation was observed to be reduced upon inhibition of p300 when compared to the untreated control, as shown in Figure 3.6A. To check for the effect of HDAC1 inhibition using romidepsin on the acetylation status of Api5, similar immunoprecipitation experiments were performed with and without romidepsin. Total as well as acetylated levels of Api5 increased upon inhibition of HDAC1 (Figure 3.6B). This confirms that Api5 undergoes acetylation and de-acetylation by p300 and HDAC1, respectively.

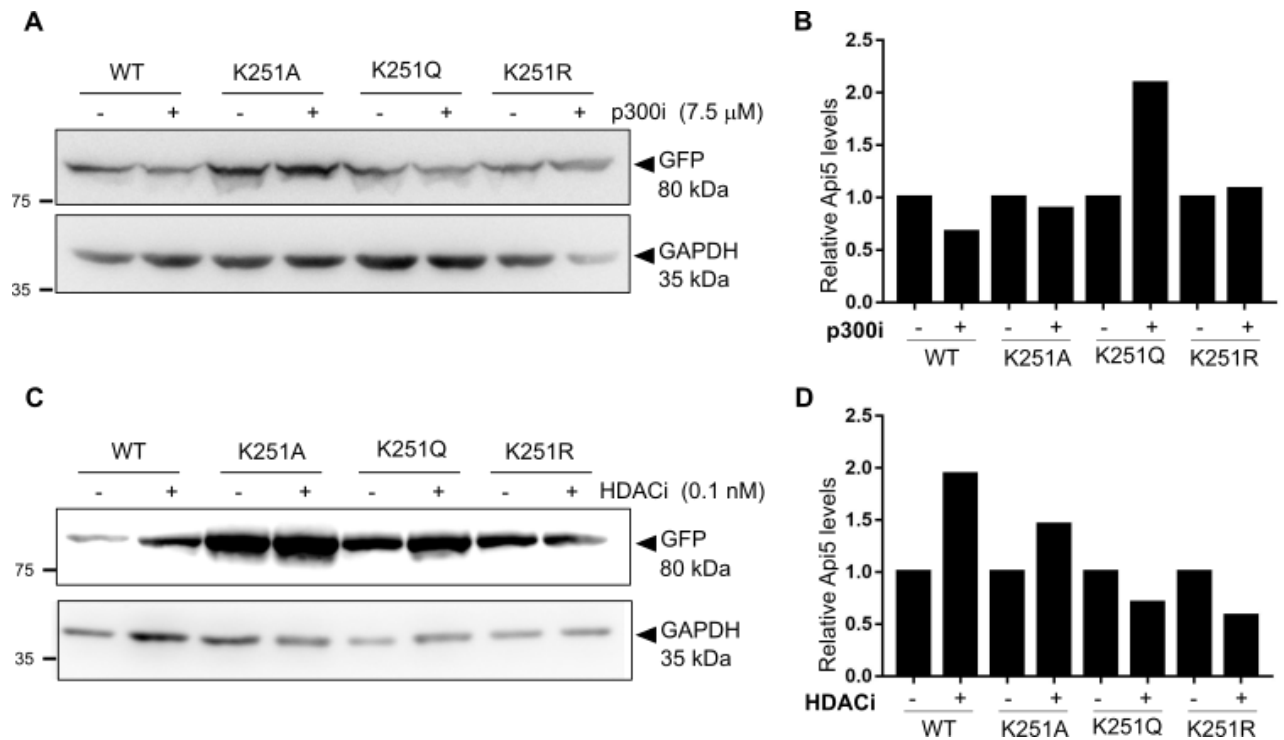


Figure 3.7: p300 and HDAC1 acetylates and deacetylates Api5 at K251

MCF 7 cells were transfected with K251 mutant constructs and treated with A) 7.5 μ M of p300i and C) 0.1 nM of romidepsin post 24 hrs of transfection for 4 and 16 hrs respectively. Western blotting was performed to check for the levels of mutant Api5 using a GFP-specific antibody. B) and D) Quantification showing the fold change in Api5 protein levels after GAPDH normalization. Data is representative of three independent experiments

3.2.4 p300 and HDAC1 acetylates- deacetylates Api5 at lysine 251

Earlier studies by Han and the group have shown that Api5 is acetylated at lysine 251 (Han et al., 2012). To identify whether Api5 acetylation and deacetylation by p300 and HDAC1 respectively occur at the lysine 251 residue, K251 mutants of Api5 were generated using site-directed mutagenesis. mVenus-tagged wild type, K251A (acetylation-deficient mutant), K251R (charge-mimic mutant), and K251Q (constitutive acetylation mimic) mutants of Api5 were ectopically expressed in cells. The expression levels of mVenus, a proxy for ectopic expression of Api5 K251 mutants, were analyzed in the presence and absence of p300i and romidepsin using a GFP-specific antibody. It was observed that levels of wild-type Api5 were reduced while the levels of K251A, K251Q, and K251R mutants of Api5 remained unaffected upon p300 inhibition (Figure 3.7A-B). However, the levels of wild-type Api5 increased

upon inhibition of HDAC1 by romidepsin, whereas that of the other mutants remained unchanged (Figure 3.7 C-D). Therefore, it was established that p300 acetylates while HDAC1 de-acetylates Api5 at the lysine 251 residue.

3.2.5 p300 mediated acetylation of Api5 is the signal for its nuclear localisation, and deacetylated Api5 is transported to the cytoplasm.

From the previous results, it can be inferred that p300 acetylates Api5 at lysine 251, thereby maintaining its stability. It has earlier been reported that Api5 is a nuclear protein. To investigate the role of acetylation on Api5 function, cells were treated with acetylation and deacetylation inhibitors, and immunofluorescence was performed.

In control cells, Api5 was observed to be present in the nucleus, while upon inhibition of p300, the percentage of cells showing Api5 to be present in the nucleus was negligible. Moreover, most cells showed nuclear and cytoplasmic localisation of Api5 (Figure 3.8A-B). Interestingly, 24% of cells showed the only cytoplasmic presence of Api5 upon p300 inhibition, which was not observed in the control cells (Figure 3.8B). Api5 localisation was also investigated upon inhibition of HDAC1. We found that, upon HDAC1 inhibition, the majority of the cells (67%) showed both nuclear and cytoplasmic localisation of Api5, whereas 29% of cells showed only nuclear localisation of Api5 (Figure 3.8C-D). Only 4% of cells showed cytoplasmic localisation of Api5, which is negligible. To follow the compartmentalisation switch of Api5, both p300 and HDAC1 were inhibited. The cytoplasmic localisation of Api5 that was observed upon p300 inhibition as well as the nuclear localisation observed upon HDAC1 inhibition was reduced upon inhibition of both p300 and HDAC1. 92% of cells showed both nuclear and cytoplasmic localisation of Api5 (Figure 3.8E-F). Interestingly it was observed that upon inhibition of HDAC1, 73% of cells showed Api5 nuclear foci while the foci were reduced to 46% when both p300 and HDAC1 inhibitors were added in concert (Figure 3.8G). These results were further confirmed in mCherry-tagged Api5 over-expressing cells (Figure 3.9). Therefore, it was concluded that Api5 acetylation at lysine 251 by p300 stabilises Api5 inside the nucleus while de-acetylation by HDAC1 at the same lysine residue leads to Api5 to locate to the cytoplasm.

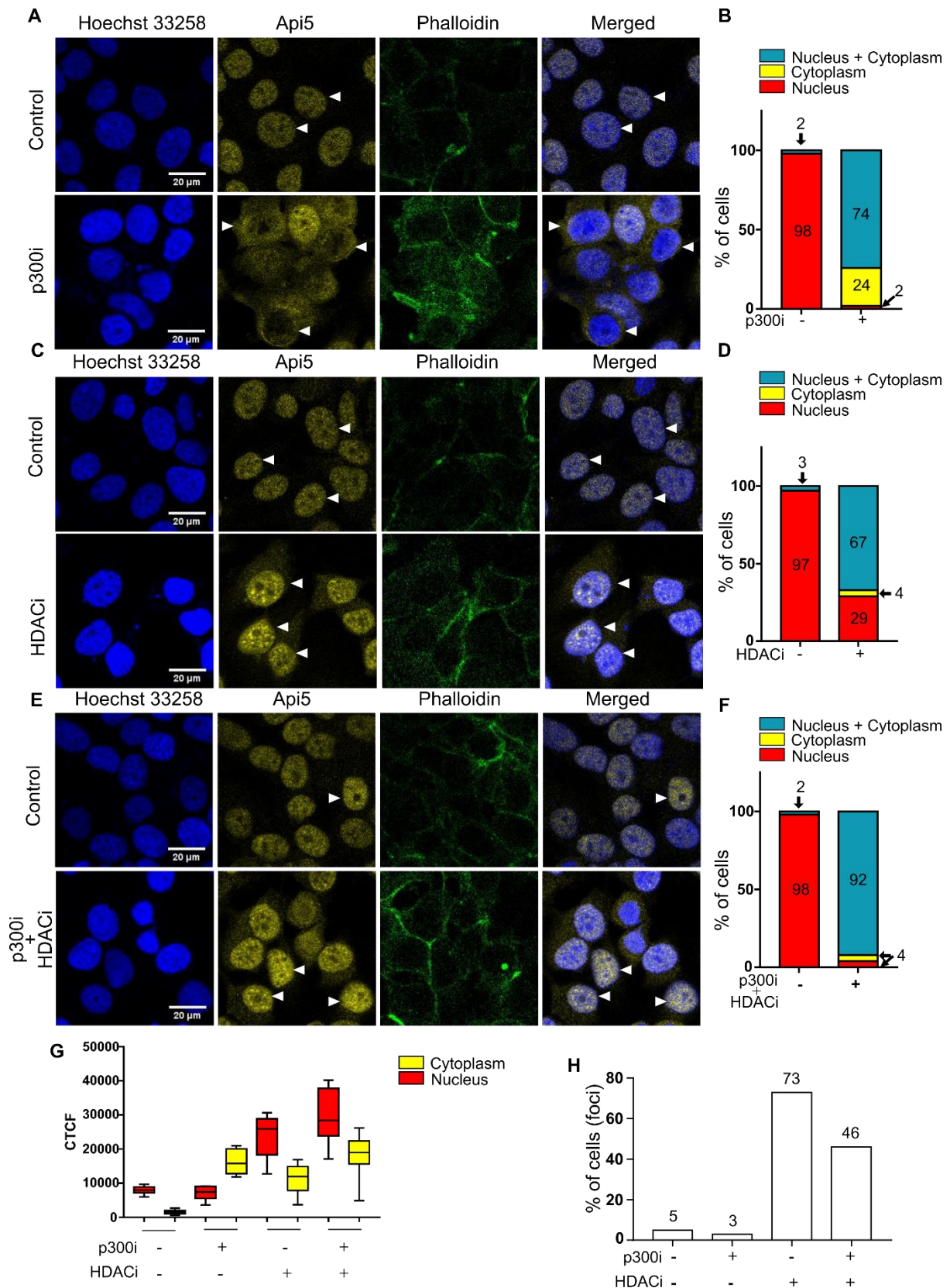


Figure 3.8: p300 and HDAC1 regulate Api5 localisation

MCF7 cells were treated with a-b) 7.5 μ M p300i, c-d) 0.1 nM HDACi and e-f) 7.5 μ M C646 and 0.1 nM romidepsin for 4 hrs and 16 hrs respectively. Immunofluorescence

assay was performed using Api5 specific antibody and cells with different Api5 staining pattern (marked with white arrow head) were manually counted and analysed. The bar graph represents the percentage of cells with Api5 staining only in the nucleus (red), only in the cytoplasm (yellow) or in both (blue). g) CTCF value of Api5 intensity in the nucleus and the cytoplasm was measured in the same experimental set up and the values are represented as box plots. Here, red corresponds to nuclear intensity and yellow corresponds to the cytoplasmic intensity of Api5. h) Cells exhibiting Api5 nuclear foci following different inhibitor treatments were manually counted and represented as bar graphs showing percentage of cells with nuclear foci. Data is representative of three independent experiments and minimum 200 cells were counted per experimental set for analysis.

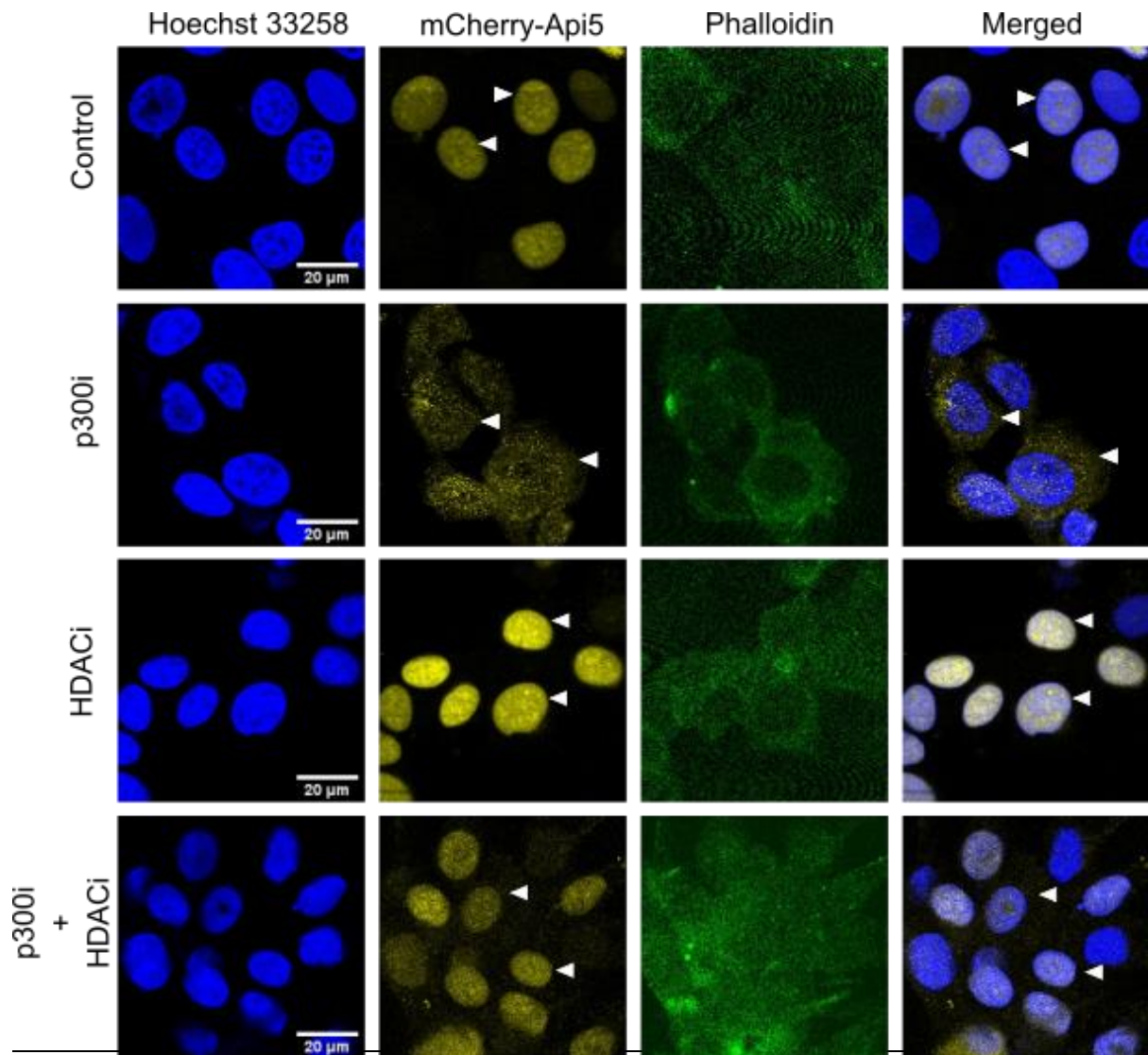


Figure 3.9: p300 and HDAC1 regulate Api5 localisation

mCherry tagged Api5 over-expressing MCF7 cells were treated with 7.5 μ M p300i, 0.1 nM HDACi, and 7.5 μ M p300 and 0.1 nM HDACi for 4 hrs and 16 hrs respectively. mCherry fluorescence (marked with white arrow head) was visualised using confocal microscopy (scale bar- 20 μ m). Data is representative of three independent experiments and minimum 200 cells were counted per experimental set.

3.2.6 AMPK and Akt regulate the p300 mediated stability of Api5

It has already been reported that AMPK can either directly or indirectly regulate G1/S cell cycle transition regulators such as p53 and p21 (Kong et al., 2016). AMPK requires the activity of AKT to regulate other cell cycle regulators (Han et al., 2018). It has been previously reported that activated Akt can activate p300 by

phosphorylating it at serine 1834 residue (Huang and Chen, 2005). Therefore to discern whether this signaling cascade led to the stability of Api5, both AMPK and Akt were inhibited using small molecule inhibitors.

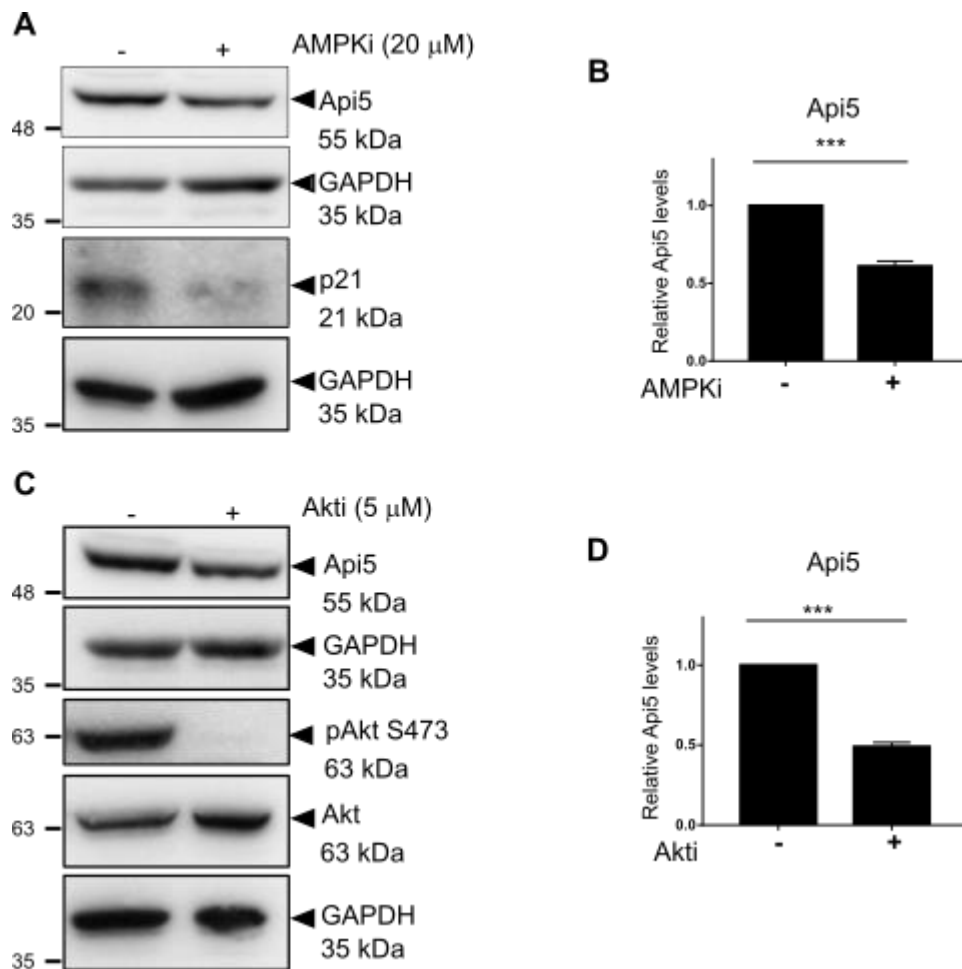


Figure 3.10: AMPK and AKT regulate Api5 stability

MCF7 cells were treated with A) 20 μ M AMPKi, and C) 5 μ M AKTi for 4 hrs and 24 hrs, respectively. Immunoblotting analysis was performed using Api5 specific antibodies. GAPDH was used as the loading control. B and D) Quantification showing the fold change in Api5 levels after normalization to GAPDH. A) p21 and C) pAkt S373 were used as positive controls to confirm AMPK and Akt inhibition, respectively. Data is representative of three independent experiments and statistical analysis for B and D was performed using T-test; *** p <0.001

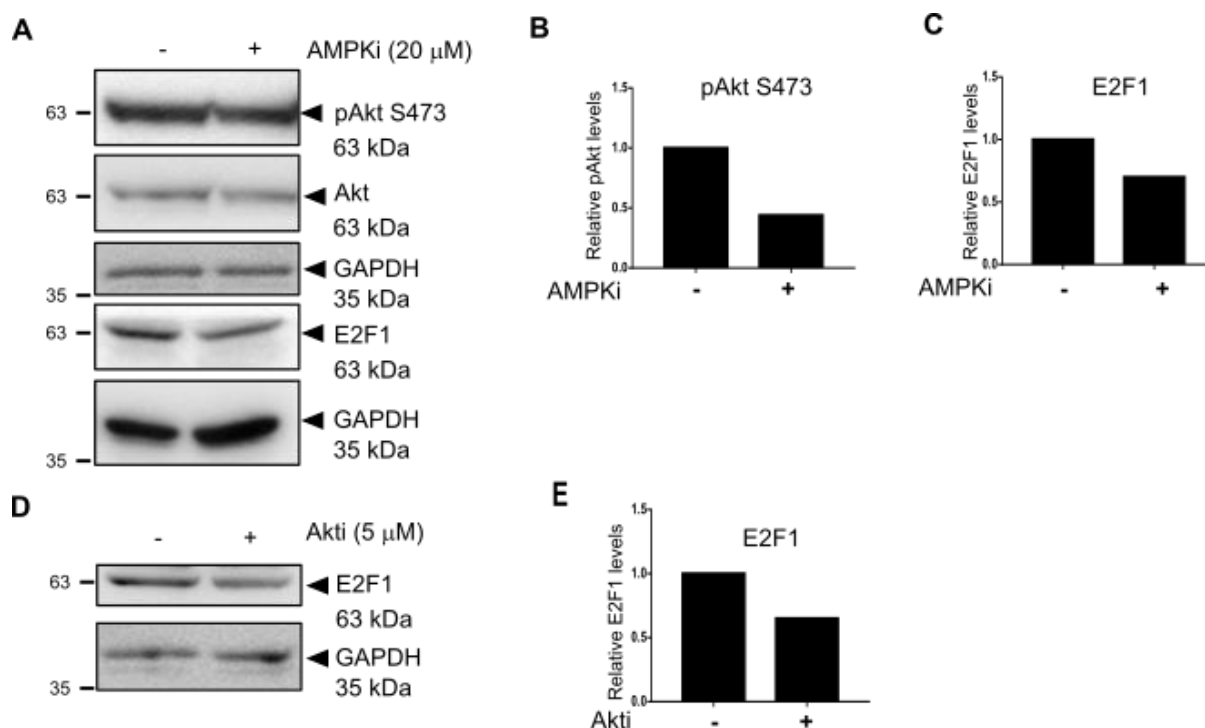


Figure 3.11: AMPK and AKT regulate Api5 stability

MCF7 cells were treated with A) 20 μ M AMPKi, and D) 5 μ M AKTi for 4 hrs and 24 hrs, respectively. A-B) pAkt S473 and A-C) E2F1 levels upon AMPK inhibition and D-E) E2F1 protein levels were analyzed following inhibition using immunoblotting. Data is representative of three independent experiments.

Api5 levels were observed to be reduced upon inhibition of both AMPK (Figure 3.10 A-B) and Akt (Figure 3.10 C-D) independently. It was also observed that upon inhibition of AMPK, Akt phosphorylation at serine 473 decreased, which is known to be required to activate p300 (Figure 3.10 E-F). Our experiments suggested that inhibition of AMPK inhibits the activation of Akt. Akt is the kinase that has been reported to phosphorylate and activate p300 histone acetyltransferase. E2F1, a transcription factor, is known to be acetylated by p300. This p300-mediated acetylation of E2F1 has been shown to regulate the stability of the protein. Reduced levels of E2F1 upon inhibition of AMPK and Akt separately confirms that both AMPK and Akt are involved in regulating the histone acetyltransferase activity of p300 (Figure 3.11 A-E). Inhibition of activation of Akt and reduction in Api5 protein levels upon inhibition of AMPK confirms that p300 mediated stability of Api5 is achieved through the AMPK-Akt pathway as illustrated in Figure 3.12.

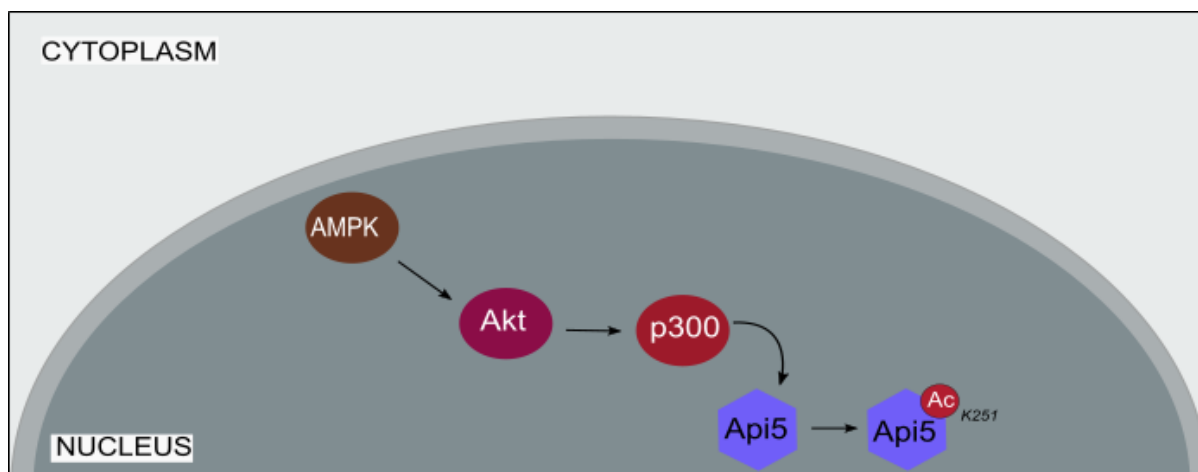


Figure 3.12: Schematic representation depicting the regulation of Api5

Regulation of Api5 via the AMPK, Akt, and p300 by signalling cascade

3.3 Discussion

In this study, we have shown that p300, a histone acetyltransferase to be responsible for acetylating and maintaining the stability of Api5. p300 is one of the most studied acetyltransferases, which acetylates not only histone proteins but also other non-histone proteins too (Ogryzko et al., 1996). E2F1 is another known protein stabilised upon acetylation by p300 (Martinez-Balbas et al., 2000). E2F1 is a transcription factor that plays a critical role during the G1/S transition of the cell cycle (Marzio et al., 2000). E2F1 levels vary during the different cell cycle phases, similar to Api5 (Garcia-Jove Navarro et al., 2013). E2F1 protein levels peak during the G1 phase of the cell cycle and start to degrade as the cell crosses the S phase. p53, the master regulator, is also acetylated by the p300 (Sakaguchi et al., 1998). Several studies show that upon acetylation, p53 stabilises and thus, can perform its different functions like activation of DNA damage response and repair pathway, cell cycle arrest, and apoptosis (Luo et al., 2000).

Histone deacetylases are one of the members of the deacetylase family of enzymes that remove acetylation from targeted proteins. Initially, HDACs were identified and reported to de-acetylate histone proteins (Inoue and Fujimoto, 1969). But extensive studies have shown that HDACs can also de-acetylate non-histone proteins. Both E2F1 and p53, which are acetylated by p300, are also de-acetylated by Class 1 HDACs (Harms and Chen, 2007; Marzio et al., 2000). E2F1 and p53, upon deacetylation become unstable and lose their activity. HDAC1 mediated de-

acetylation of E2F1 also aids in its localisation shift from the nucleus to the cytoplasm.

Earlier studies have shown Api5 acetylation at K251 residue to stabilise Api5 inside the nucleus (Han et al., 2012). However, the enzymes involved in the acetylation process were unknown. Our study is the first to identify p300 and HDAC1 as the acetylating and de-acetylating enzymes of Api5, respectively, that work in concert, thus leading to the stability or instability of the protein during normal cell functioning. In this study, we report that Api5, a nuclear protein under normal physiological conditions, is acetylated by histone acetyltransferase p300. p300 is an essential regulator of Api5 as the acetylated protein is localized in the nucleus while the de-acetylated protein switches its localisation to the cytoplasm. As Api5 levels reduce upon inhibition of p300, it suggests that Api5 might be undergoing post-translational degradation in the cytoplasm. As the acetylated Api5 is stable in the nucleus and is not transported to the cytoplasm, it may also be inferred that acetylation prevents Api5 from binding to translocator/transporter proteins that are responsible for its translocation/ transportation from the nuclear compartment to the cytoplasm. However, de-acetylation might be exposing the Api5 binding site to transporter protein(s) that enables the protein to translocate to the cytoplasm.

As mentioned earlier, Api5 levels peak during the G1 and S phase of the cell cycle. They are thereby able to play a role in the regulation of cell cycle progression and the proliferation of cells by possibly functioning as a transcription factor. During the G1 and S phases of the cell cycle, Api5 may be interacting with chromatin to transcribe those genes that regulate the progression of the cell cycle. Transcription of those genes may also regulate the proliferation of cells. Thus it may be inferred that acetylation of Api5 by p300 possibly provides additional functionality to Api5 that includes the transcription of cell cycle regulators.

It has also been reported that Api5 is overexpressed in many cancers. This overexpression of Api5 promotes angiogenesis and metastasis in cancerous cells. The oncogenic properties of tumour-promoting proteins can also be considered as an outcome of their stability. In this case, Api5 is being stabilised by the action of p300 histone acetyltransferase. Inhibition of p300 and induction of HDAC1 can reduce the oncogenic potential of Api5. Thus inhibitors of p300 and inducers of

HDAC1 have therapeutic implications in the treatment of cancers that have high expression of Api5.

As K251 acetylation of Api5 is the only known post-translational modification conserved from protists to mammals (Han et al., 2012), suggesting that conservation of this acetylation may play a key role during normal development. AMPK and Akt are essential regulators of various cellular processes like cell division, transcription regulation, and apoptosis (Fogarty and Hardie, 2010; Musi and Goodyear, 2002; Towler and Hardie, 2007) (Jin et al., 2016; Ouyang et al., 2017). Reduced levels of Api5 upon inhibition of these key regulators also indicate its role in normal physiological and developmental processes. However, our study suggests that p300 is the acetylating and stabilising regulator of Api5. At the same time, HDAC1 is the de-acetylating and negative regulator of stability; one cannot ignore the possibility of other enzymes playing a role in the regulation of Api5 stability.

Thus taken together, our data suggest that p300 and HDAC1 are the novel regulators of Api5 that interact and mediate the localisation and activity of Api5 by acetylating and de-acetylating at lysine 251, respectively, as illustrated in Figure 3.13.

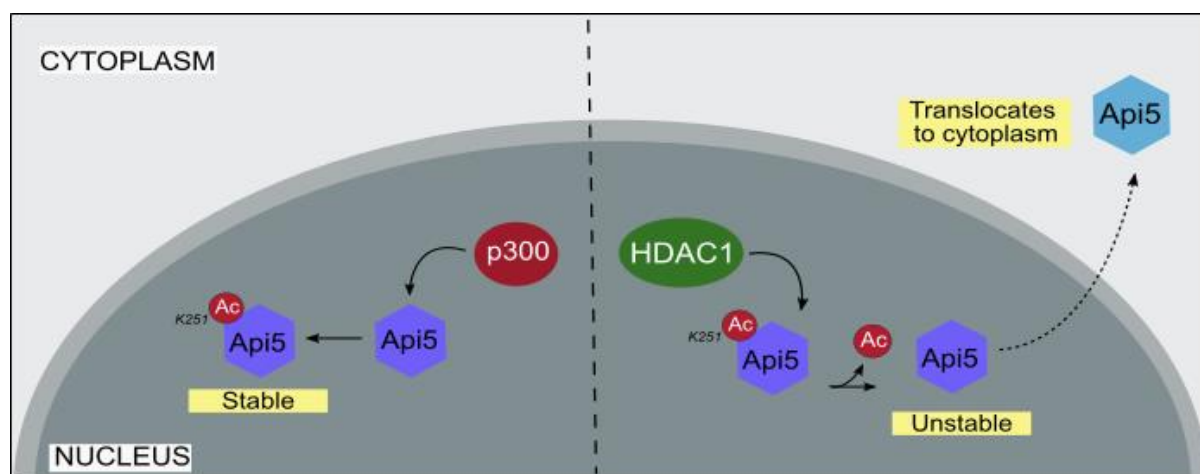


Figure 3.13: Schematic representation depicting the regulation of Api5 following acetylation and deacetylation

p300 acetylation of Api5 at lysine residue 251 stabilises the protein in the nucleus, whereas de-acetylation of Api5 at K251 by HDAC1 de-stabilises the protein, enabling it to get translocated to the cytoplasm.

Chapter 4

**Api5 undergoes ubiquitination by FBXW2,
FBXO3, and FBXO6**

4.1 Background

In chapter 3, we identified that p300 stabilises Api5 inside the nucleus while HDAC1 makes the protein susceptible to post-translational degradation by facilitating its transportation to the cytoplasm. The spatial localisation of proteins also regulates their stability. After being transported out of their original cellular compartments, proteins may be inactive or non-functional in the new sub-cellular localisation. These non-functional proteins are cleared off inside the cells to maintain cellular homeostasis. Such proteins undergo degradation before their clearance. In this chapter, we investigated the molecular pathway and pathway players involved in the degradation of Api5. There are two main pathways by which proteins get degraded: lysosomal pathway-mediated degradation and proteasomal pathway-mediated degradation. In lysosomal pathway-mediated degradation, lysosomes, the suicidal bags of the cells become active (Dunn, 1994). Inactive proteins are first encapsulated in the vesicles. These vesicles fuse with the lysosomes. Lysosomes contain lysozymes which are pH-dependent hydrolases. These hydrolases perform the proteolysis of the encapsulated proteins. Thus the target protein gets converted into multiple small fragments. These small fragments are later taken up by the antigen-presenting cells and hence cleared by phagocytosis by the phagocytic cells like neutrophils, monocytes and macrophages. Multiple proteins undergo degradation through the lysosomal pathways. Nuclear Lamins like Lamin B1, which are the main component of nuclear architecture, are amongst these proteins. The process of lysosomal pathway-mediated degradation of proteins is non-selective. However, some small stretches of amino acids make the proteins a target for lysosomal pathway-mediated degradation. The initial phase of this protein degradation pathway is also called autophagy (Eskelinen and Saftig, 2009). Proteasomal pathway-mediated degradation of proteins is the other method of clearing off the non-functional proteins. For the proteasomal pathway-mediated degradation of proteins, a post-translational modification, i.e., ubiquitination, is a mandatory step (Lecker et al., 2006). Proteasomal degradation of cellular proteins mainly involves poly-ubiquitination of cellular proteins. Poly-ubiquitination is the process of conjugation of ubiquitin (Ub) to the substrate proteins. The nature of Ub-Ub conjugation determines the fate of poly-ubiquitylated proteins. For instance, lysine-48 (K-48) linked poly-ubiquitination of proteins is marked for proteasomal degradation.

The Proteasome system is a complex of multiple proteins with proteolytic activity. Proteins get fragmented by proteasomal machinery and cleared by phagocytosis similar to the lysosomal pathway. p53 (Farnebo et al., 2010) and E2F1, a crucial regulator of apoptosis (Ginsberg, 2002) and cell cycle (Zhang et al., 2010), are amongst the proteins which undergo ubiquitin-proteasome pathway-mediated degradation (Budhavarapu et al., 2012; Moll and Petrenko, 2003).

From the results of the previous chapter, it was observed that Api5 upon de-acetylation by HDAC1 is translocated to the cytoplasm. It has already been reported by Han and the group that de-acetylated protein also undergoes degradation at the post-translational level (Han et al., 2012). By keeping these points in mind, it was hypothesised that the translocation of Api5 in the cytoplasm is somehow linked with its degradation. However, the molecular mechanism and pathway of its degradation were not known. In this chapter, we identified the pathway and regulators involved in the post-translational degradation of Api5.

4.2 Results

4.2.1 Api5 degradation is not through the lysosomal pathway

To identify the potential pathway of Api5 degradation, cells were treated with different concentrations of chloroquine, a lysosomal pathway inhibitor. Inhibition of the lysosomal pathway did not show any change in Api5 levels suggesting that Api5 degradation is not through the lysosomal pathway, whereas Lamin B1, a positive control for lysosomal degradation, showed accumulation (Figure 4.1), thus suggesting that Api5 does not undergo post-translational degradation through lysosomal pathway.

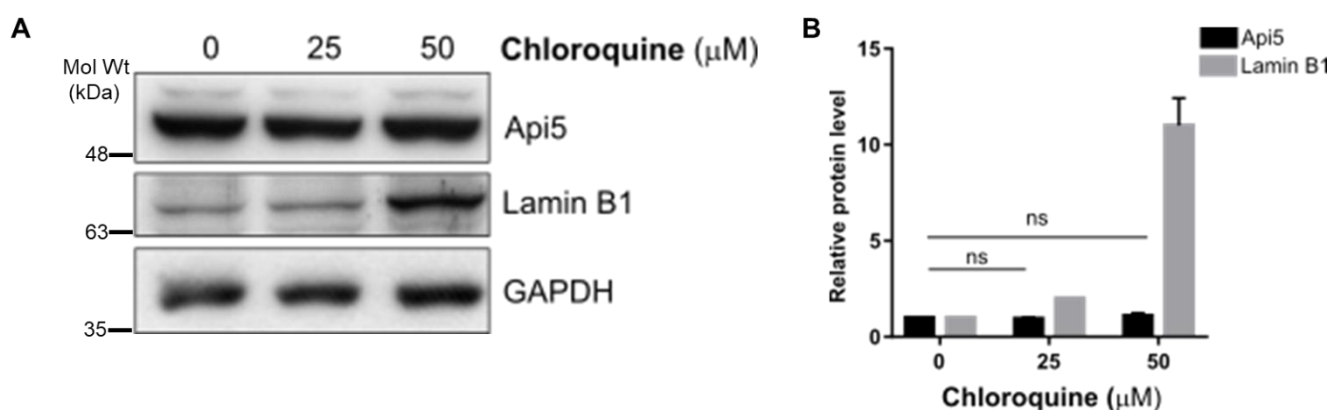


Figure 4.1: Api5 degradation is not through the lysosomal pathway

A). Western blot analysis showing the level of Api5 in MCF7 cells upon treatment with different dosages of chloroquine for 8hrs. B) The fold change of respective proteins after normalised with GAPDH. Lamin B1 was used as a positive control, and GAPDH was used as a loading control. Data is representative of three independent experiments and statistical analysis was performed using One way ANOVA; ns $p > 0.05$

4.2.2 Api5 degradation is through proteasomal pathway

To analyse whether Api5 is undergoing proteasomal degradation, levels of Api5 were examined in the absence and presence of proteasome inhibitors. MG-132, a potent inhibitor of the 26S proteasome system, was used. To estimate the optimum concentration of MG-132, MCF7 cells were treated with different concentrations of MG-132 viz. 5 μM, 10 μM, 15 μM, 20 μM, 25 μM for 8 hrs. Immunoblotting data revealed that the maximum accumulation of Api5 occurred upon treatment of 10 μM MG-132 for 8 hrs (Figure 4.2A-B). To determine the optimal time of MG-132 treatment, MCF7 cells were incubated with 10 μM MG-132 for 4, 8, and 12 hrs. Maximum accumulation of Api5 was observed following treatment of 10 μM MG-132 for 4 hrs (Figure 4.2C-D).

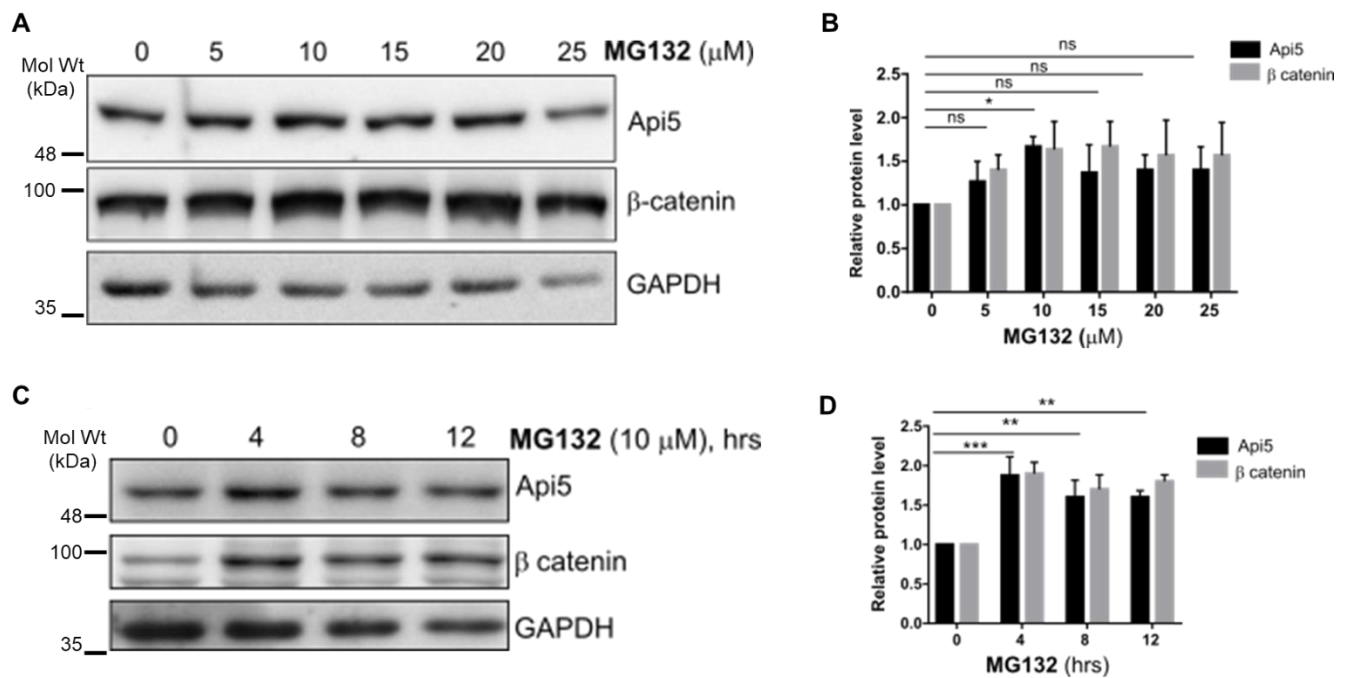


Figure 4.2: Api5 degradation is through proteasomal pathway

Western blot analysis showing accumulation of Api5 in MCF7 cells upon treatment with A) different doses of MG-132 and B) 10 μM MG132 for different time points. C- D) The fold change of respective proteins after normalisation with GAPDH. β-catenin was used as a positive control, and GAPDH was used as a loading control. Data is representative of three independent experiments and statistical analysis was performed using One way ANOVA; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

To further validate the proteasomal regulation of Api5, a cycloheximide chase assay was performed. MCF7 cells were treated with cycloheximide, a protein synthesis inhibitor, for 1, 2, and 4 hrs. Immunoblotting results revealed that levels of Api5 declined with time following treatment of cycloheximide chase, suggesting that the expression level of Api5 is regulated at the post-translational level (Figure 4.3A-B).

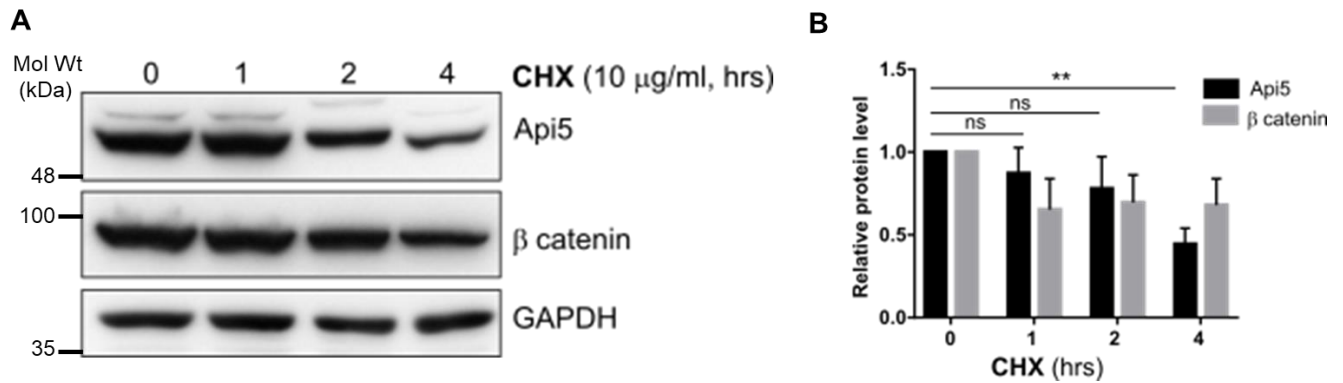


Figure 4.3: 10 μg/ml of cycloheximide inhibits Api5 synthesis

A) Western blot analysis to standardise the cycloheximide time duration. B) Quantification of the western blot for the effect of 10 μg/ml of cycloheximide in MCF7 cells. β-catenin was used as a positive control, and GAPDH was used as a loading control. Data is representative of three independent experiments and statistical analysis was performed using One way ANOVA; ns p > 0.05 , ** p < 0.01,

To prove that accumulation of Api5 was due to inhibition of proteasomal degradation and not due to increased protein synthesis, MCF7 cells were treated with cycloheximide and MG-132 for 4 hours. Accumulation of Api5 is observed upon treatment with MG132 even when protein synthesis is blocked (Figure 4.4), indicating that degradation of Api5 is via the proteasomal pathway.

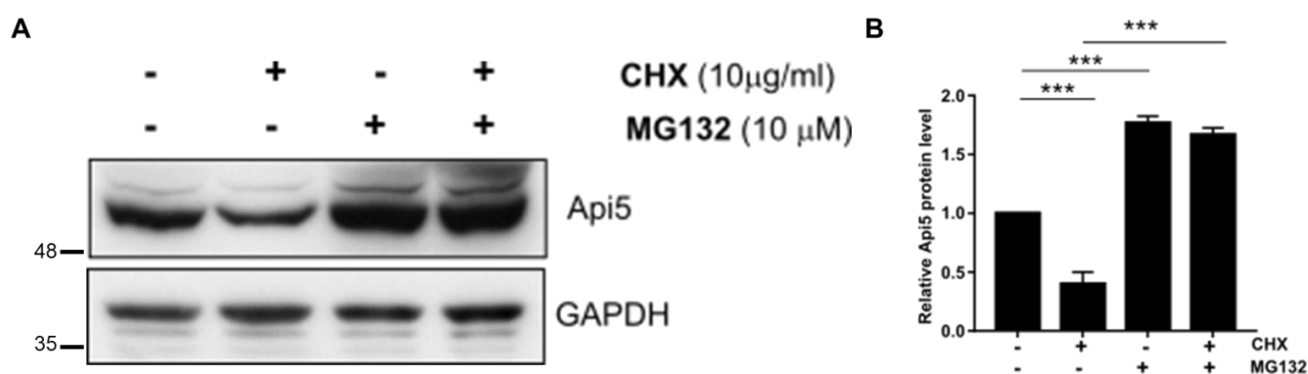


Figure 4.4: Accumulation of Api5 upon inhibition of proteasome pathway is the result of its reduced post-translational degradation

A) Western blot analysis B) quantification to check the effect of 10 μg/ml of cycloheximide and 10 μM MG-132 in MCF7 cells treated for 4 hrs. GAPDH was used as a loading control. Data is representative of three independent experiments and statistical analysis was performed using One way ANOVA; *** p < 0.001

4.2.3. Api5 undergoes poly-ubiquitination

Ubiquitination majorly marks the proteins for degradation by proteasomal pathway. In this study, Api5 has been shown to undergo degradation by this pathway. To check whether Api5 gets ubiquitinated before undergoing degradation, endogenous Api5 in MCF7 cells was pulled down using Api5 specific antibody and immunoblotting was performed to check the presence of ubiquitination by the ubiquitin-specific antibody. It was observed that Api5 undergoes poly-ubiquitination (Figure 4.6).

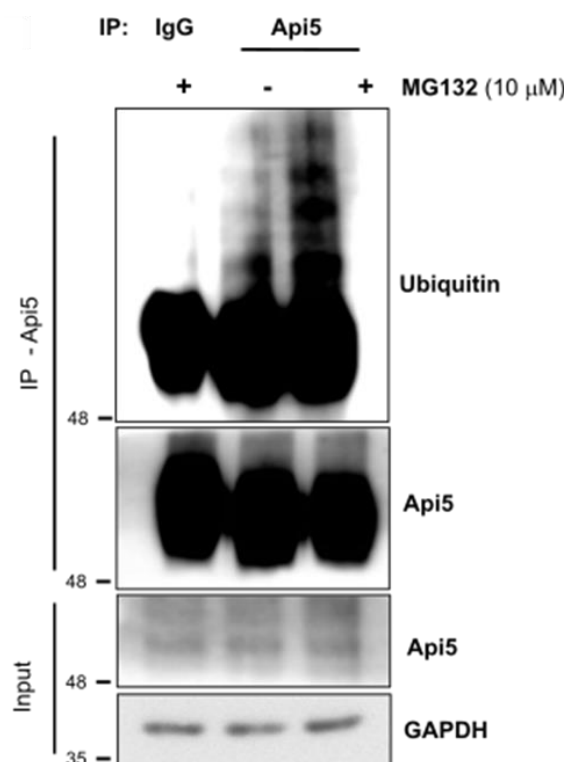


Figure 4.6: Api5 undergoes poly-ubiquitination

MCF7 cells treated with 10 μ M MG132 for 4 hrs were lysed, and immunoprecipitation was performed using Api5 specific antibody and immunoblotting using ubiquitin antibody.

4.2.4 Screening of F-Box proteins involved in the degradation of Api5

F-box proteins are the key players in the Ubiquitin-Proteasome pathway. F-box proteins are responsible for directing the poly-ubiquitination of cellular proteins and are typically marked for proteasomal degradation through 26S Proteasome. Moreover, many F-box proteins are also involved in cell cycle progression, apoptosis, DNA damage response and development. We hypothesised that the fluctuation of cellular levels of Api5 during cell cycle progression might be due to the involvement of F-box proteins.

To find which F-box proteins were involved in the degradation of Api5, MCF7 cells were transfected with plasmids containing cDNAs of F-box genes individually. Each cDNA will express FLAG and Myc-tag F-box proteins, and we checked the levels of Api5 following ectopic expression of F-box protein by immunoblotting. FBXW2, FBXW4, and FBXW9 from the WD family (Figure 4.7), FBXL6 and FBXL8 from the Leucine family (Figure 4.8), and FBXO3 and FBXO6 (Figure 4.9) from the other

family were found to degrade Api5 to its minimum levels. So these candidates were selected for further experiments.

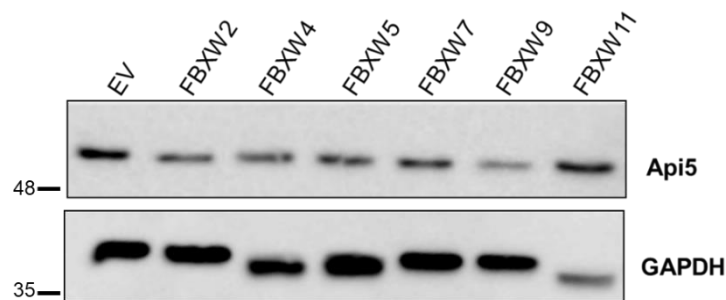


Figure 4.7: FBXW2, FBXW4, and FBXW9 degrade Api5

Plasmids containing various Flag-tagged FBXW genes were ectopically expressed in MCF7 cells by transient transfection. These cells were lysed, and Api5 levels were analysed using immunoblotting. GAPDH was used as the loading control. Data is representative of three independent experiments.

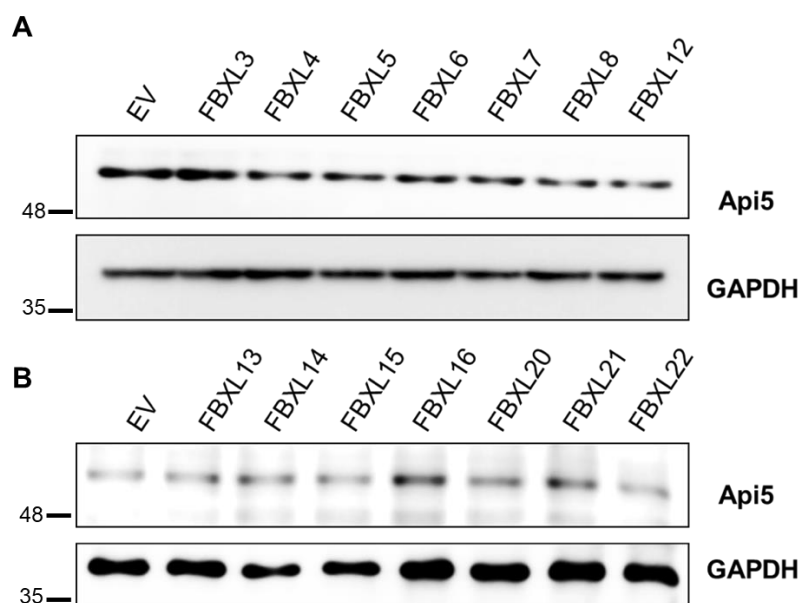


Figure 4.8: FBXL6 and FBXL8 degrade Api5

Plasmids containing various Flag-tagged FBXL genes were ectopically expressed in MCF7 cells by transient transfection. These cells were lysed, and Api5 levels were analysed using immunoblotting. GAPDH was used as the loading control. Data is representative of three independent experiments.

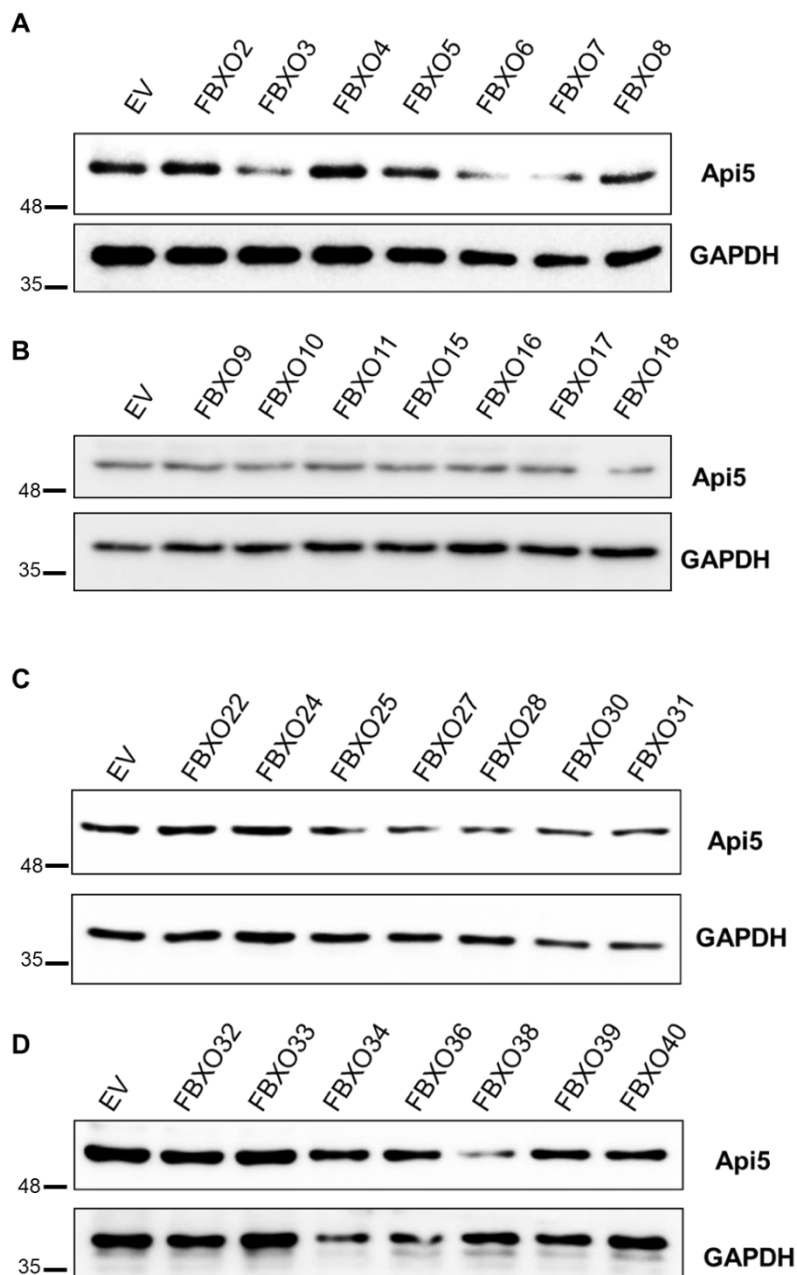


Figure 4.9: FBXO3 and FBXO6 degrade Api5

Plasmids containing various Flag-tagged FBXO genes were ectopically expressed in MCF7 cells by transient transfection. These cells were lysed, and Api5 levels were analysed using immunoblotting. GAPDH was used as the loading control. Data is representative of three independent experiments.

4.2.5 FBXW2, FBXO3, and FBXO6 degrades Api5

Of the 56 FBOX proteins screened, Api5 levels were significantly decreased following ectopic expression of FBXL6, FBXL8, FBXW2, FBXW4, FBXW9, FBXO3, and FBXO6 (Figure 4.9A). Quantification of Api5 levels revealed that the ectopic expression of FBXW2, FBXO3, and FBXO6 resulted in the reduction of Api5 to its minimal level (Figure 4.9B). These F-box proteins were selected for further studies.

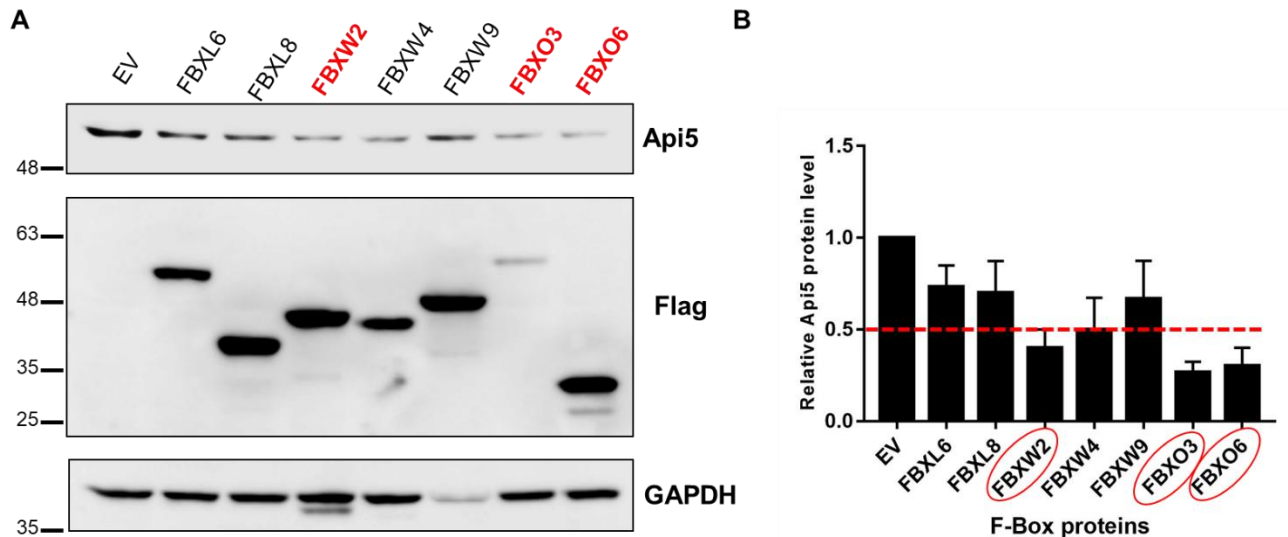


Figure 4.9: FBXL6, FBXL8, FBXW2 FBXW4, FBXW9, FBXO3, and FBXO6 degrade Api5

Plasmids containing various Flag-tagged F-box genes were ectopically expressed in MCF7 cells by transient transfection. These cells were lysed, and Api5 levels were analysed using A) immunoblotting and B) quantified after normalising with GAPDH. Data is representative of three independent experiments.

4.2.6 FBXW2, FBXO3, and FBXO6 degrade Api5 in a dose-dependent manner

To demonstrate a dose-dependent degradation of Api5 by the selected FBOX proteins, cells were transfected with either vector control or plasmids containing FBXW2, FBXO3 and FBXO6 genes, and Api5 protein levels were analysed. It was observed that FBXW2, FBXO3 and FBXO6 degrade Api5 in a dose-dependent manner (Figure 4.10).

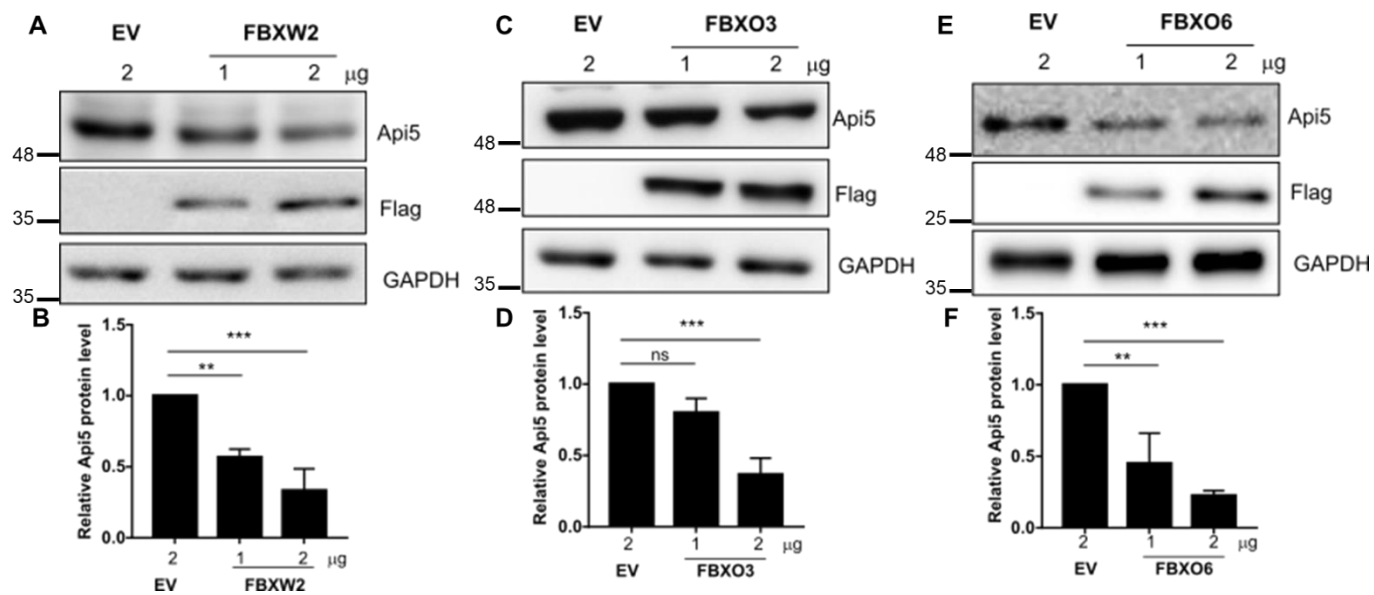


Figure 4.10: FBXW2, FBXO3, and FBXO6 degrade Api5 in a dose-dependent manner

MCF7 cells transfected with 1 and 2 μ g of A) FBXW2, C) FBXO3, and E) FBXO6 and Api5 levels were analysed using immunoblotting and B, D, F) quantified after normalising with GAPDH. EV is the empty vector (2 μ g) transfected cells lysates were used as control. Data is representative of three independent experiments and statistical analysis was performed using One way ANOVA; ns> 0.05, **p<0.01, *** p<0.001.

4.2.7 FBXW2, FBXO3, and FBXO6 degrade Api5 through proteasomal pathway

From the previous studies, it was clear that FBXW2, FBXO3 and FBXO6 degrade Api5. To prove that FBOX-mediated degradation is through the proteasomal pathway, cells were treated with MG132 upon overexpression of FBXW2, FBXO3 and FBXO6. Api5 levels were restored upon MG132 treatment in the transfected cells suggesting that FBOX proteins degrade Api5 through the proteasomal pathway (Figure 4.11)

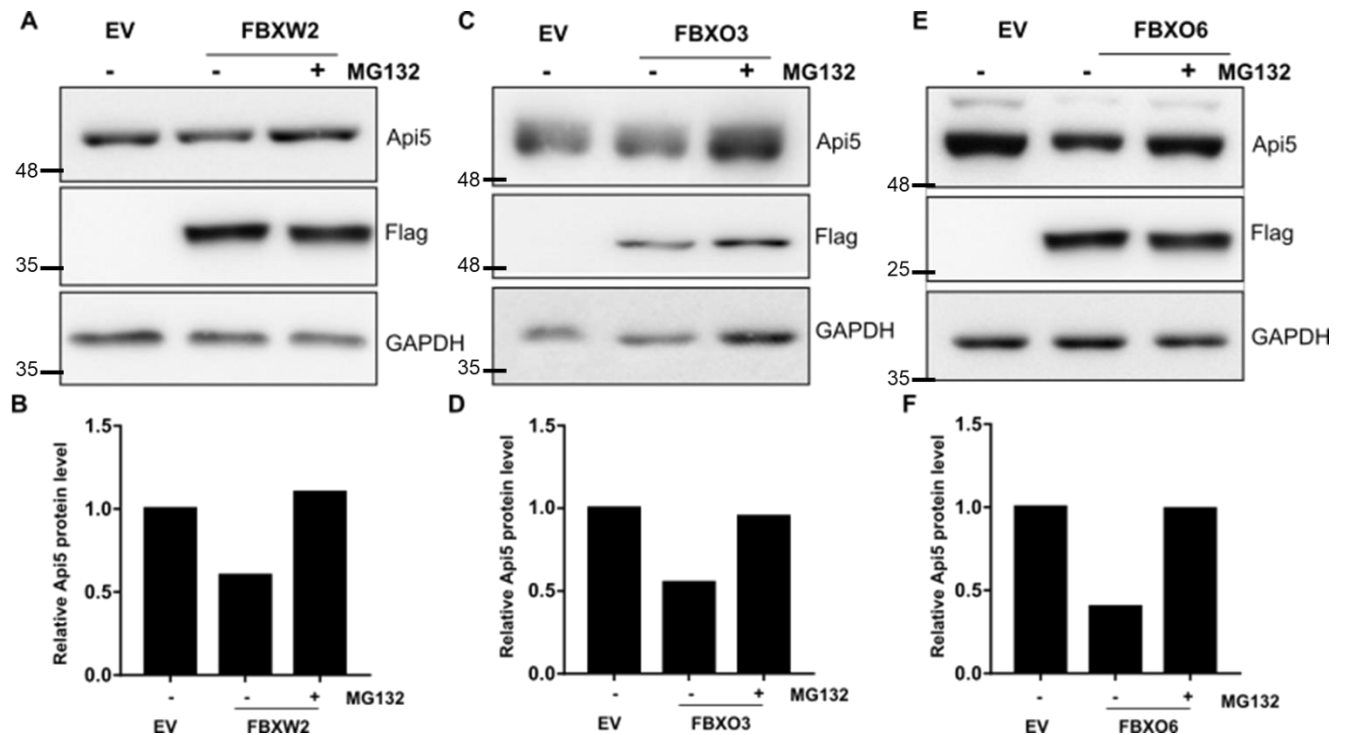


Figure 4.11: FBOX proteins degrade Api5 through the proteasomal pathway

MCF7 cells transfected with 1 μ g of the plasmid containing A) FBXW2, C) FBXO3 and E) FBXO6 were treated with 10 μ M of MG132 for 4 hrs post 24 hrs of transfection were lysed, and Api5 levels were analysed using immunoblotting. B, D and F) Api5 levels were quantified and normalised to GAPDH. Data is representative of three independent experiments.

4.2.8 FBXW2, FBXO3, and FBXO6 interact with Api5

For Api5 to undergo ubiquitin-proteasomal degradation, the selected FBOX proteins must interact with Api5. Therefore, to demonstrate the interaction between Api5 and the selected FBOX proteins, myc-tagged FBXW2, FBXO3 and FBXO6 were ectopically expressed, and immunoprecipitation was carried out using myc-specific antibody. The presence of Api5 in the pulled-down lysates confirmed that FBXO3, FBXO6 and FBXW2 interact with Api5 (Figure 4.12).

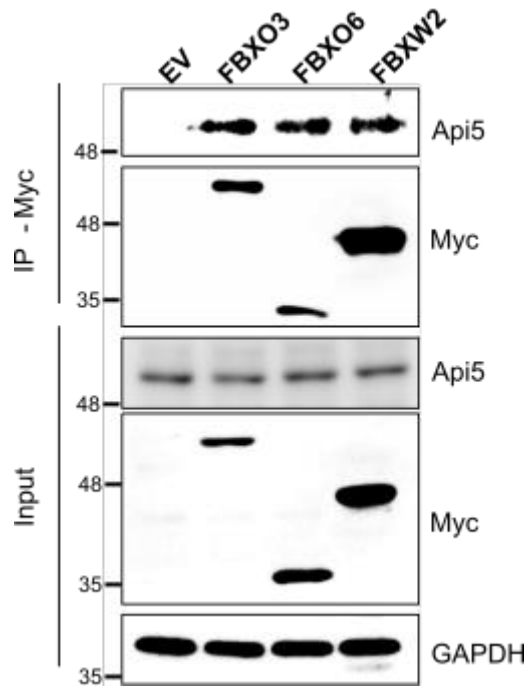


Figure 4.12: FBXW2, FBXO3, and FBXO6 interact with Api5

MCF7 cells transfected with myc tagged FBXW2, FBXO3, and FBXO6 containing plasmids were lysed, and immunoprecipitation was carried out using myc antibody. Api5 levels were analysed by immunoblotting using Api5 specific antibody.

FBXW2 has been reported to act as a tumour suppressor in lung carcinomas by degrading oncogenic SKP2 (Xu et al., 2017). Moreover, multiple studies suggest that Api5 can act as a potential oncogene. So it may be hypothesised that the degradation of Api5 is through its interaction with FBXW2. To prove that the degradation of Api5 is by FBXW2, the interaction between FBXW2 and Api5 was analysed at the endogenous level. Immunoprecipitation was performed using Api5 specific antibody, and the presence of FBXW2 in the Api5 pull-down lysates confirmed that Api5 and FBXW2 interact at the endogenous level (Figure 4.13).

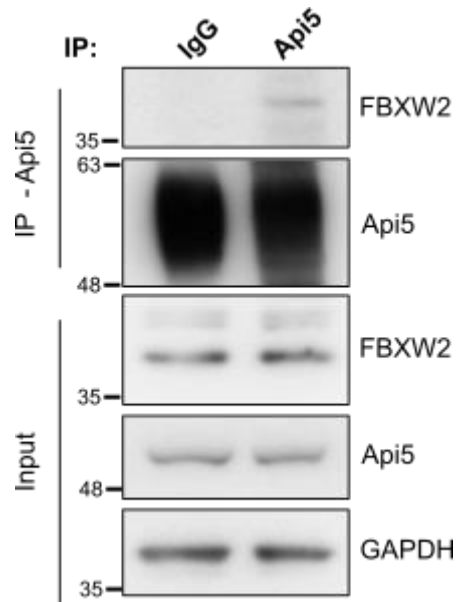


Figure 4.13: Api5 interacts with endogenous FBXW2

Endogenous Api5 was pull down in MCF7 cells, and FBXW2 interaction was analysed using immunoblotting.

4.2.9 FBXW2 degrades Api5

The role of FBXW2 in the degradation of Api5 was further confirmed using knock-down studies. FBXW2 was stably knocked down in cells, and Api5 levels were analysed. Knock-down of FBXW2 in cells resulted in the accumulation of Api5 (Figure 4.14). Taken together, it may be concluded that FBXW2 is the main SCF type E3 ubiquitin ligase that plays a critical role in the degradation of Api5.

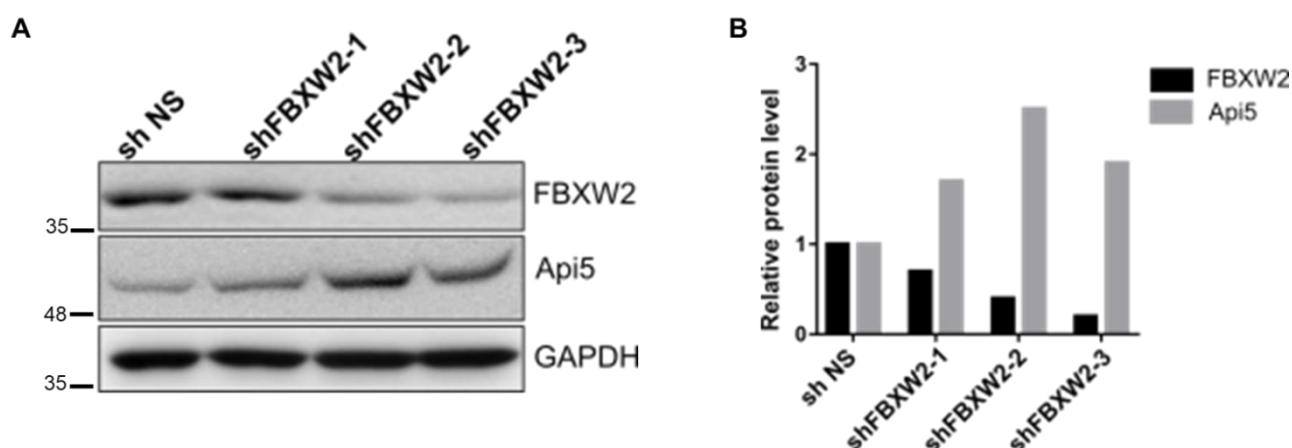


Figure 4.14: FBXW2 degrades Api5

A) HeLa cells stably expressing various shRNA constructs of FBXW2 were lysed, and Api5 and FBXW2 levels were analysed using immunoblotting. B) FBXW2 and Api5 levels were quantified and normalised to GAPDH. Data is representative of three independent experiments.

4.3. Discussion

Accumulation of Api5 upon inhibition of the proteasomal pathway suggests that Api5 undergoes post-translational degradation through the proteasomal pathway.

Accumulation of Api5 upon inhibition of protein synthesis along with inhibition of proteasomal pathway also proves and strengthens our finding that Api5 undergoes posttranslational degradation by the proteasomal pathway. These studies also confirm that Api5 undergoes post-translational modification, ubiquitination. This ubiquitination makes Api5 a target for proteasomal pathway-mediated degradation, while no change in protein levels of Api5 upon the treatment of chloroquine rules out the possibility for its post-translational degradation by lysosomal pathway. There are multiple types of ubiquitin additions to the target substrate. It was observed that Api5 undergoes K-48 linked ubiquitination. K-48 linked ubiquitination is required to degrade the targeted proteins. E3 ligases are the key enzymes that provide specificity in the ubiquitin attached to the substrate proteins. E3 ligase is an enzyme that has multiple protein subunits. FBOX is the main component of the E3 ligase enzyme which has the domain that can bind to the target proteins. Our studies revealed that FBXL6, FBXL8, FBXW2 FBXW4, FBXW9, FBXO3 and FBXO6 decreased the levels of Api5 upon ectopic expression. Out of these, FBXW2, FBXO3 and FBXO6 decreased the Api5 levels to their lowest. To attain their molecular

function, FBOX proteins need to interact with substrate proteins. Interaction of Api5 with FBXW2, FBXO3 and FBXO6 proved that Api5 is degraded by these selected FBOX proteins by the proteasomal pathway. FBOX proteins are novel enzymes in the field of ubiquitination and proteasomal pathways.

FBXW2 is an SCF type of E3 ubiquitin ligase involved in the ubiquitin-mediated degradation of potential cell cycle regulator SKP2 (Xu et al., 2017). It has already been reported that in lung carcinomas, SKP2, a potential oncogenic protein, gets degraded by FBXW2, which thus acts as a tumour suppressor (Xu et al., 2017; Yang and Sun, 2019; Yin et al., 2019). Api5 is also found to be upregulated in different types of cancers like cervical, oesophageal and lung cancers (Bousquet et al., 2019; Cho et al., 2014; Lawrenson et al., 2015; Xu et al., 2017). Multiple studies by various groups suggest that Api5 can also be a potential oncogenic factor whose downregulation makes cells vulnerable towards DNA damage mediated cell death (Rigou et al., 2009). Our studies confirmed that FBXW2 interacts with Api5 and promotes its degradation through ubiquitination in physiological conditions (Figure 4.15).

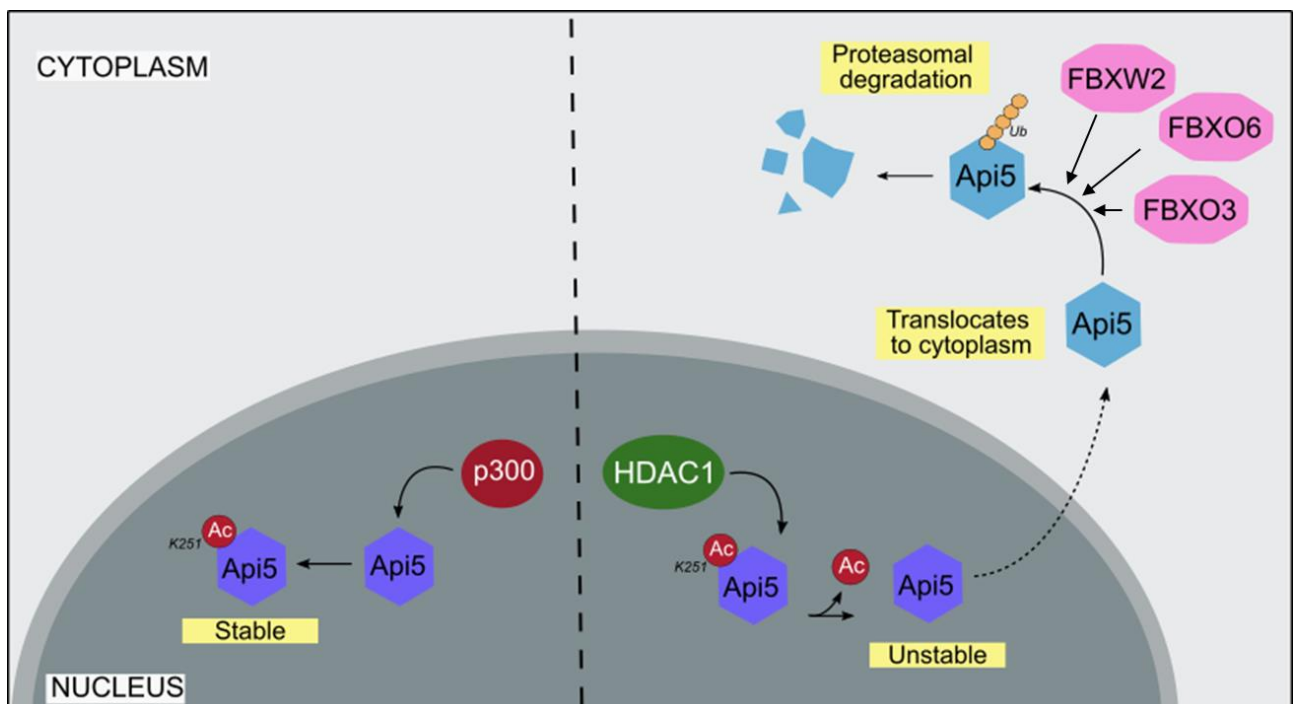


Figure 4.15: Schematic representation summarising the degradation of Api5 p300 acetylation of Api5 at lysine residue 251 stabilizes the protein in the nucleus, whereas de-acetylation of Api5 at K251 by HDAC1 de-stabilizes the protein, enabling

it to get translocated to the cytoplasm. In the cytoplasm, Api5 undergoes ubiquitination and proteasomal degradation by FBXW2, FBXO3 and FBXO6

Chapter 5

Regulation of Api5 during cell cycle progression

5.1. Background

Cell division is when a cell duplicates its contents, segregates them equally, and then divides to give rise to two daughter cells (Schafer, 1998). The time interval between two successive divisions constitutes a cell cycle. The completion of a cell cycle requires the functioning of various proteins that will guide cells to specific sequences to achieve two daughter cells. Central to all, Cyclin-dependent kinases Cdk and cyclins are the crucial regulators of cell division (Satyanarayana and Kaldis, 2009). These sequential events have been categorised as the different phases of the cell cycle. In each phase, the cell has various ways to control this cycle, such as checkpoints at different stages, a series of switches, and many external and internal cues. All this makes up the cell cycle control system, and it is essential in controlling the number of cells.

The major components that regulate various events in the cell cycle are the cyclin-CDK complexes. Cdk are kinases that are always present in cells, but their activity depends on various other proteins, the most prominent being the cyclins, whose presence in the cell varies during the cell cycle (Schafer, 1998). When a certain cyclin level is at its peak, that leads to the formation of the cyclin-CDK complex. This complex then phosphorylates many other proteins, which then perform the required functions during the progression of the cell cycle (Vigneron et al., 2018). For example, degradation of Rb upon phosphorylation by cyclin E-cdk2 is required for the progression of the cell cycle from G1 to S phase (Narasimha et al., 2014). SCF and the anaphase-promoting complex (APC/C) are the crucial E3 ubiquitin ligases in cells that target various proteins for degradation, which eventually leads to successful cell division (Vodermaier, 2004).

It has been reported that Api5 protein levels fluctuate while its mRNA levels remain unchanged during the cell cycle progression (Garcia-Jove Navarro et al., 2013). It has been studied that Api5 protein expression is at its peak during the G1 and S phase of the cell cycle while Api5 protein levels reduce in the G2-M phase. It has also been reported that Api5 knockdown induces a significant decrease in cyclin D (G1 cyclin), cyclin E1 (G1/S cyclin), cyclin A (S phase cyclin), and Cdk mRNA levels, which is the same effect observed upon knockdown of E2F1. This suggests that both Api5 and E2F1 could be the regulators of the same pathway as Api5 has been

shown to facilitate the E2F1 recruitment to E2F1's target promoters (Garcia-Jove Navarro et al., 2013).

As earlier reports have shown Api5 to undergo post-translational degradation, it was pertinent to investigate the mechanism of its degradation during the cell cycle.

5.2 Results

5.2.1. Api5 degradation is cell cycle-dependent

According to the study of Garcia and his group, Api5 levels oscillate during the cell cycle progression (Garcia-Jove Navarro et al., 2013). However, mRNA levels of Api5 remain unchanged during cell cycle progression indicating the involvement of post-translational regulation. To analyse the levels of Api5 during cell cycle phases, MCF7 cells were synchronized in the G2-M phase and released for different time points to obtain the cells in G2-M, G1, and S phases of the cell cycle, and synchronisation were confirmed by analysing the cell cycle profile of cells using fluorescence-activated cell sorting (FACS). In the phase arrested cells, Api5 protein levels were analysed using immunoblotting (Figure 5.1A). It was observed that Api5 levels were low in the G2-M phase while its levels were maintained during the G1 phase and were at their peak in the S phase of the cell cycle that is in agreement with the previous study. To confirm the fluctuation in Api5 levels during the cell cycle, cells were arrested in the G1 phase by serum starvation, the S phase using double thymidine, and in G2-M by using nocodazole (Figure 5.1B). Immuno-fluorescence was carried out to check the levels of Api5 in each phase, and it was observed that Api5 levels remained high during the G1 and S while Api5 was found to be reduced in G2-M phase arrested cells (Figure 5.1B). Thus it was confirmed that the regulation of Api5 is cell cycle-dependent. To confirm the post-translational degradation of Api5, cells were arrested in the G2-M phase of the cell cycle using Nocodazole. Api5 protein expression and *Api5 mRNA* levels were analysed (Figure 5.2A-C). It was observed that Api5 protein levels were reduced significantly while its transcript levels were not affecting in the G2-M phase of the cell cycle that concluded that G2-M phase-dependent degradation of Api5 is at post-translational level (Figure 5.2A-D).

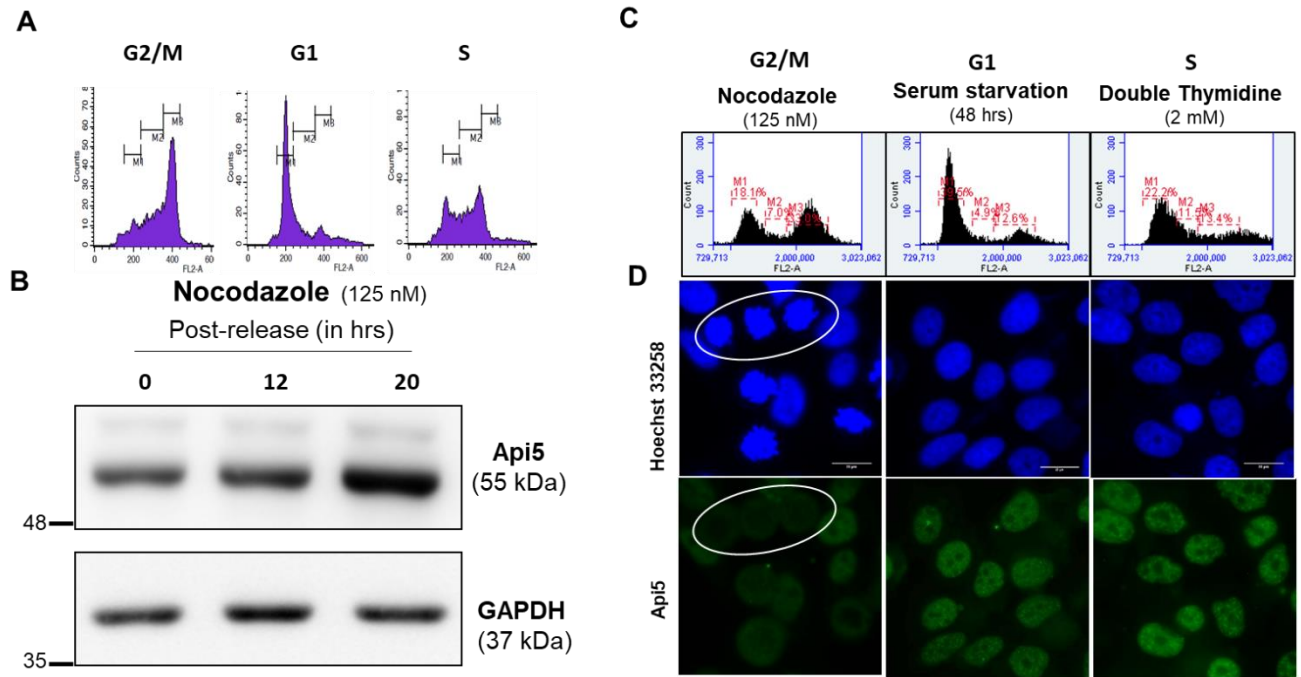


Figure 5.1: Regulation of Api5 is cell cycle-dependent

A) MCF7 cells were treated with nocodazole (100 ng/ml) for 16 hrs, and cells were released into fresh media and collected at 0, 12, and 20 hrs. Cells were fixed in ethanol and stained with propidium iodide, and cell cycle profile was monitored, or western blotting was performed to check the levels of Api5. GAPDH was used as a loading control for immunoblotting. B) MCF7 cells were treated with nocodazole (100 ng/ml) for 16 hrs for G2-M arrest, Serum starved for 48 hrs for G1 arrest, and double thymidine (2mM) for S phase arrest. Cells were fixed, and immunofluorescence was performed using Api5 specific antibody and visualised using Leica Sp8 confocal microscopy at 63X (scale bar- 20 μ m). Data is representative of three independent experiments.

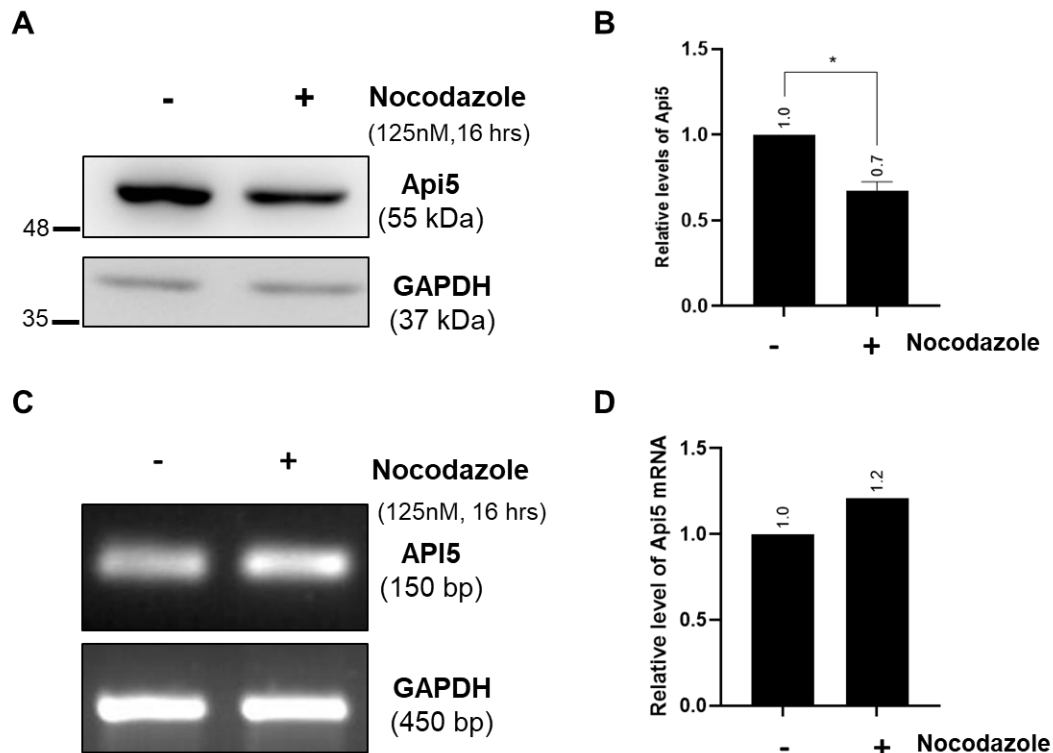


Figure 5.2: Api5 protein level decreases during G2-M phase

A) MCF7 cells were treated with nocodazole (125nM) for 16 hrs were lysed, and immunoblotting was performed to analyse the levels of Api5 and B) quantified after normalising to the loading control GAPDH. C) API5 transcript levels upon nocodazole (125nM for 16 hrs) treatment were analyzed using semi-quantitative PCR and D) quantified after normalising with GAPDH. Data is representative of three independent experiments and statistical analysis was performed using T-test; * $p < 0.05$.

After confirming the degradation of Api5 in the G2-M phase of the cell cycle, it was interested to identify the stage of mitosis in which the Api5 levels were showing the maximum reduction. For that, cells stably expressing mCherry tagged Api5 were first synchronised in the G2-M phase using cdk1 inhibitor and then released for subsequent hrs to obtain the cells in different stages of mitosis. It was observed that Api5 protein levels start reducing from the prophase (Figure 5.3A). This reduction in Api5 was also persistent in metaphase cells while cells in anaphase were completely devoid of Api5, suggesting that Api5 undergoes complete post-translation degradation during the anaphase of cells (Figure 5.3A-B).

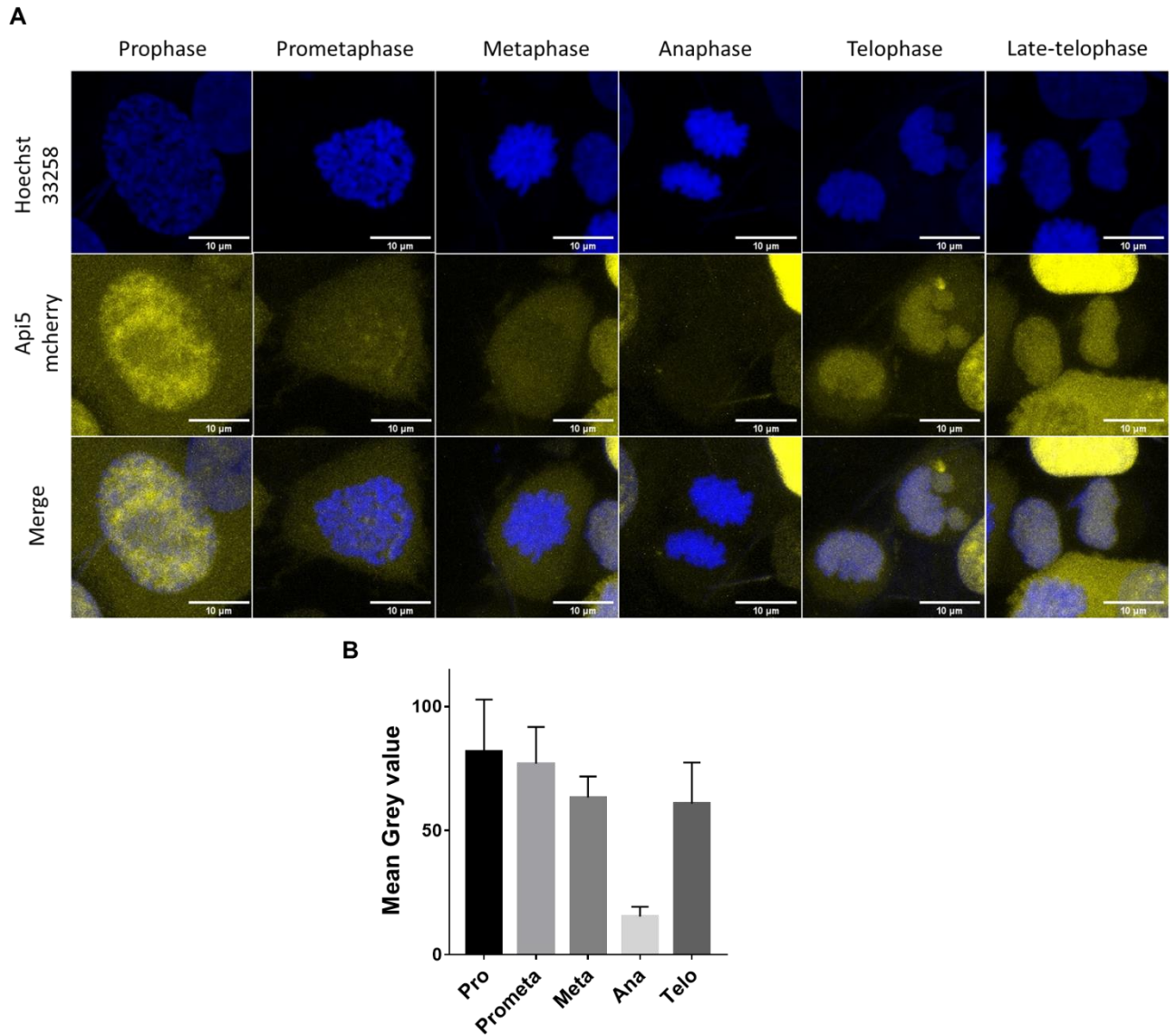


Figure 5.3: Api5 protein level decreases during sub-stages of mitosis

A) mCherry tagged Api5 over-expressing MCF7 cells were synchronised in G2-M phase using cdk1 inhibitor (10 μ M for 24 hrs) and then released for subsequent hrs to obtain the cells in different stages of mitosis. Cells were fixed, and mCherry fluorescence was imaged in Leica Sp8 confocal microscope at 63X with 2X zoom (scale bar- 10 μ m). Data is representative of three independent experiments.

5.2.2. Pathway of regulation of Api5 during cell cycle progression

To investigate whether Api5 undergo proteasomal pathway-mediated degradation in the G2-M phase, cells were arrested in the G2-M phase by nocodazole in the presence and absence of MG-132, and levels of Api5 were analysed by immunoblotting. The reduced levels of Api5 in G2-M were restored upon inhibition of the proteasomal pathway of degradation (Figure 5.4A-C). These results were further corroborated using an Immuno-fluorescence assay (Figure 5.5). This also proves that cell cycle-dependent degradation of Api5 is also through the proteasomal pathway.

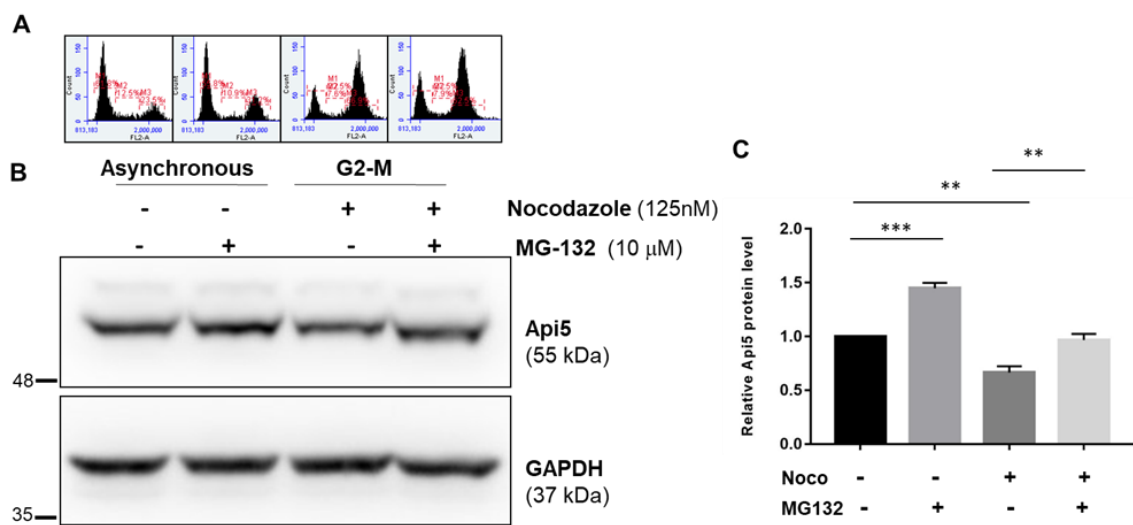


Figure 5.4: Cell cycle-dependent regulation of Api5 is through proteasomal pathway

MCF7 cells were treated with 125nM nocodazole for 16 hrs for arresting in the G2-M phase, and 10 μ M MG132 was added for 4 hrs before collection. A) Cells were fixed in ethanol and stained with propidium iodide, and cell cycle profile was monitored, or B) western blotting was performed to check the levels of Api5. GAPDH was used as a loading control for immunoblotting. C) The fold change of Api5 was quantified after normalising to GAPDH. Data is representative of three independent experiments and statistical analysis was performed using One way Anova; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

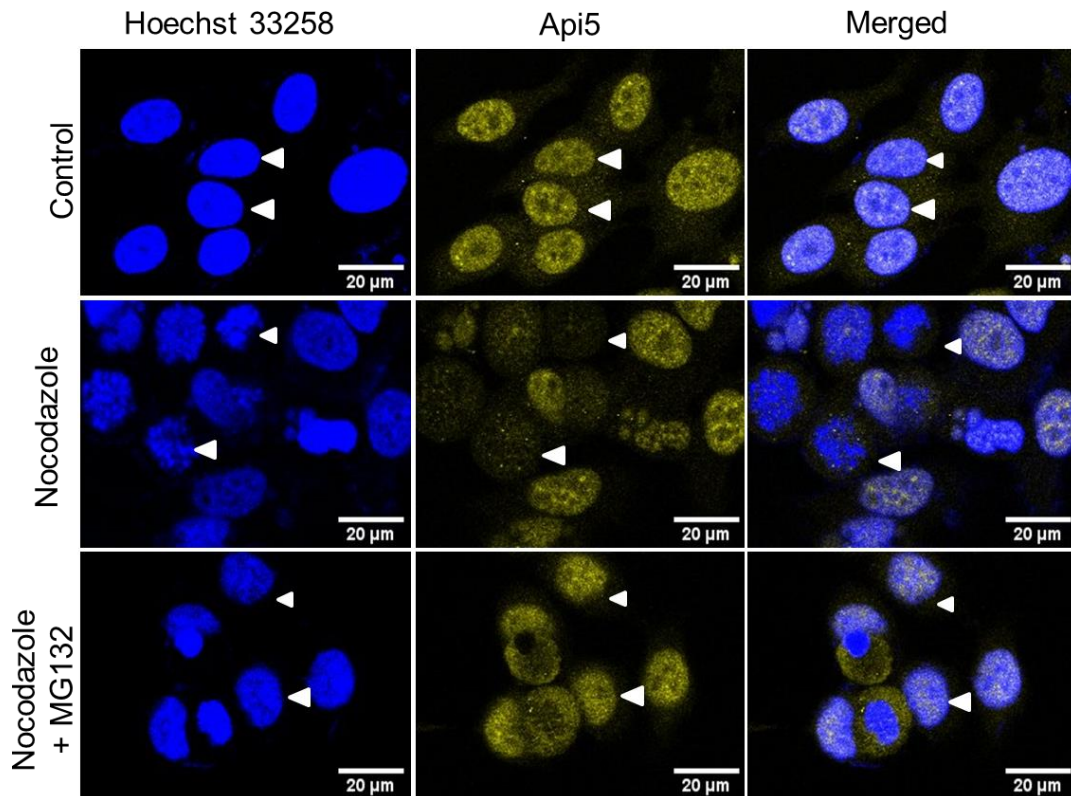


Figure 5.5: Cell cycle-dependent regulation of Api5 is through proteasomal pathway

MCF7 cells were seeded on coverslips and treated with 125nM nocodazole for 16 hrs for arresting in the G2-M phase and 10 μ M MG132 for 4 hrs before fixation. Immunofluorescence was performed using Api5 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

Ubiquitination is a marker for the proteins that undergo degradation through the proteasomal pathway (Swatek and Komander, 2016). As Api5 was found to be degraded through the proteasomal pathway, it was interested to find whether Api5 undergo ubiquitination. To illustrate whether Api5 undergoes ubiquitination before undergoing degradation via the proteasomal pathway in the G2-M phase of the cell cycle, Api5-mCherry cells were synchronised in the G2-M phase using nocodazole. These cells were lysed, and the whole-cell lysates were immune-precipitated using GFP-specific antibody. Western blotting was performed using a ubiquitin-specific antibody. The presence of a high molecular mass ladder in immune-precipitates of G2-M arrested cells confirmed that Api5 was undergoing poly-ubiquitination in the G2-M phase of the cell cycle (Figure 5.6).

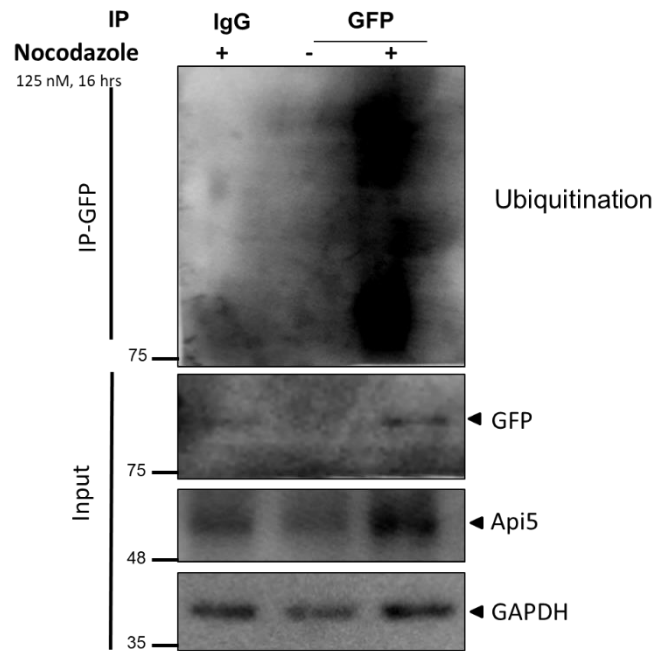


Figure 5.6: Api5 undergoes polyubiquitination in the G2/M phase of cell cycle mCherry-tagged Api5 over-expressing MCF7 cells treated with 125 nM nocodazole for 16 hrs and 10 μ M MG132 for 4 hrs were lysed, and immunoprecipitation was performed using GFP specific antibody. Western blotting analysis was performed to analyse the ubiquitination status of Api5 using ubiquitin-specific antibody

In chapter 4, we found that proteasomal degradation of Api5 in asynchronous cells is carried out by FBXW2, an SCF type E3 ubiquitin ligase. To find out whether G2-M phase mediated degradation of Api5 was also through FBXW2, Api5 levels were analyzed in FBXW2 knockdown stable cells after arresting in G2-M phase using nocodazole. It was observed that control cells showed a reduction in Api5 protein levels in the G2-M phase while Api5 levels did not reduce upon FBXW2 knockdown cells (Figure 5.7A-B), suggesting that Api5 degradation in the G2-M phase requires FBXW2 E3 ubiquitin ligase activity.

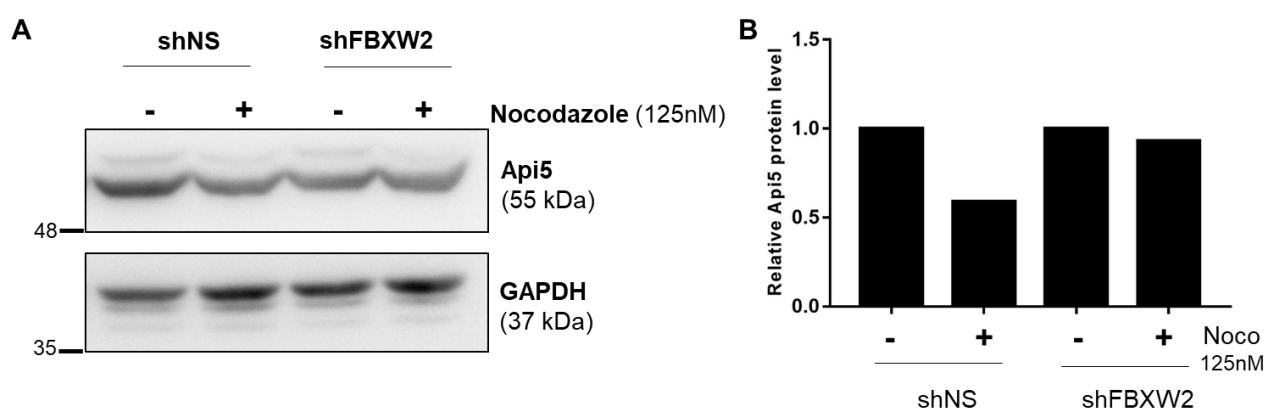


Figure 5.7: Cell cycle-dependent degradation of Api5 is by FBXW2

A) HeLa cells stably expressing shNS and shFBXW2#2 were lysed after 125nM nocodazole treatment for 16hrs, and immunoblotting was performed to analyse Api5 levels. B) Api5 levels were quantified after normalising with GAPDH. Data is representative of three independent experiments.

Like ubiquitination, phosphorylation is also a mandatory but secondary signal for proteasomal-mediated degradation (Wang et al., 2019). Proteins undergoing proteasomal degradation by ubiquitination need to be phosphorylated by certain kinases. As it has been shown that Api5 undergoes proteasomal degradation during cell cycle progression, it might be possible that Api5 is getting phosphorylated by some kinase that plays a pivotal role in cell cycle progression. As the levels of Api5 were low in the G2-M phase, the kinases whose functioning are known in G2-M progression were selected to understand their role in regulating levels of Api5. Cells were treated with small chemical inhibitors of different kinases like Polo-like kinases, Cdks, and Aurora Family kinases. It was observed that cells treated with cdk1, Polo-like kinase, or Aurora kinase A inhibitor did not show any change in Api5 levels (Figure 5.8A-C), while cells treated with Aurora kinase B inhibitor showed higher levels of Api5 in the asynchronous population of cells (Figure 5.9A-B). Henceforth Aurora kinase B was selected for further studies.

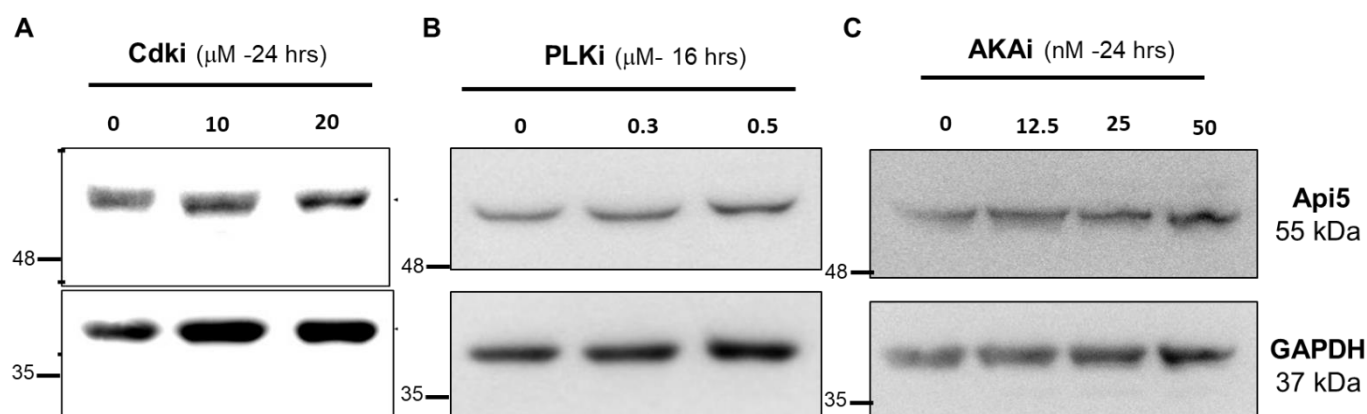


Figure 5.8: Api5 levels upon inhibition of Cdk, PLK, and Aurora Kinase A

MCF7 cells were treated with varying doses of A) Cdk for 24 hrs, B) Polo-like kinase inhibitor (PLKi) for 16 hrs, and C) Aurora kinase A inhibitor (AKAi) for 24 hrs. Immunoblotting was performed to analyse Api5 levels. GAPDH was used as a loading control. Data is representative of three independent experiments.

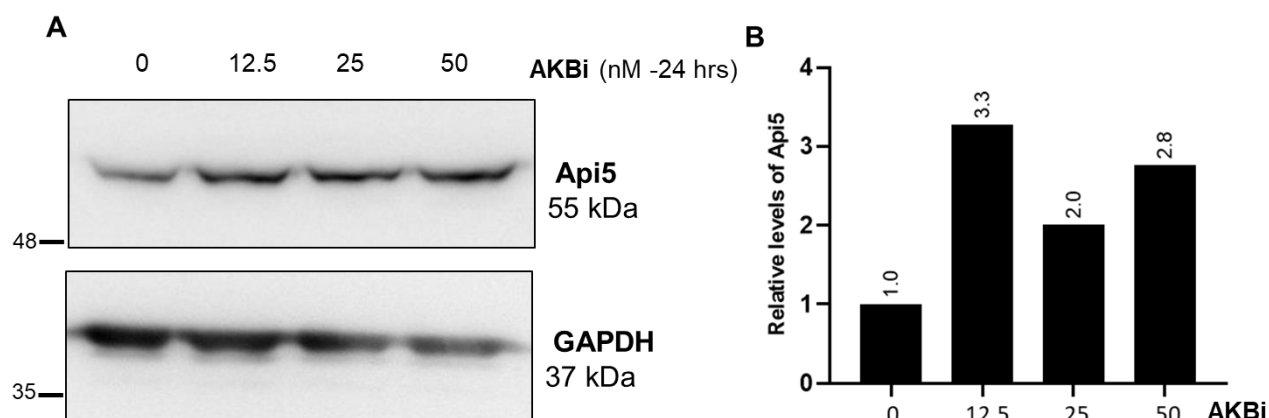


Figure 5.9: Api5 levels increase upon inhibition of Aurora Kinase B

A) MCF7 cells were treated with varying doses of Aurora kinase B inhibitor (AKBi) for 24 hrs. Immunoblotting was performed to analyse Api5 levels. B) Api5 levels were quantified after normalising with GAPDH. Data is representative of three independent experiments.

Next, we wanted to know whether aurora kinase B inhibition in the G2-M phase of the cell cycle affects levels of Api5. To prove that cells were treated with Nocodazole alone and in combination with Aurora kinase B inhibitor to arrest the cells in the G2-M phase of the cell cycle, and levels of Api5 were analysed. It was observed that cells treated nocodazole showed reduced levels of Api5 in the G2-M phase;

however, Api5 levels remain unaltered in G2-M cells where Aurora kinase B activity was inhibited (Figure 5.10A-C). These results were further corroborated using Immunofluorescence (Figure 5.11). These data prove that Aurora kinase B regulates G2-M mediated degradation of Api5.

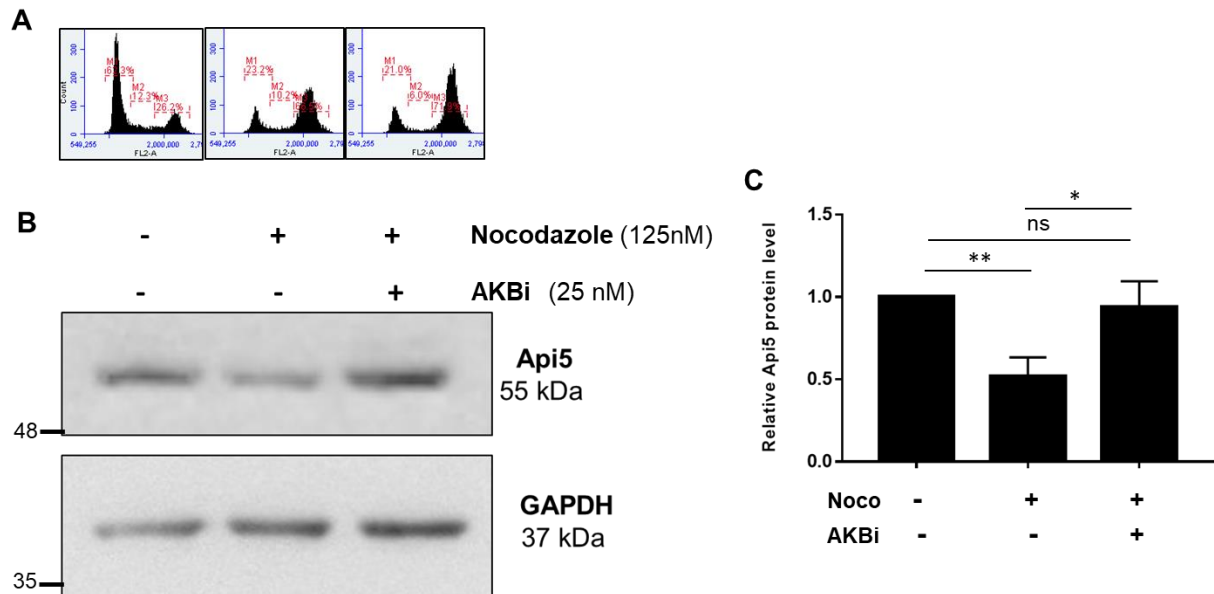


Figure 5.10: Api5 degradation in G2-M phase is through Aurora Kinase B

MCF7 cells were treated with 25 nM of Aurora kinase B inhibitor (AKBi) for 24 hrs and 125nM nocodazole for 16 hrs. A) Cells were fixed in ethanol and stained with propidium iodide, and cell cycle profile was monitored, or B) western blotting was performed to check the levels of Api5. C) The fold change of Api5 was quantified after normalising to GAPDH. Data is representative of three independent experiments and statistical analysis was performed using One way Anova; ns $p > 0.05$ * $p < 0.05$, ** $p < 0.01$.

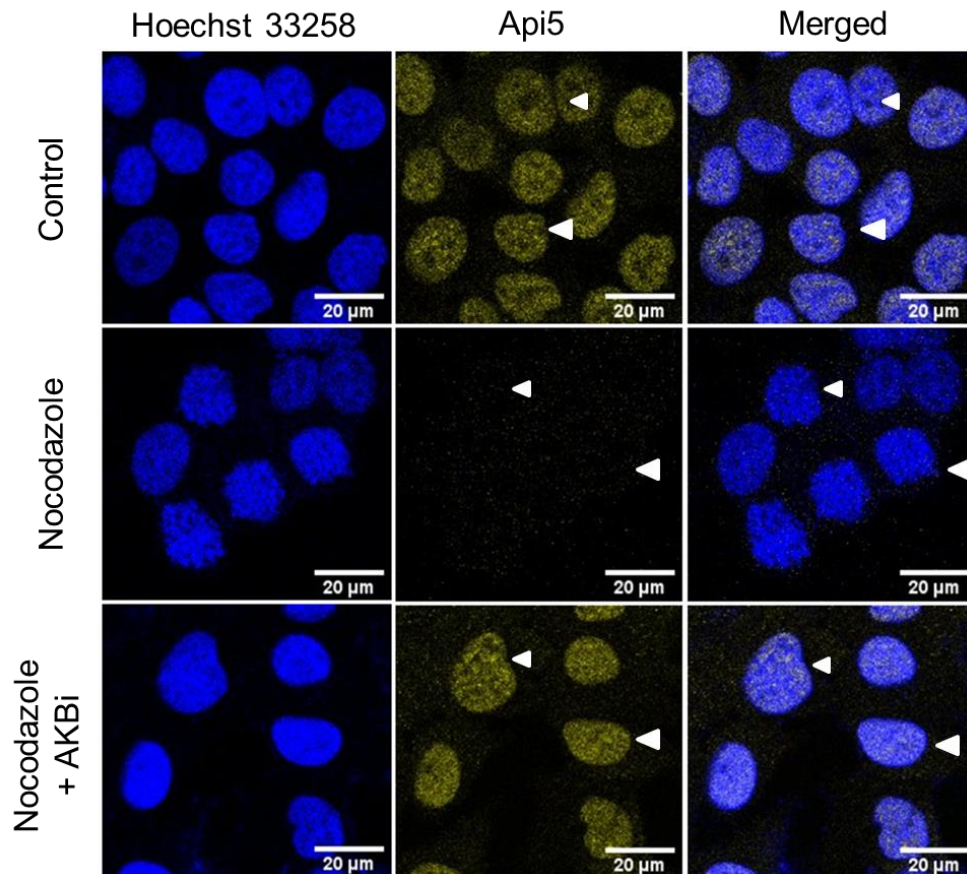


Figure 5.11: Api5 degradation in G2-M phase is through Aurora Kinase B

MCF7 cells seeded on coverslips were treated with 25 nM of Aurora kinase B inhibitor (AKBi) for 24 hrs and 125nM nocodazole for 16 hrs. Immunofluorescence was performed using Api5 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μm). Data is representative of three independent experiments.

In order to regulate and phosphorylate substrate proteins, the kinase must interact with its substrate. To identify the interaction between Api5 and Aurora kinase B, G2-M arrested mCherry-Api5 cells were immune-precipitated using GFP specific antibody, and western blotting was performed using Aurora Kinase B antibody. The presence of Aurora kinase B in GFP pull-down lysates confirmed that Aurora kinase B interacts with Api5 (Figure 5.12A). This interaction was further confirmed by reverse immunoprecipitation, where immunoprecipitation was carried out using Aurora kinase B antibody, and western blotting was performed using GFP specific antibody. The presence of Api5 in AKB pull-downs confirmed the interaction of Api5 with Aurora kinase B in the G2-M phase of the cell cycle (Figure 5.12B). This overall

suggested that Aurora kinase B might phosphorylate and regulate Api5 levels during the progression of the cell cycle.

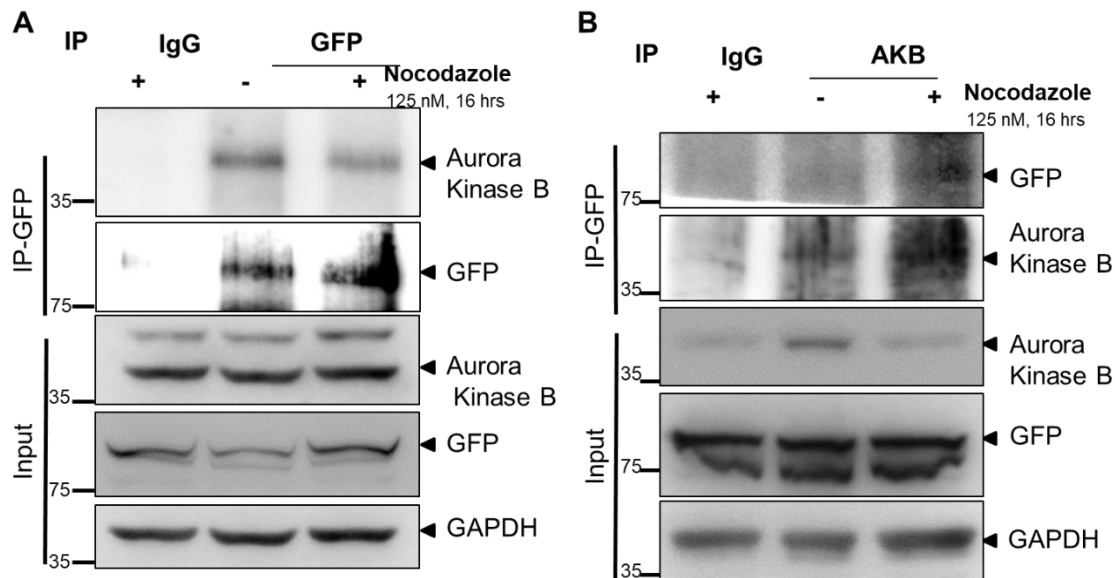


Figure 5.12: Aurora Kinase B interacts with Api5

mCherry-tagged Api5 over-expressing MCF7 cells treated with 125nM nocodazole for 16hrs were lysed, and immunoprecipitation was performed using A) GFP and B) Aurora kinase B (AKB) specific antibody. Western blotting analysis was performed to analyse the presence of ATR and Api5, respectively.

5.2.3. Regulators of Api5 during G2-M phase of cell cycle

In chapter 3, it was observed that p300, a histone acetyltransferase enzyme, catalyzes the acetylation of p300 while HDAC1 de-acetylates Api5. In an asynchronous population, p300 mediated acetylation led to the stabilization while HDAC1 mediated de-acetylation led to the degradation of Api5. Thus, it was interested to observe the levels of these two critical regulators of Api5 during the G2-M phase of the cell cycle. Cells were arrested in the G2-M phase by nocodazole, and protein levels of p300 and HDAC1 were analysed. It was observed levels of p300 were reduced while HDAC1 were unaffected in the G2-M phase of the cell cycle (Figure 5.13A-C). As p300 was discovered to acetylate and stabilize Api5, the reduction of Api5 upon G2-M could partly be because of the reduction in p300.

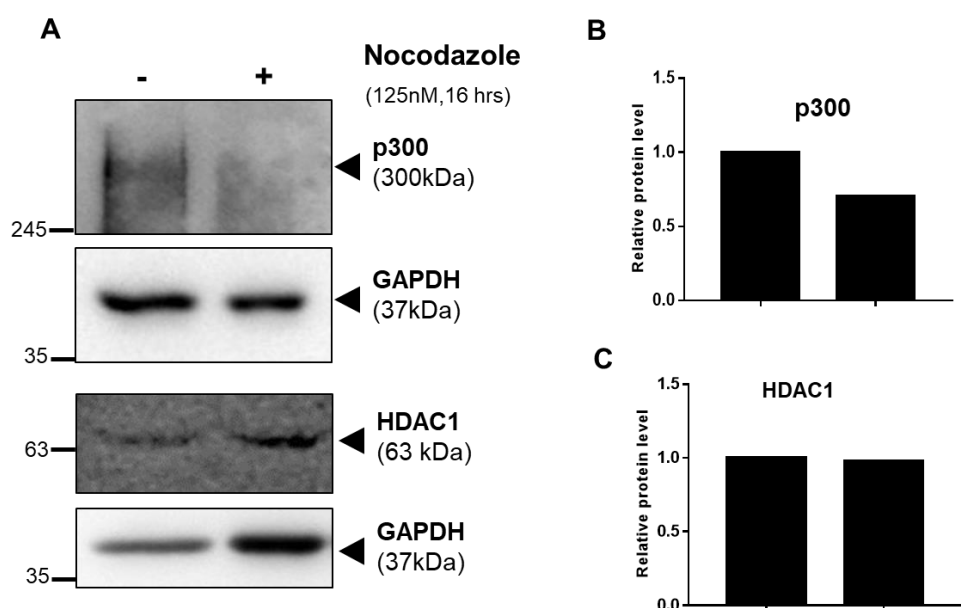


Figure 5.13: Regulators of Api5 in G2-M phase of cell cycle

A) MCF7 cells were treated 125nM nocodazole for 16 hrs, and western blotting was performed to check the levels of p300 and HDAC1. The fold change of B) p300 and C) HDAC1 was quantified after normalising to GAPDH. Data is representative of three independent experiments.

5.2.4. Acetylation of Api5 is critical for normal progression of the cell cycle

The study by Garcia and the group has shown that Api5 protein undergoes degradation in the G2/M phase of the cell cycle while the transcript levels remain unaltered. Our recent data and study from Han's group showed that acetylation is responsible for maintaining the stability of Api5. It was aimed to find whether the Api5 acetylation affects the cell cycle profile. To prove this, mVenus-tagged K251 mutants of Api5 (K251A: charge deficient mutant, K251R: charge mimic mutant and K251Q: constitutive acetylation mimic mutant) were ectopically expressed along with wild-type Api5 in Api5 knockdown background, and cell cycle profile was analysed. Interestingly it was observed that cells transfected with K251R, which is a charge mimic mutant of Api5, were found to be arrested at the S phase of the cell cycle (Figure 5.14A). PCNA, an S phase cell cycle marker, was also found to be upregulated in K251R transfected cells while other phase cell cycle markers were not affected, which again confirmed the S phase arrest of the cell cycle (Figure 5.14B-F). This result was further corroborated with the observation that the Api5-K251R mutant expressing cells showed higher proliferation when compared to the

other Api5 acetylation mutants (Figure 5.14G). This increase in proliferation may be due to an alteration in the cell cycle functioning resulting from the change in the acetylation status of Api5. Therefore it was confirmed that the acetylation status of Api5 also regulated proliferation and cell cycle progression.

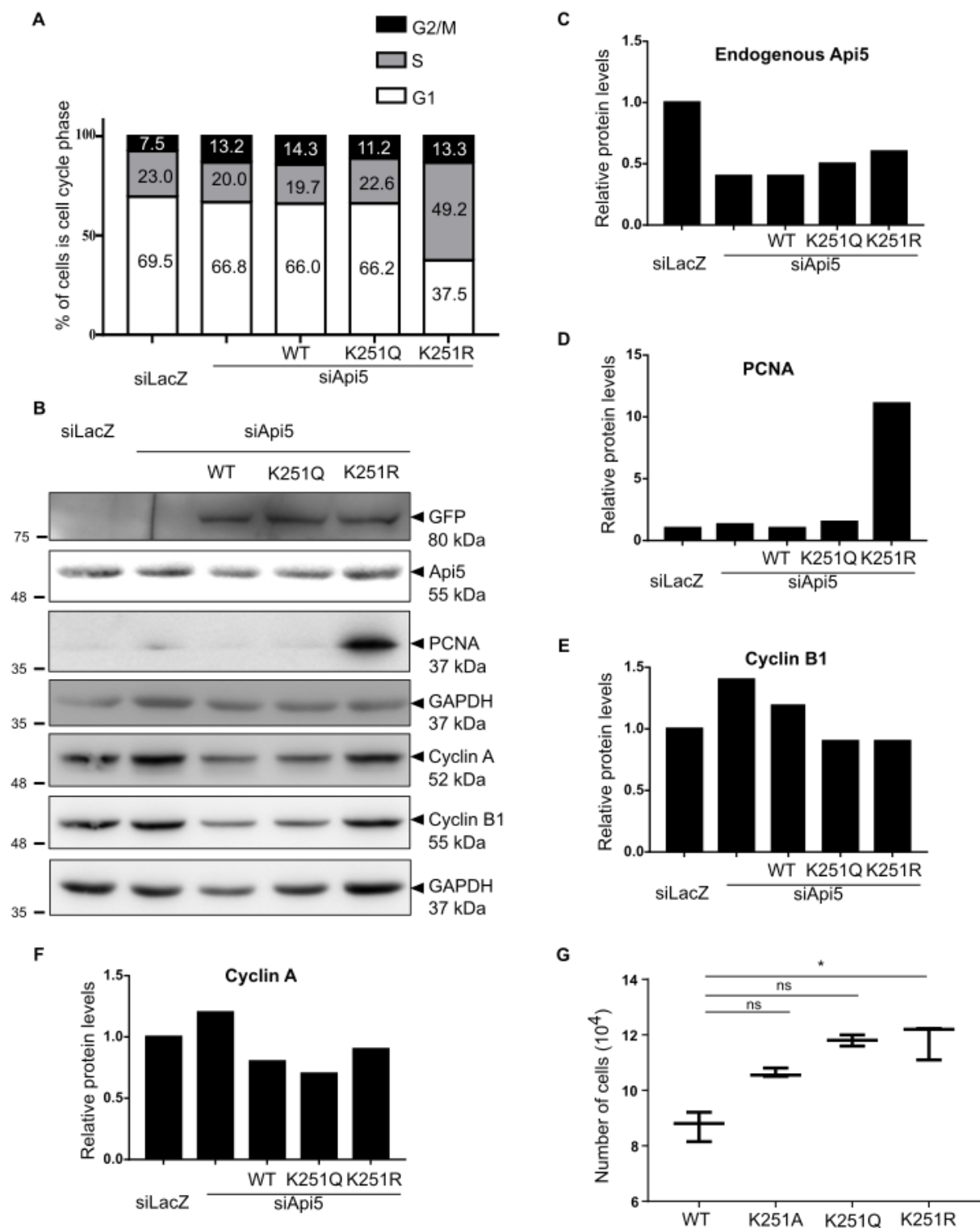


Figure 5.14: Acetylation status of Api5 affects cell cycle

MCF7 cells transfected with siRNA-resistant Api5-K251 mutants 24 hrs post Api5 knockdown. Cells were fixed in ethanol and stained with propidium iodide, and cell cycle profile was monitored. a) Percentage of cells in the different cell cycle phases were analysed in a flow cytometer and plotted as box plots. b) Western blotting was performed to check the levels of b) Api5, PCNA, Cyclin A, and Cyclin B1 and c-f) quantified after normalising to the loading control GAPDH. g) MCF7 cells transfected with mVenus-tagged Api5 WT and K251 mutants were counted using Sceptar™ to check for cell proliferation. Data is representative of three independent experiments.

To establish the role of acetylation on the stability of Api5 during the G2-M phase of the cell cycle, cells were transfected with K251 mutants of Api5, and the levels of these proteins were analysed in the G2-M phase using western blotting. Wild Type Api5 levels were reduced. However, there was a slight upregulation in the K251R while other mutants did not show any difference in their protein expression in the G2-M phase of the cell cycle, suggesting that dynamics of acetylation plays a significant role in the regulation of the stability of Api5 (Figure 5.15A-B). Next, it was decided to analyse the levels of cyclins upon expression of different mutants of Api5 in the G2-M phase of the cell cycle. It was observed that wild type Api5 transfected cells showed higher levels of cyclin A levels similar to K251Q and K251R cells, suggesting that wild type, K251Q, and K251R regulate the S phase of cells as cyclin A is an S phase cyclin (Figure 5.16 A-B). This result was similar to previous results that were showing that K251R mutant cells show a maximum number of cells in the S phase of the cell cycle. Wild type and K251A cells showed maintained levels of cyclin D, suggesting more number of cells in the G1-S boundary as cyclin D is a G1-S transition marker (Figure 5.16 A and C). Wild type, K251A, and K251R cells showed an increase in the levels of cyclin B1, that is, G2-M phase cyclin (Figure 5.16 A and D). Wild type and K251Q cells showed a reduction in the levels of cyclin E, that is, G1 phase cyclin (Figure 5.16 A and E). PCNA that is an S phase marker, was found to be upregulated in wild-type cells (Figure 5.16 A and F). This experiment suggested that acetylation at K251 regulates the cell cycle differentially by regulating the protein expression of cyclins, as the different mutants. Api5 showed differential expression in almost all the cyclins in the G2-M phase of the cell cycle.

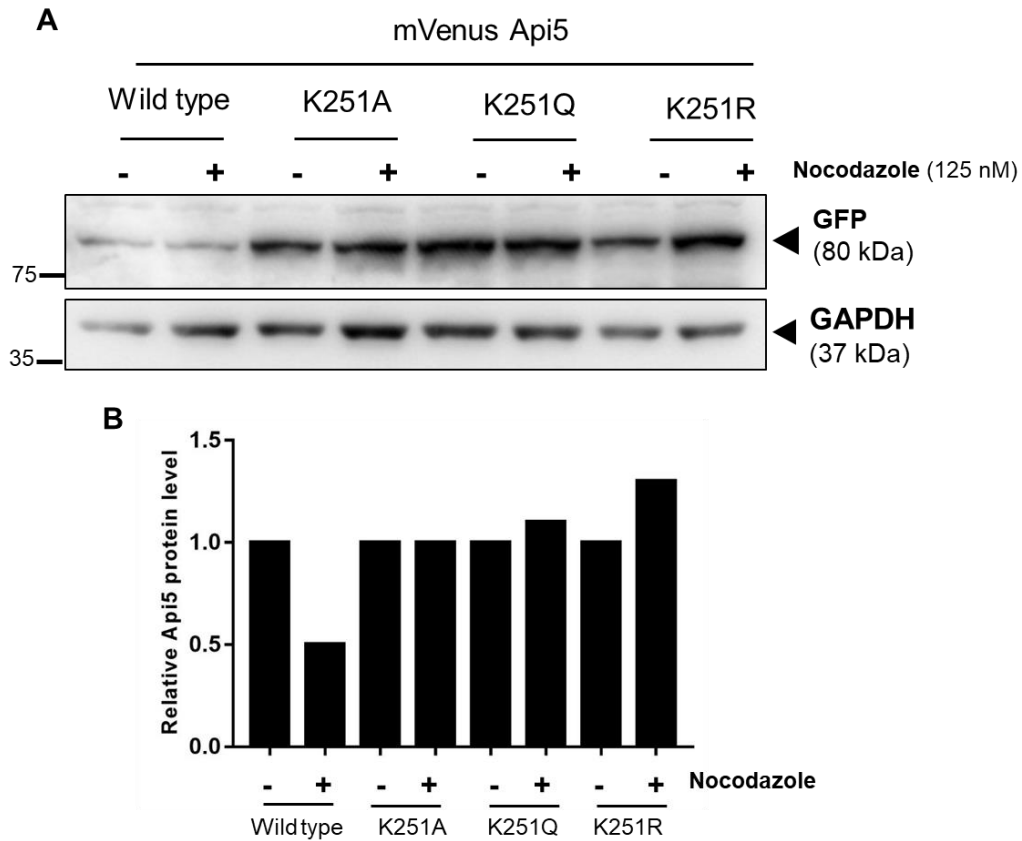


Figure 5.15: Acetylation status affects the degradation of Api5 in the G2-M phase

MCF7 cells were transfected with mVenus tagged K251 mutant constructs and treated with 125nM of nocodazole for 16hrs post 24 hrs of transfection. Western blotting was performed to check for the levels of A) GFP (mVenus Api5) and B) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

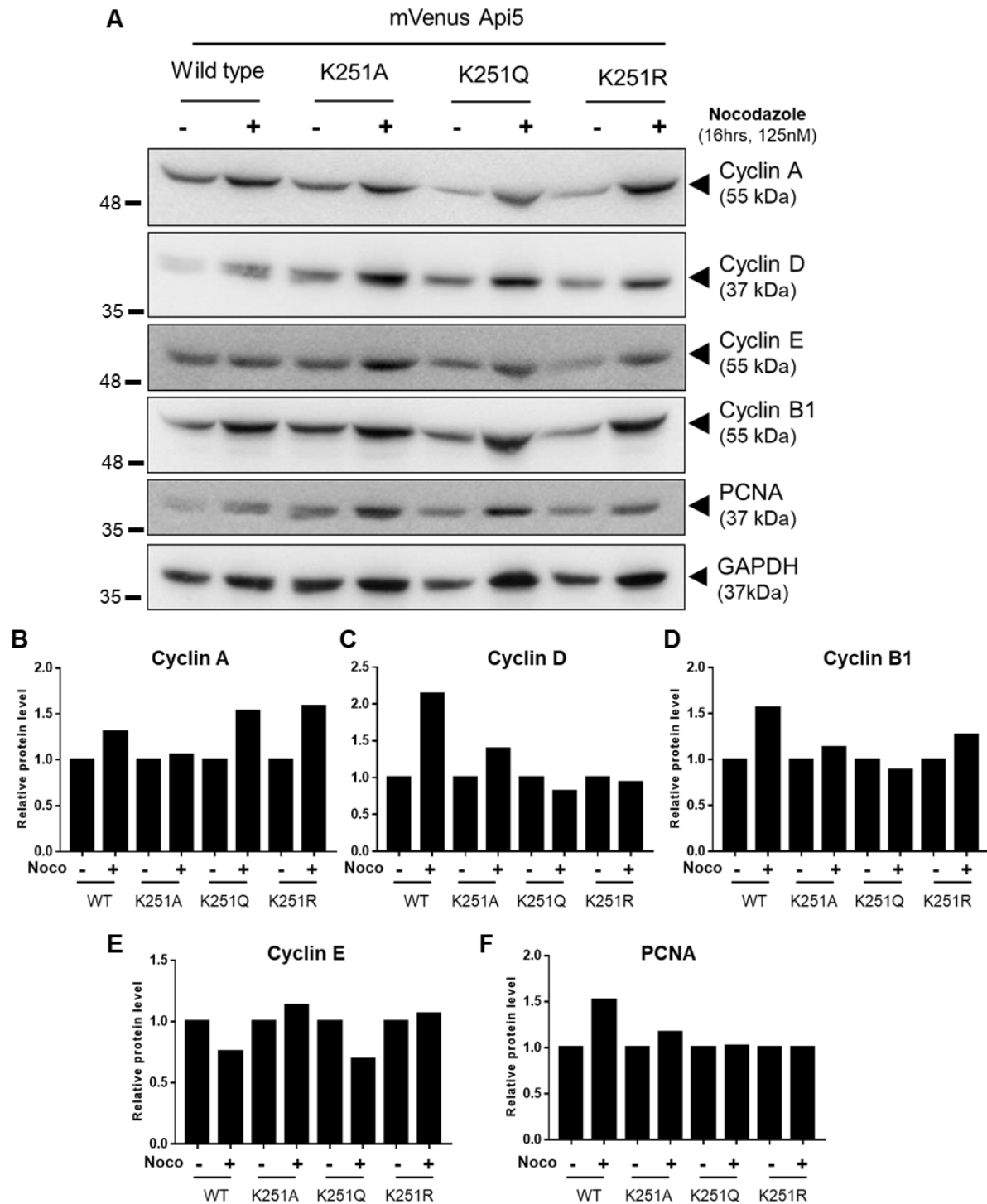


Figure 5.16: Acetylation status of Api5 affects cell cycle

MCF7 cells were transfected with mVenus tagged K251 mutant constructs and treated 125nM of nocodazole for 16 hrs post 24 hrs of transfection and lysed. Western blotting was performed to check for the levels of A) Cyclin A, Cyclin D, Cyclin B1, Cyclin E, and PCNA and B-F) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

5.2.5 Knockdown of Api5 inhibits proliferation of cells

It was observed that Api5 protein expression fluctuates during the cell cycle. Levels of Api5 were at their peak during the G1 and S phases of the cell cycle. To illustrate the function of Api5, the proliferation of cells was analysed upon its knockdown. To demonstrate whether Api5 affected cell proliferation, cells stably expressing shRNA against Api5 were seeded equally, and their proliferation was measured in normal physiological and serum starvation conditions. It was observed that the proliferation of cells was reduced upon knockdown of Api5 in both conditions (Figure 5.17A).

To further understand the role of Api5 in cellular proliferation, colony formation assay was performed with the cells expressing stable Api5 knockdown. An equal number of cells were sparsely seeded, and crystal violet staining was done after 7 days of seeding to measure the proliferation of cells. It was observed that Api5 knockdown led to a decrease in colony numbers (Figure 5.17B-C).

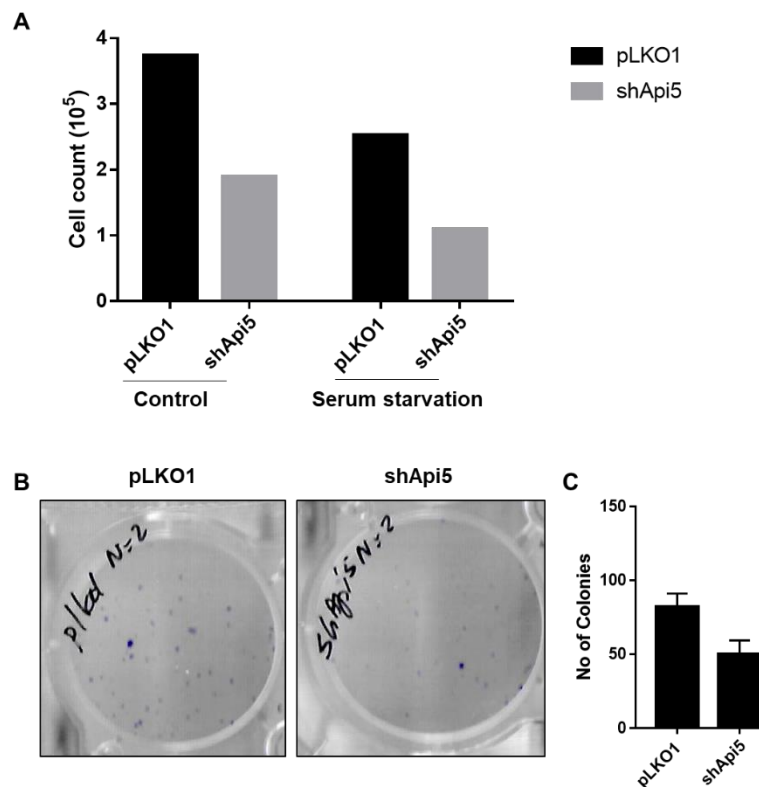


Figure 5.17: Knockdown of Api5 reduces proliferation of cells

A) 1×10^5 MCF7 cells stably expressing pLKO1 or shApi5 were seeded on 35 mm dishes. 16 hrs post-seeding complete media was replaced with serum-free media for serum starvation. Cells were trypsinised and counted using Sceptar™ to check for

cell proliferation post 48 hrs of serum starvation. B) 1000 MCF7 cells stably expressing pLKO1 or shApi5 were seeded on a 35 mm dish, and colony-forming ability was monitored after 7 days by staining with crystal violet. C) Number of colonies were counted manually. Data is representative of three independent experiments.

These results showing a reduction in proliferation of Api5 knockdown cells were further corroborated by using BrdU assay. For that, pLKO1 and shApi5 cells were seeded and incubated overnight at 37⁰ C. BrdU was added for 6 hrs, and immunofluorescence was performed using the BrdU specific antibody. It was observed that the percentage of BrdU positive cells was reduced upon knockdown of Api5 (Figure 5.18). These results overall suggested that knockdown of Api5 inhibits the proliferation of cells.

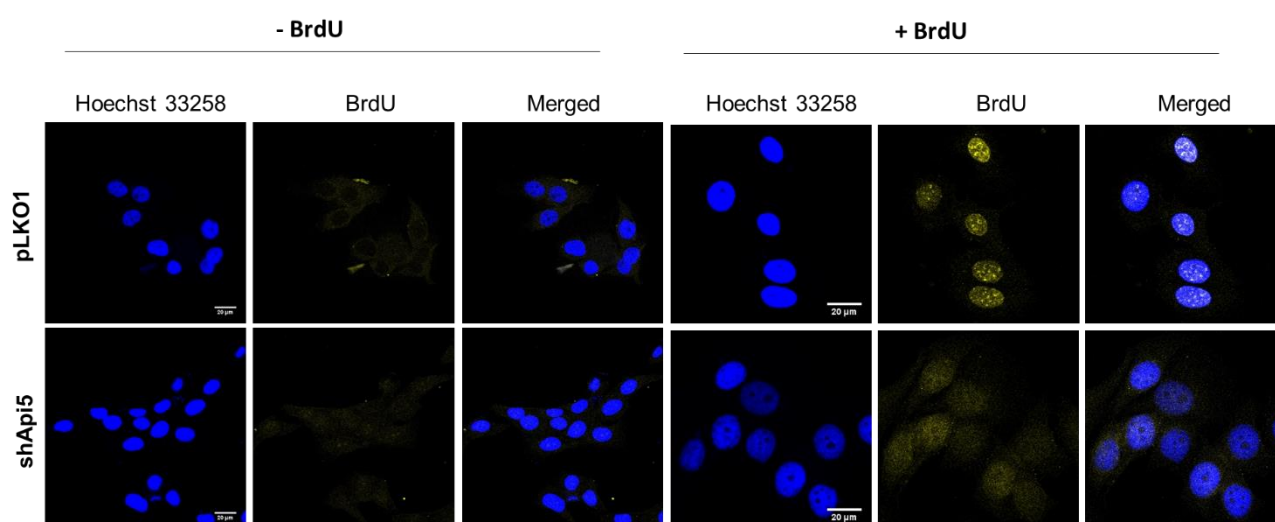


Figure 5.18: Knockdown of Api5 reduces proliferation of cells

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslips. 16 hrs post-seeding BrdU was added for 6hrs, and immunostaining was performed using BrdU specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μm). Data is representative of three independent experiments.

To demonstrate whether Api5 acetylation can affect cell proliferation, cells stably expressing the K251 mutants of Api5 in an Api5 knock-down background were seeded equally, and their proliferation was measured in physiological conditions. It was observed that proliferation of cells was reduced upon knockdown of Api5, and expression of wild type of Api5 rescued the proliferation of shApi5 cells (Figure 5.19).

Similarly, the expression of K251Q and K251A individually led to the increase in proliferation of Api5 knockdown cells. Interestingly it was also observed that expression of K251A mutant of Api5 showed maximum proliferation of cells. Similar experiment when carried out in serum-starved conditions, it was observed that knockdown of Api5 led to a reduction in proliferation which was restored upon expression of wild type of Api5, but the expression of K251Q and K251A could not restore the proliferation upon serum starvation (Figure 5.19). It suggested that Api5 and its acetylation at K251 play a significant role in the proliferation of cells.

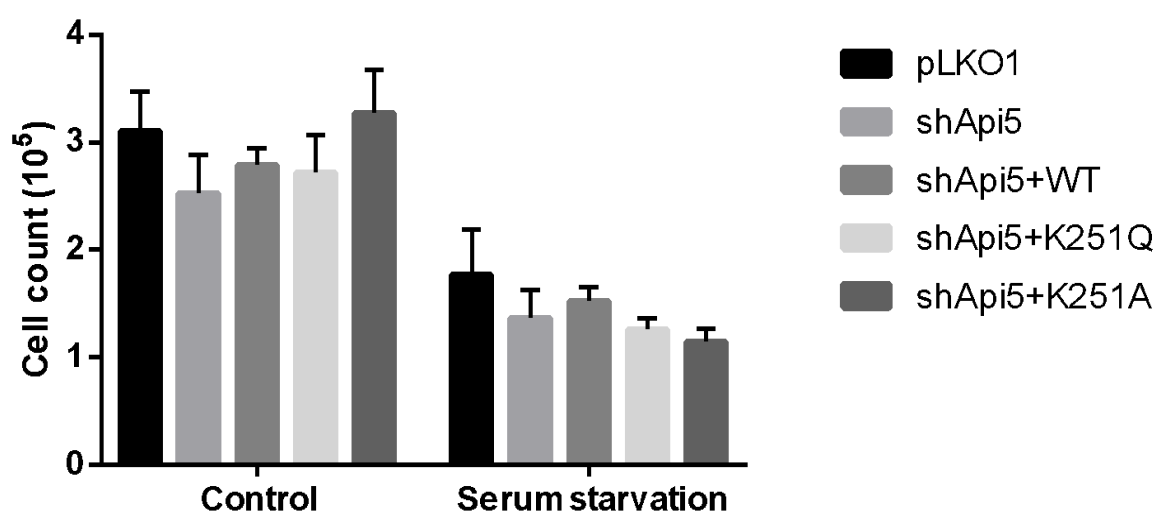


Figure 5.19: Role of Api5 K251 acetylation in the proliferation of cells

A) 1×10^5 MCF7 cells stably expressing pLKO1 or shApi5 or with Api5 K251 mutants were seeded on a 35 mm dish. 16 hrs post-seeding complete media was replaced with serum-free media for serum starvation. Cells were trypsinised and counted using Sceptar™ to check for cell proliferation post 48 hrs of serum starvation. Data is representative of three independent experiments.

To further understand the role of Api5 and its acetylation at K251 in cell proliferation, colony formation assay was performed with the cells expressing K251 mutants of Api5 in an Api5 knock-down background. An equal number of cells were seeded, and crystal violet staining was done after 7 days of seeding to measure the proliferation of cells. It was observed that Api5 knockdown led to a decrease in colony numbers, and these reductions in colony number were rescued with the expression of wild type Api5 (Figure 5.20A-B). The expression of K251A and K251Q independently also led to the increase in colony numbers of Api5 knock-down cells.

This experiment also confirmed that the K251A mutant of Api5 had more impact on the proliferation of cells as the cells expressing the K251 mutant of Api5 showed a maximum number of colonies (Figure 5.20A-B). This confirmed that Api5 and its acetylation regulate the proliferation of cells during physiological conditions.

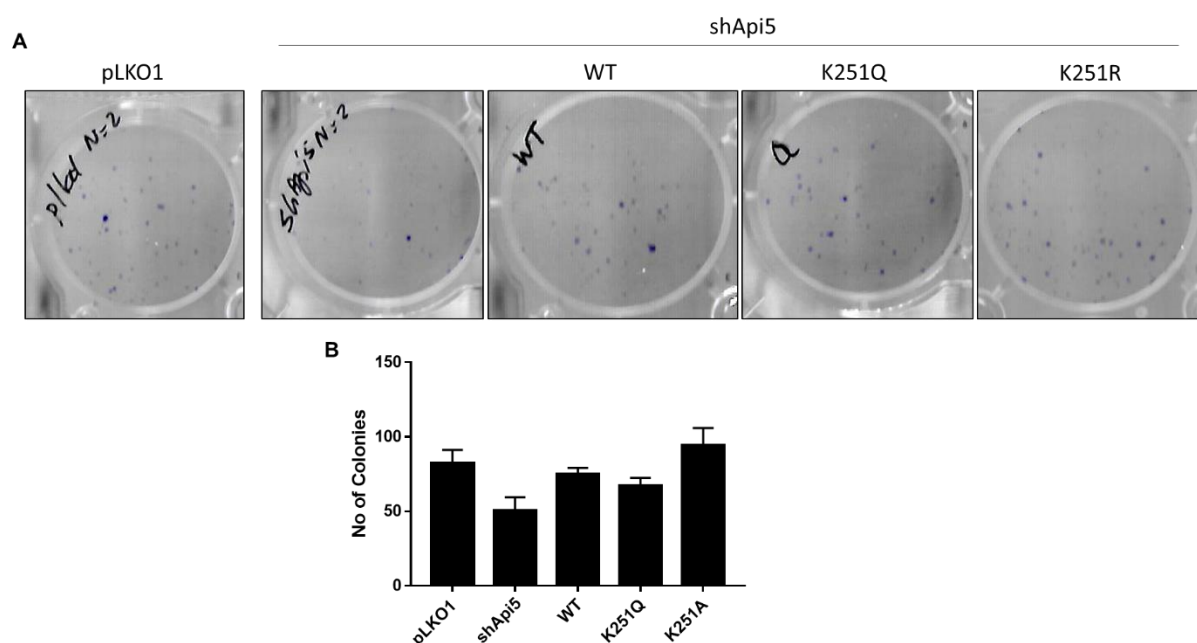


Figure 5.20: Role of Api5 K251 acetylation in the proliferation of cells

A) 1000 MCF7 cells stably expressing pLKO1 or shApi5 or Api5 K251 mutants in shApi5 background were seeded on 35 mm dish, and colonies formed were monitored after 7 days by staining with crystal violet. B) Number of colonies were counted manually. Data is representative of three independent experiments.

5.2.6 Knockdown of Api5 makes cells susceptible to undergo senescence

Reduction in proliferation observed upon knockdown of Api5 also directed our idea towards senescence as the known down of Api5 was showing a change in morphology of the cells. As compared to the control cells, Api5 knockdown cells were showing some stressed phenotype. It has been reported that during the onset of senescence, cells prefer to inhibit the ongoing cell cycle progression and enter in the G0 phase (Braumuller et al., 2013). To determine the role of Api5 in senescence, the cell cycle profile of control as well as shApi5 cells after serum starvation was analysed using FACS after staining with propidium iodide. It was observed that shApi5 cells showed a significant increase in the population of cells at

G0 compared to the control cells (Figure 5.21) upon serum starvation. This suggested that Api5 knockdown could direct the cells towards senescence. To further prove the role of Api5 in senescence, protein levels of p53 and p21 reported as senescence markers were analysed, and it was observed that upon knockdown of Api5, levels of p53 and p21 were increased (Figure 5.22A-D). It was observed that knockdown of Api5 could trigger the phenomenon of senescence, thus confirmed that Api5 plays a pivotal role in the regulation of senescence.

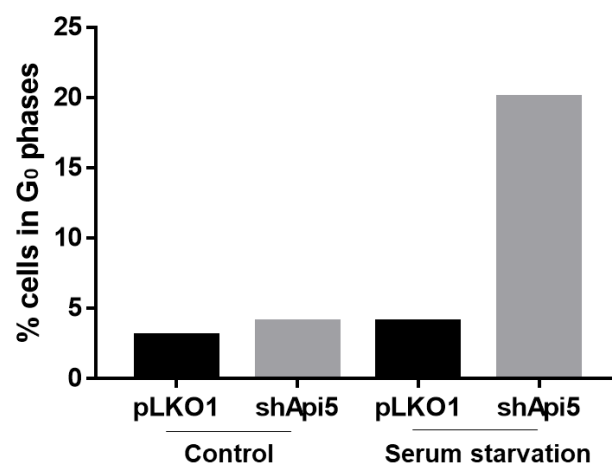


Figure 5.21: Api5 knockdown leads to increase in G0 population of cells upon serum starvation

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on a 35 mm dish. 16 hrs post-seeding complete media was replaced with serum-free media for serum starvation. Cells were fixed and stained using propidium iodide post 48 hrs of serum starvation, and the cell cycle profile was monitored using FACS. Data is representative of three independent experiments.

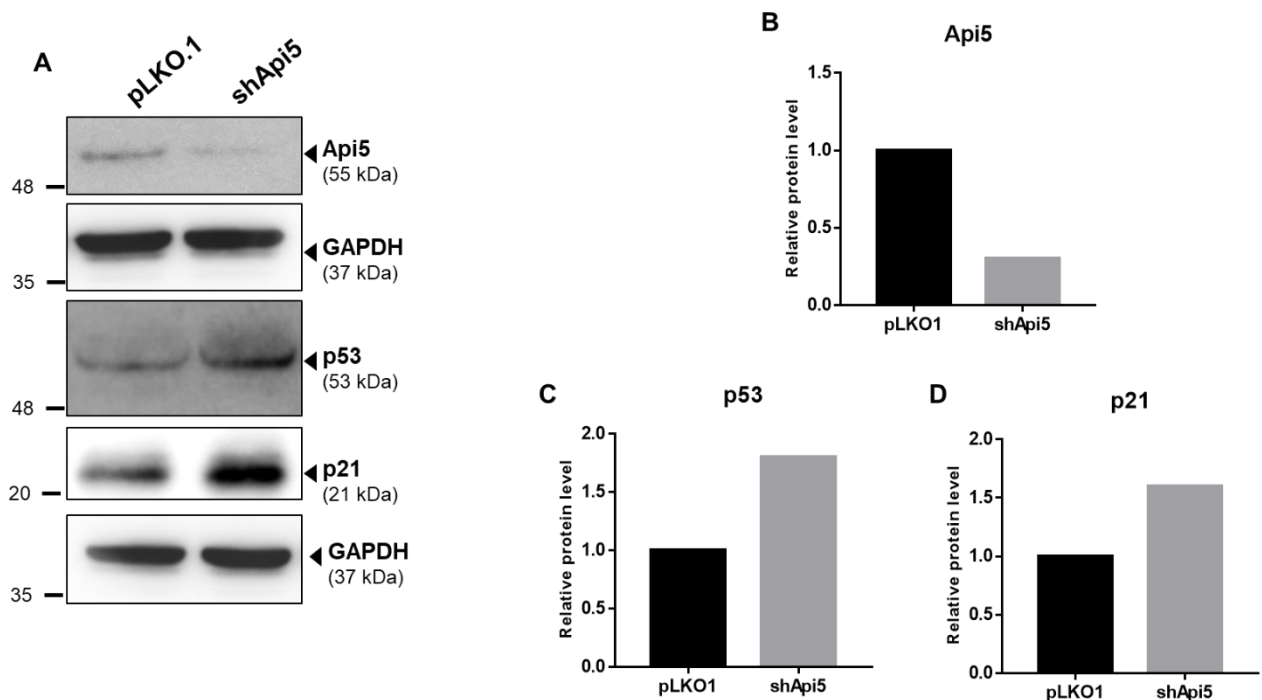


Figure 5.22: Knockdown of Api5 induces cells to undergo senescence

A) MCF7 cells stably expressing pLKO1 or shApi5 were lysed, and immunoblotting was performed to analyse the levels of Api5, p53, and p21 and B-D) quantified after normalising to GAPDH. Data is representative of three independent experiments.

5.2.7 Api5 knockdown may induce genomic instability

It was confirmed from the previous results that depletion in the endogenous levels of Api5 caused reduced proliferation as well as induction of senescence. These phenotypes like reduced proliferation and senescence are generally an outcome of the accumulation of genomic instabilities. Thus it was aimed to understand the status of genomic instabilities in Api5 depleted cells. To understand the molecular mechanism of reduction in cell proliferation, knock-down cells were screened for the presence of RPA32 activation, BRCA1, and Rad51, the markers for genomic instability. It was observed that knockdown of Api5 led to an increase in the activation of RPA 32 levels that is a DNA damage sensing agent and bound to the single-stranded breaks (Figure 5.23). This suggested that Api5 knockdown in cells led to the accumulation of genomic instabilities. Interestingly it was also observed that Rad51 and BRCA1 proteins which are involved in DNA damage repair in S and G2 phase, were reduced upon knockdown of Api5 (Figure 5.24 and 5.25). In Api5 depleted cells, BRCA1 and Rad 51 were not able to form foci as efficiently as control

cells. This suggested that reduction in proliferation and induction of senescence of Api5 knockdown cells could be an outcome of the accumulation of genomic instabilities as cells were failing in repairing the damaged DNA.

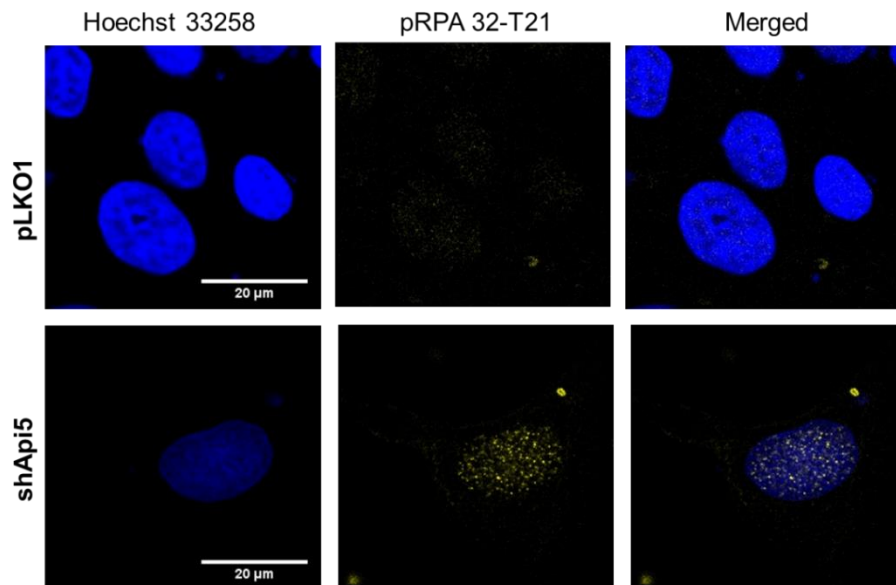


Figure 5.23: Api5 knockdown leads to RPA activation

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip, and immunostaining was performed using pRPA32-T21 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μm). Data is representative of three independent experiments.

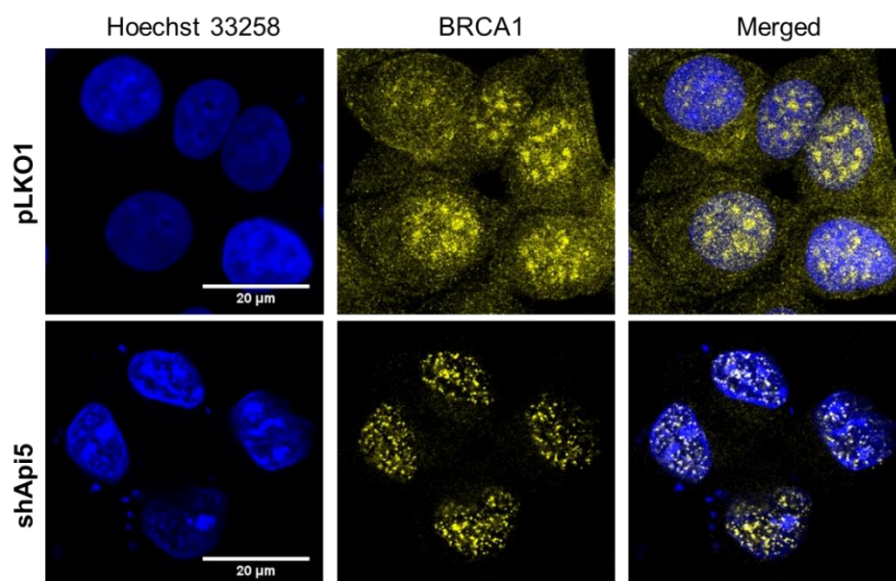


Figure 5.24: BRCA1 levels decrease upon Api5 knockdown

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslip, and immunostaining was performed using BRCA1 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

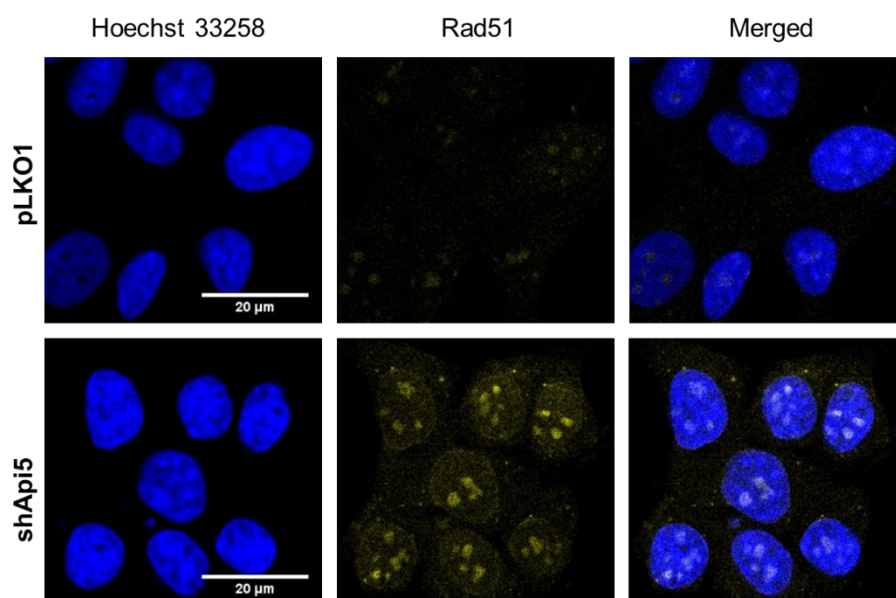


Figure 5.25: Rad51 levels increase upon Api5 knockdown

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslip, and immunostaining was performed using Rad51 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

5.2.8. Reduction of Api5 levels caused nuclear architecture defects

In the previous experiment, it was proved that Api5 depletion caused the accumulation of DNA damage and senescence. Multiple studies suggest the correlation between DNA damage, senescence, and defects in nuclear architecture. The nucleus is a membranous organelle that is composed of two plasma membranes, i.e. outer plasma membrane and inner plasma membrane. Beneath the inner plasma membrane, there is a crucial layer that is called the nuclear lamina. The nuclear lamina is a protective layer that protects the chromatin component of the cells (Stephens et al., 2019). The nuclear lamina is composed of different lamin proteins like Lamin A, B, and C. To understand the role of Api5 on nuclear architecture, an immunofluorescence assay was performed using Lamin A and Lamin B1 antibodies. It was observed knockdown of Api5 resulted in a differential pattern of lamins. Lamin A was found to be increased upon the knockdown of Api5, while Lamin B1 was showing loss from the nuclear lamina (Figure 5.26 and 5.27). To confirm the differential expression of these Lamin proteins in Api5 depleted cells, immunoblotting was performed. Lamin A/C levels were observed to be upregulated, while Lamin B1 showed reduction upon the knockdown of Api5 (Figure 5.28A-D). This overall suggested that Api5 helps in maintaining the nuclear lamina.

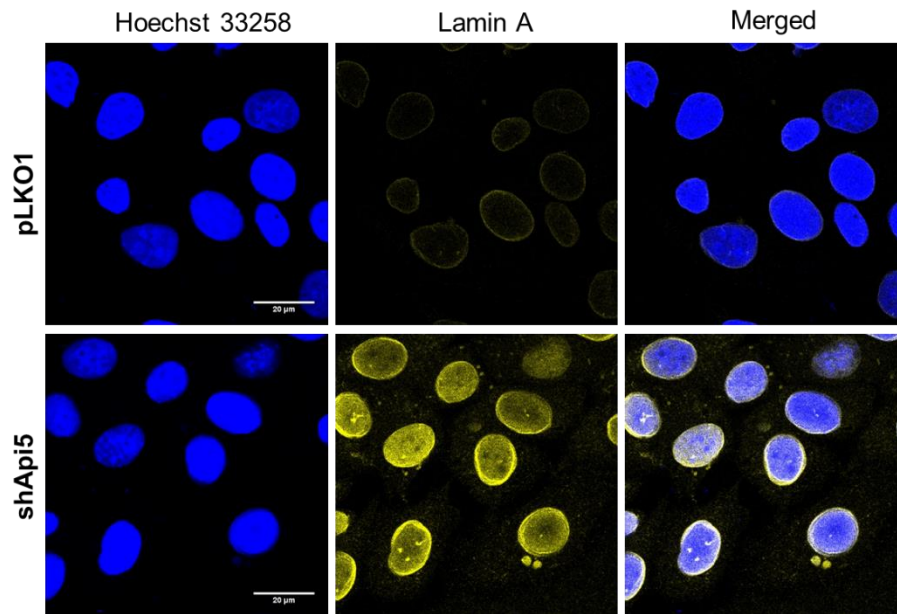


Figure 5.26: Lamin A levels increases upon Api5 knockdown

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslip, and immunostaining was performed using Lamin A specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

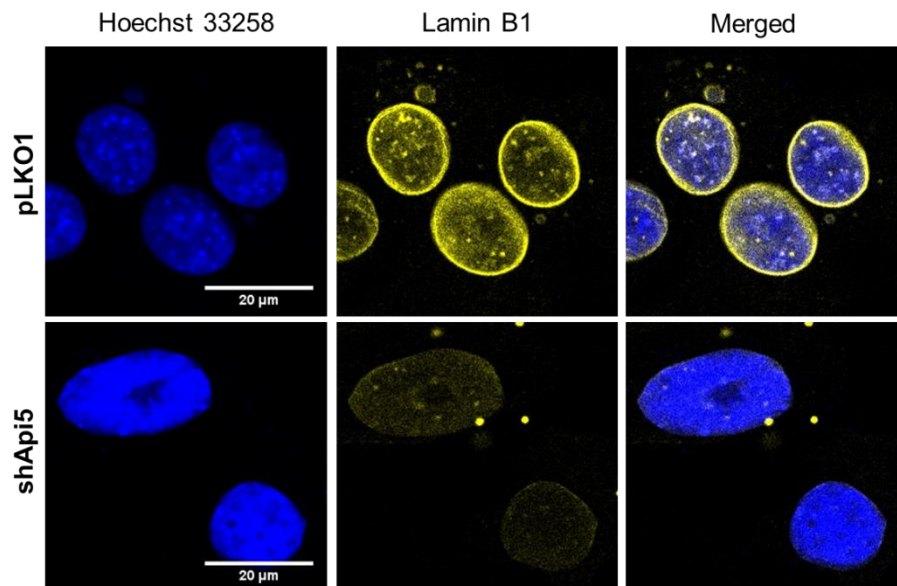


Figure 5.27: Lamin B levels decrease upon Api5 knockdown

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslip, and immunostaining was performed using Lamin B1 specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

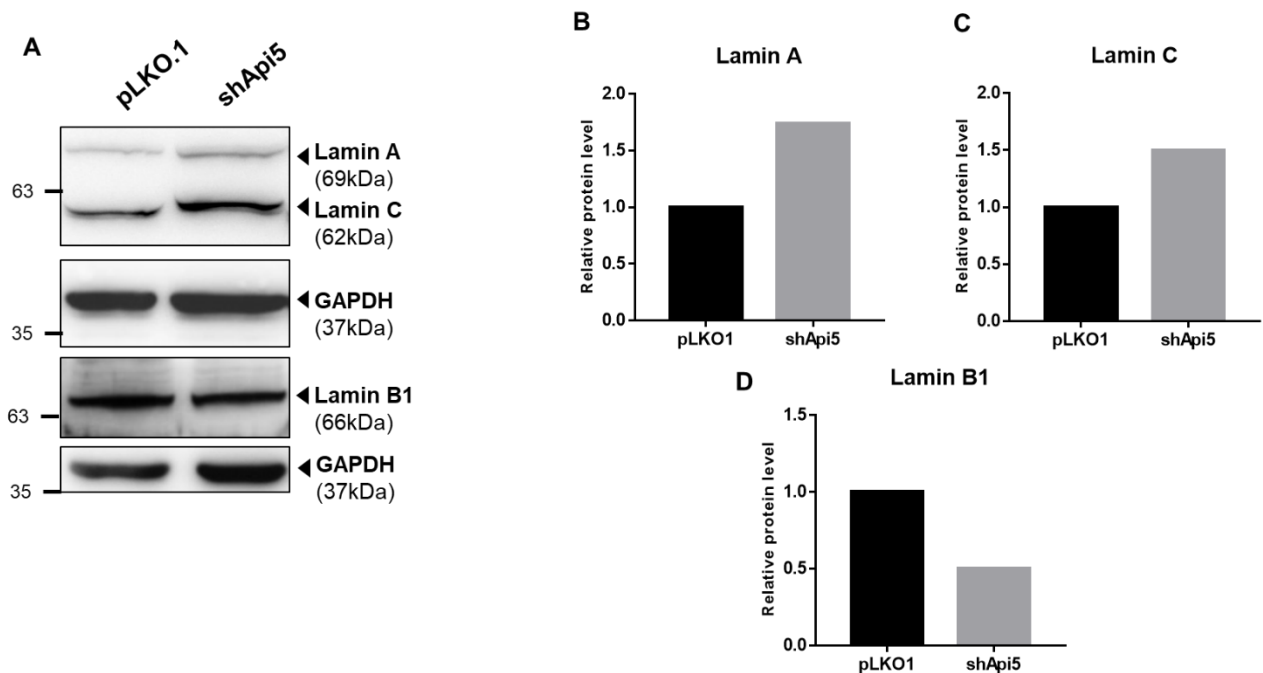


Figure 5.28: Api5 knockdown affects the levels of nuclear lamins

MCF7 cells stably expressing pLKO1 or shApi5 were lysed, and immunoblotting was performed to analyse the levels of Lamin A, Lamin C, and Lamin B1 and B-D) quantified after normalising to GAPDH. Data is representative of three independent experiments.

5.2.8 Reduced Api5 levels are associated with G1 phase-specific defects

In the previous results, we observed that knockdown of Api5 led to cells towards reduction in their proliferative potential. As Api5 was found to be a cell cycle phase-associated protein, it was interesting to understand the function of Api5 in the G1 phase of the cell cycle as the levels of Api5 were enhanced in the G1 phase. To begin with that, the first cell cycle profile of both Api5 knockdown as well as control cells were analysed upon synchronisation in the G1 phase of cell cycle using the serum starvation method by FACS after staining with PI. It was observed that both the cells were showing similar arrest at G1 (Figure 5.29). However, Api5 knockdown cells showed more cells in S and less in the G2-M phase of the cell cycle (Figure 5.29). This suggested that Api5 could play some role in the regulation of the cell cycle. To further prove the role of Api5 cells in the G1 phase, Api5 knockdown cells were stained for Lamin A and B. It was observed that shApi5 cells showed an increase in the protein expression while a reduction in Lamin B1 during the G1

phase of the cell cycle (Figure 5.30 and 5.31). These results overall suggested that Api5 helps in the regulation of the G1 phase of the cell cycle.

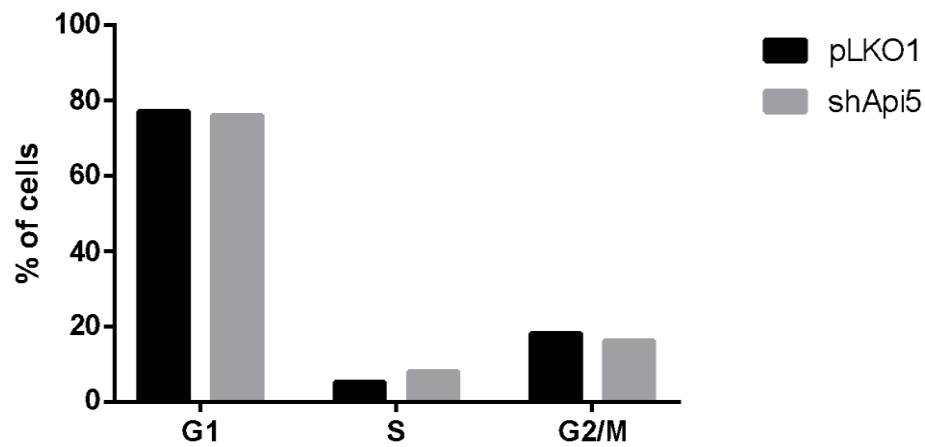


Figure 5.29: Api5 knockdown affects the cell cycle upon G1 arrest

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on a 35 mm dish. 16 hrs post-seeding complete media was replaced with serum-free media for arresting cells in the G1 phase. Cells were fixed and stained using propidium iodide post 48 hrs of serum starvation, and the cell cycle profile was monitored using FACS. Data is representative of three independent experiments.

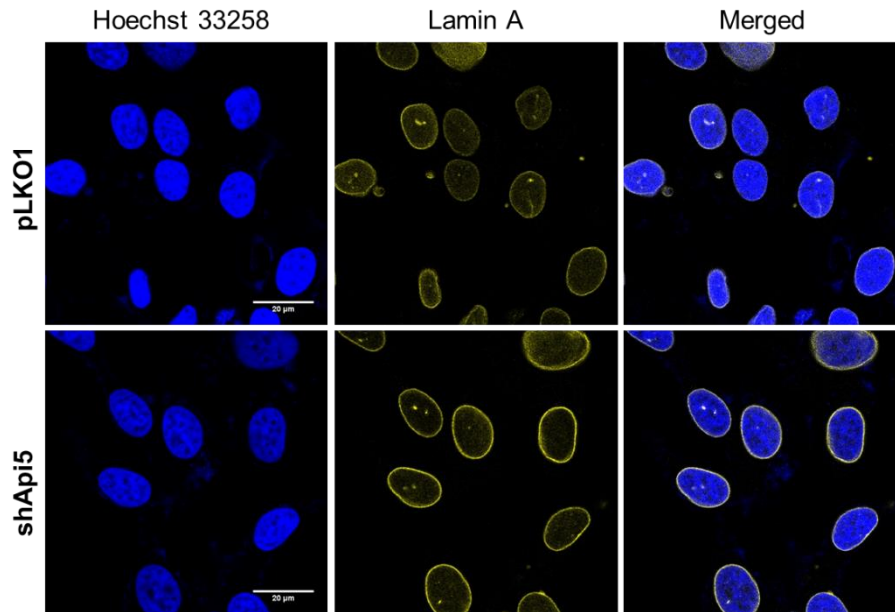


Figure 5.30: Lamin A levels increases upon Api5 knockdown in the G1 phase
MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip and serum-starved for 48 hrs to arrest cells in the G1 phase. Immunostaining was performed using Lamin A specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

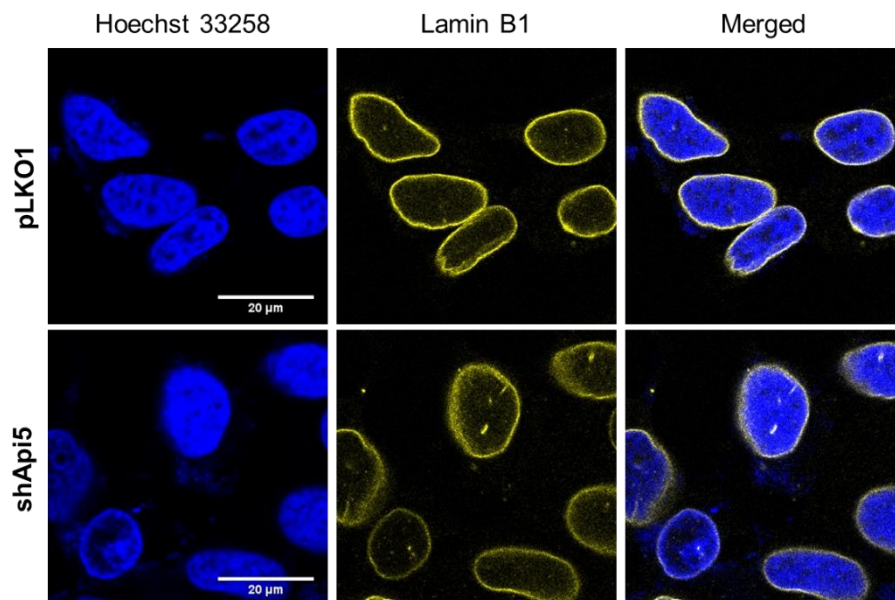


Figure 5.31: Lamin B1 levels decrease upon Api5 knockdown in the G1 phase
MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip and serum-starved for 48 hrs to arrest cells in the G1 phase. Immunostaining was performed using Lamin B1 specific antibody and imaged in Leica Sp8 confocal

microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

5.2.9 Reduced Api5 levels are associated with S phase-specific defects

To understand the role of Api5 in the S phase of the cell cycle, both control and shApi5 cells were synchronised in the S phase using the double thymidine method. FACS profiles of the cells were analysed upon staining with PI. It was observed that cells showing knockdown of Api5 were not showing a proper arrest at the S phase as the significant fraction of cells were still in the G1 phase of the cell cycle (Figure 5.32). That led less shApi5 cells to be in S and G2-M phase cell cycle. To further prove the role of Api5 in the S phase, control and shApi5 cells were arrested in the S phase and stained for nuclear Lamins. It was observed that both Lamin A and B1 showed a reduction in their protein expressions in Api5 depleted cells during the S phase of the cell cycle (Figure 5.33-5.34). This suggested that Api5 can also play a significant role in the S phase of the cell cycle.

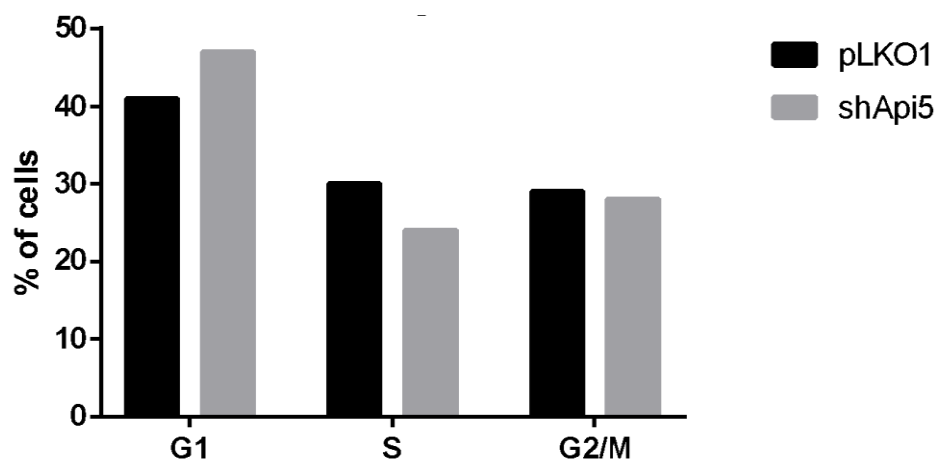


Figure 5.32: Api5 knockdown affects the cell cycle upon S phase arrest

MCF7 cells stably expressing pLKO1 or shApi5 were arrested in the S phase using double thymidine (2mM) treatment. Arrested cells were fixed and stained using propidium iodide post, and the cell cycle profile was monitored using FACS. Data is representative of three independent experiments.

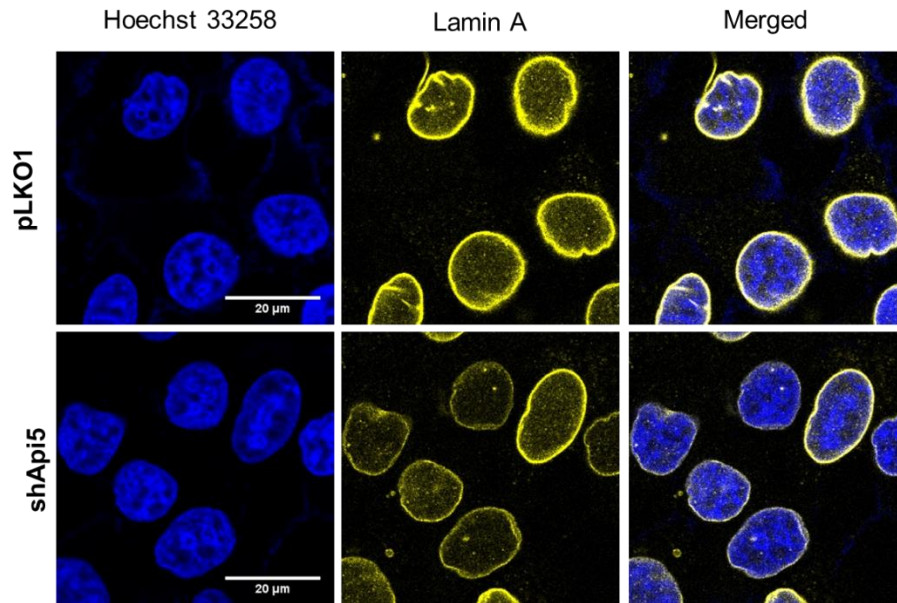


Figure 5.33: Lamin A levels decrease upon Api5 knockdown in G1 phase

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip and arrested in the S phase using double thymidine (2mM) treatment. Immunostaining was performed using Lamin A specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

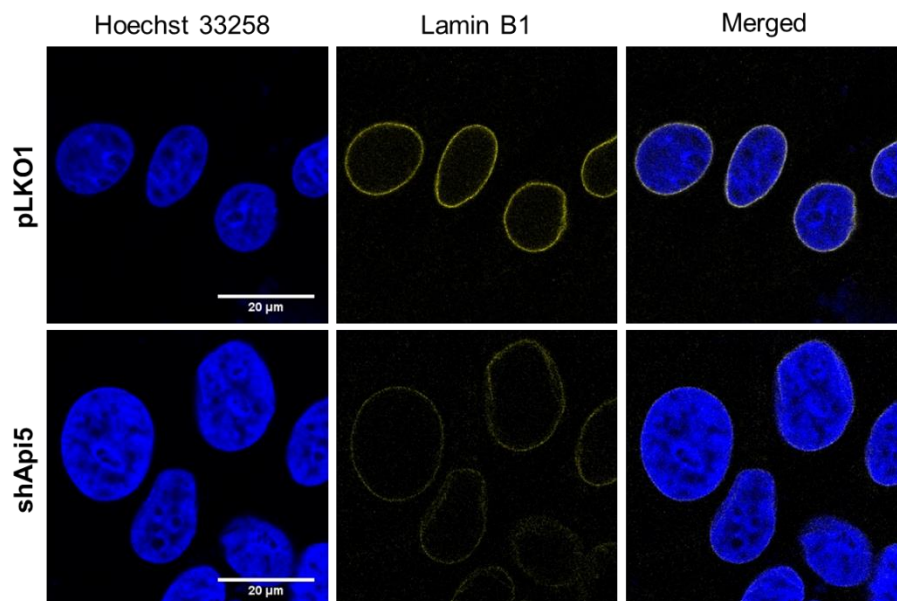


Figure 5.34: Lamin B1 levels decrease upon Api5 knockdown in the G1 phase

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip and arrested in the S phase using double thymidine (2mM) treatment. Immunostaining was performed using Lamin B1 specific antibody and imaged in Leica Sp8 confocal

microscope (scale bar- 20 μ m). Data is representative of three independent experiment.

5.2.10 Reduced Api5 levels are associated with G2 phase defects

To understand the role of Api5 in the G2 phase of the cell cycle, cells were synchronised in the S phase using the double thymidine method and later cells were released in thymidine-free media for 6 hrs. By this significant number of cells were obtained in the G2 phase of the cell cycle. It was observed that Api5 knock-down cells could not synchronise at G2 compared to control (Figure 5.35). These cells were stained for the Lamin A. It was observed that Lamin A protein levels increased upon the knockdown of Api5 (Figure 5.36). These results overall suggested that Api5 helps in maintaining the G2 phase of the cell cycle.

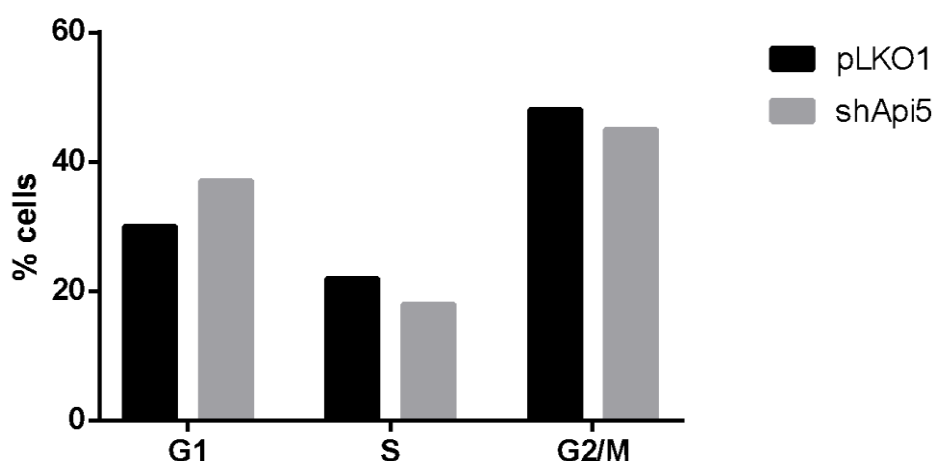


Figure 5.35: Api5 knockdown affects the cell cycle upon G2 arrest

MCF7 cells stably expressing pLKO1 or shApi5 were arrested in the G2 phase by using double thymidine (2mM) treatment and release to fresh media for 6 hrs. Arrested cells were fixed and stained using propidium iodide post, and the cell cycle profile was monitored using FACS. Data is representative of three independent experiments.

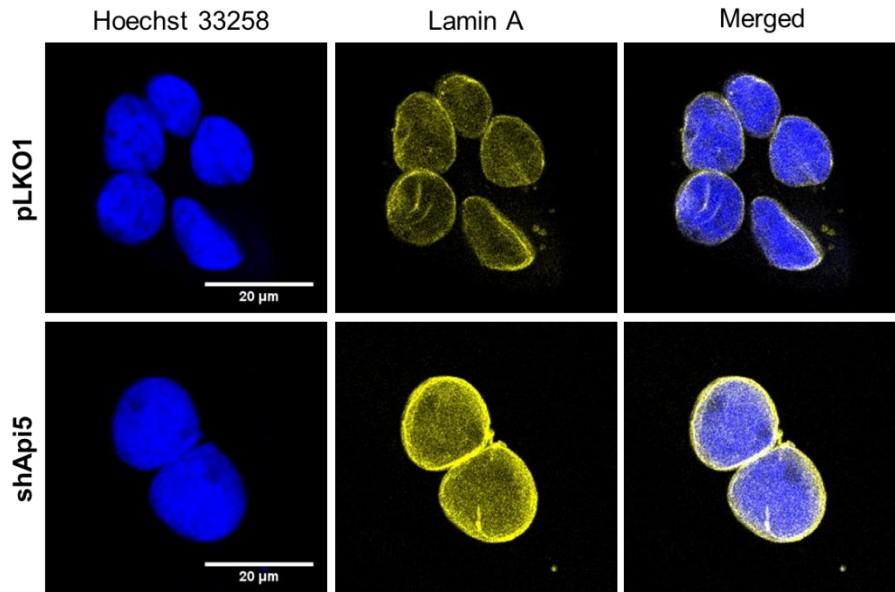


Figure 5.36: Lamin A levels increases upon Api5 knockdown in the G1 phase

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on the coverslip and arrested in the G2 phase by using double thymidine (2mM) treatment and release to fresh media for 6 hrs. Immunostaining was performed using Lamin A specific antibody and imaged in Leica Sp8 confocal microscope (scale bar- 20 μ m). Data is representative of three independent experiments.

5.2.12 Reduced Api5 levels are associated with M phase defects.

To understand the function of Api5 in the cell cycle, stable cells with Api5 knockdown were synchronised in the G2-M phase of the cell cycle using Cdk1 inhibitor and released on post subsequent hrs to get a maximum number of cells in the mitosis. Immunofluorescence assay was performed to detect the cells in different sub-stages of mitosis. It was observed that a significant number of metaphase cells showed chromosome aligning defects. Most of such cells showed defects like acentric chromosomes (Figure 5.37A-B). Apart from a metaphase population showing acentric chromosomes, some cells were also observed with other chromosomal defects like the multipolar organisation of chromosomes during the metaphase (Figure 5.37A-B). Multipolar structures could also be an outcome of the initiation of aneuploidy inside the cells. Thus it was confirmed that Api5 plays a significant role in the regulation of alignment of chromosomes at the metaphase plate. Interestingly during the anaphase also some defects were observed in Api5 knockdown cells. A significant number of cells showed phenotypes like bridging and lagging of the

chromosomes during the anaphase (Figure 5.37A and C). These chromosomes were not positioned to their respective poles. Henceforth could turn out to be the micronuclei alongside the main nucleus body (Figure 5.38). Thus, these experiments confirmed that Api5 plays a significant role in the regulation of proper cell division in the M phase of the cell cycle.

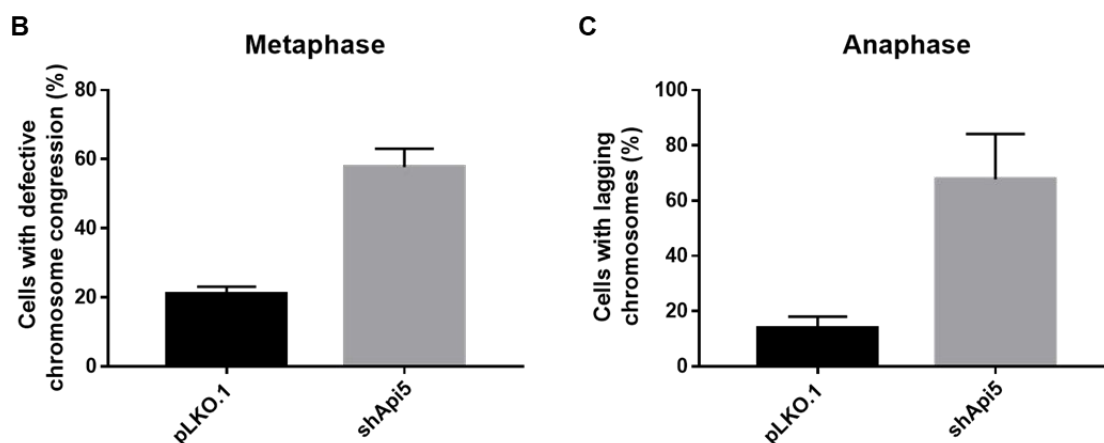
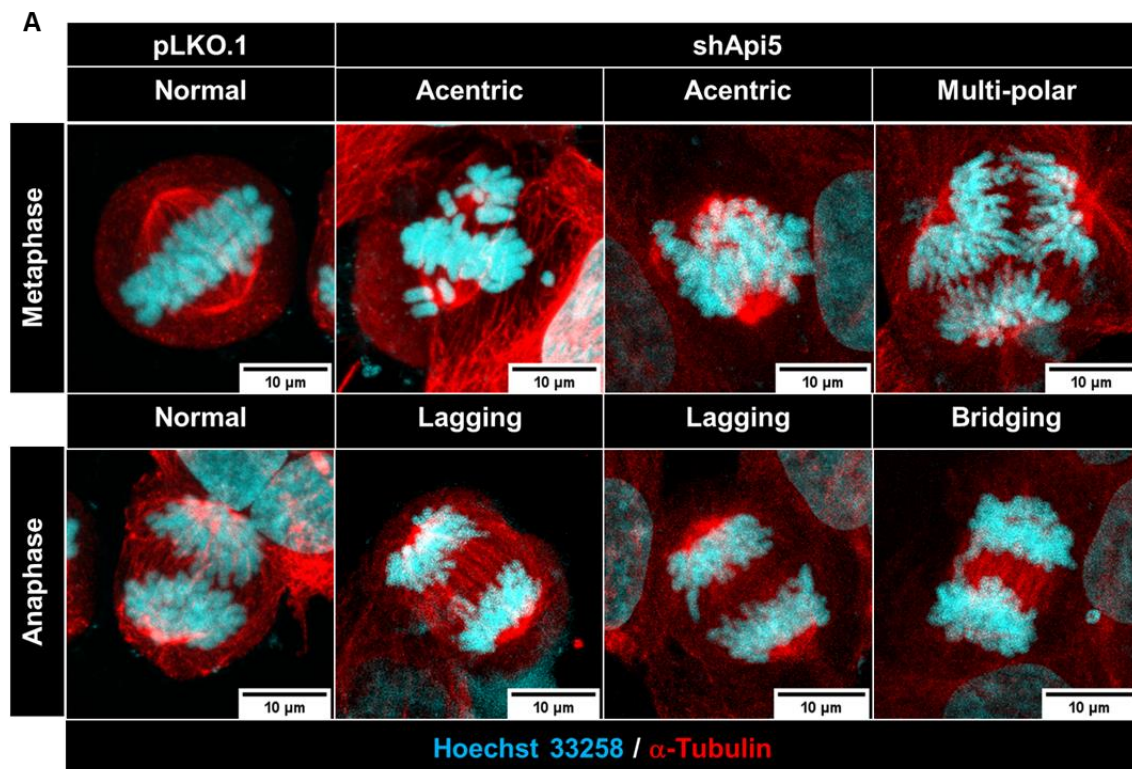


Figure 5.37: Api5 knockdown leads to mitotic defects

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslips and arrested in M phase by Cdk1 (10 μ M) treatment for 24 hrs and release to fresh media for subsequent hrs. Immunostaining was performed using tubulin and Hoechst

(nuclear) and imaged in Leica Sp8 confocal microscope (scale bar- 10 μ m). Cells with chromosomal defects in B) metaphase and C) anaphase were counted manually. Data is representative of three independent experiments.

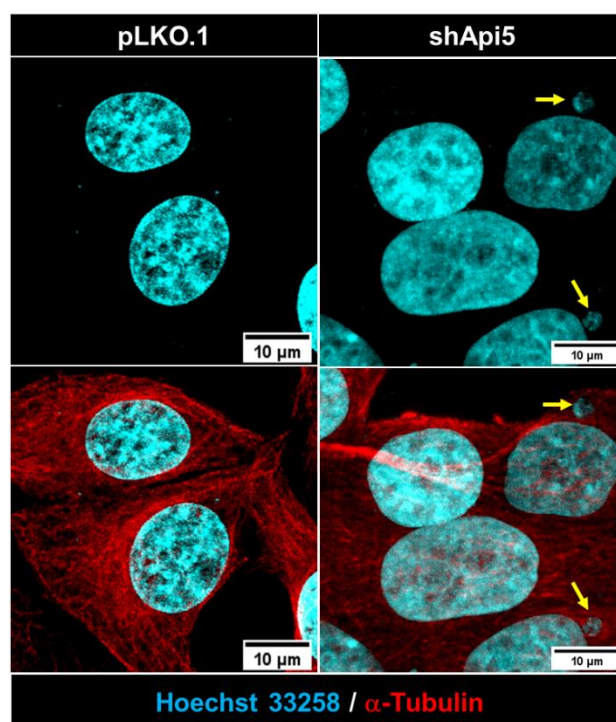


Figure 5.38: Api5 knockdown leads to the micro-nuclei formation in MCF7 cells

MCF7 cells stably expressing pLKO1 or shApi5 were seeded on coverslip, and immunostaining was performed using tubulin and Hoechst (nuclear) and imaged in Leica Sp8 confocal microscope (scale bar- 10 μ m). Data is representative of three independent experiments.

5.2.13 Mechanism of regulation of cell cycle by Api5

To find the overall mechanism behind the regulation of the cell cycle by Api5, Api5 knockdown cells along with control cells were lysed, and immunoblotting was performed to check the levels of p53 as a master regulator and involved almost all the cellular processes. p53 has been reported to be associated with DNA damage, cell cycle arrest, and all the obtained phenotypes in each phase of the cell cycle (Chen et al., 2017; Lindemann et al., 2018). It was observed that p53 protein levels were increased in the Api5 knockdown cells (Figure 5.22). p21 is the downstream player of p53 and has also been associated with most of the biological functions of

Api5. It was observed that along with p53, p21 protein expression was also enhanced upon knockdown of Api5 in the G1 and S phase of the cell cycle (Figure 5.22). In all cell cycle phases also, p53 levels were found to be enhanced along with p21 suggesting that p53 is the key regulator of Api5 mediated cell cycle regulation (Figure 5.39 and 5.40).

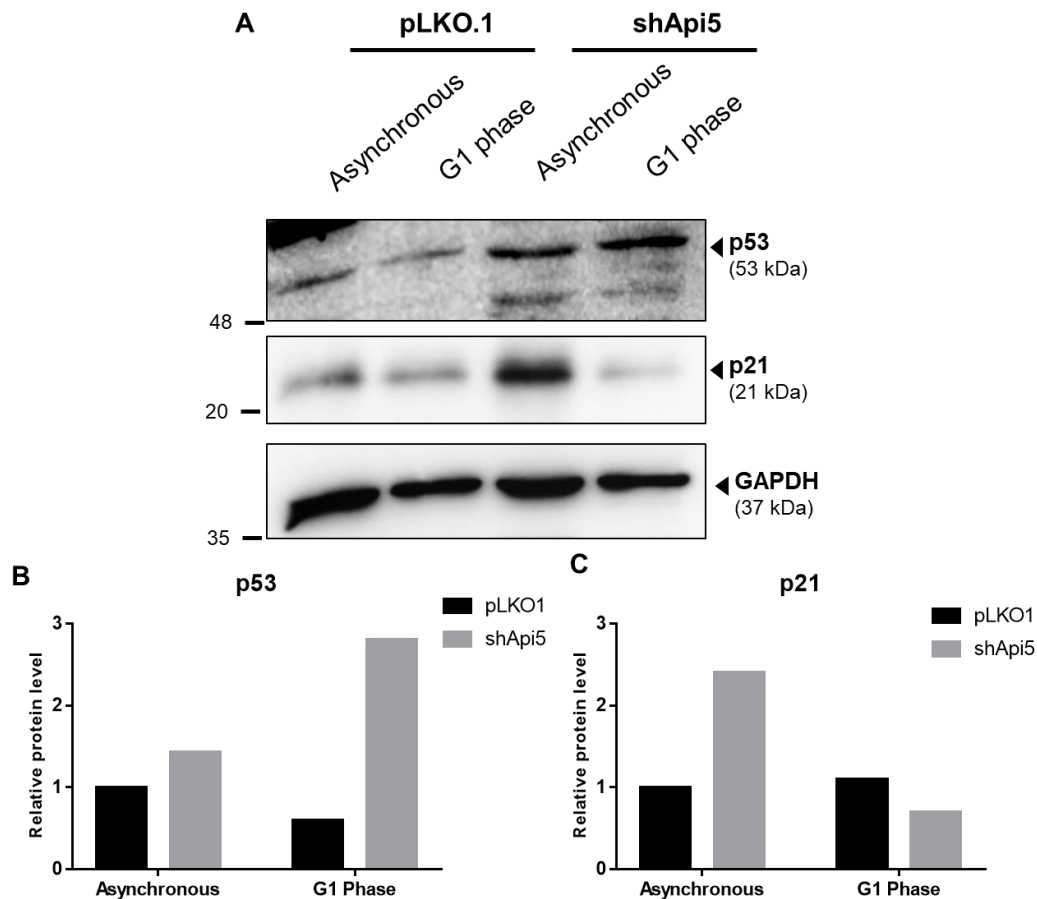


Figure 5.39: p53 and p21 levels increases upon Api5 knockdown in the G1 phase

A) MCF7 cells stably expressing pLKO1 or shApi5 were arrested in the G1 phase by serum starvation for 48 hrs and lysed, and immunoblotting was performed to analyse the levels of p53 and p21 and B-C) quantified after normalising to GAPDH. Data is representative of three independent experiments.

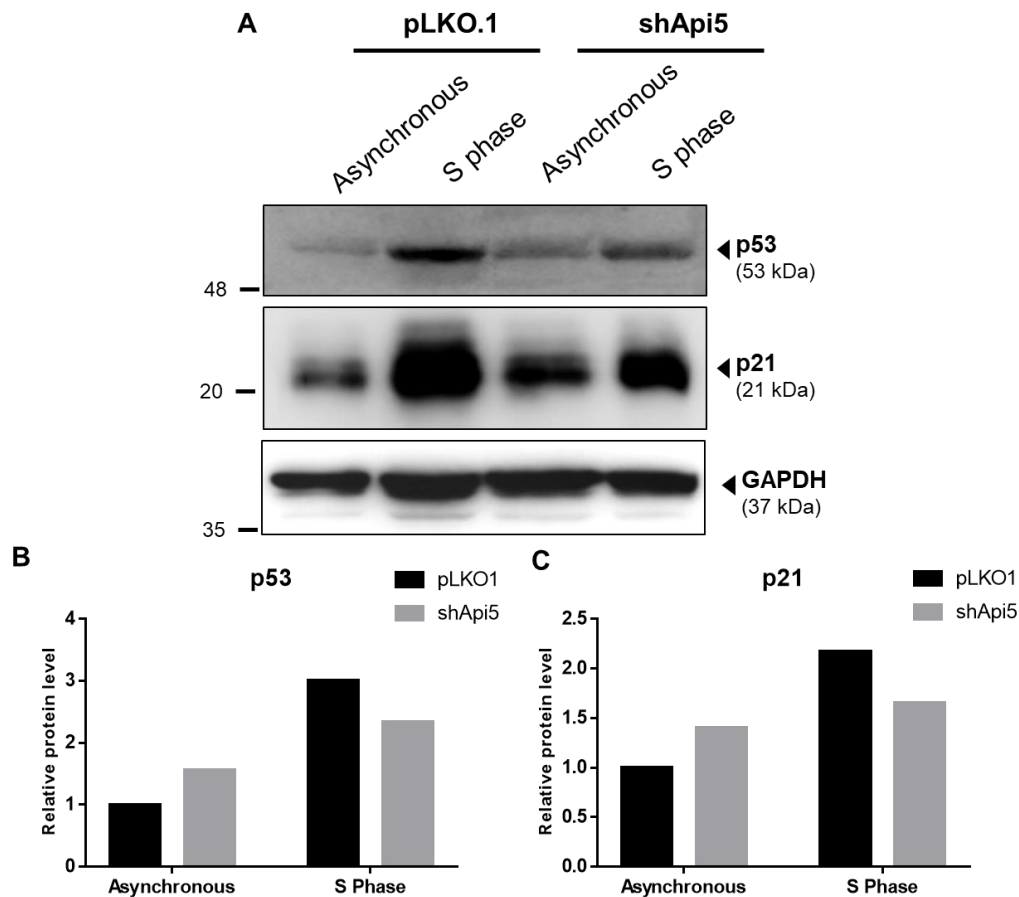


Figure 5.40: p53 and p21 levels increases upon Api5 knockdown in the S phase

A) MCF7 cells stably expressing pLKO1 or shApi5 were arrested in the S phase by double thymidine treatment and lysed, and immunoblotting was performed to analyse the levels of p53 and p21 and B-C) quantified after normalising to GAPDH. Data is representative of three independent experiments.

5.3 Discussion

Api5 was initially discovered as a protein that inhibited apoptosis upon serum starvation (Tewari et al., 1997). Later it was also studied that Api5 could also regulate apoptosis in different contexts like DNA damage and certain infections (Rigou et al., 2009). Garcia and his group reported that Api5, an inhibitor of apoptosis, regulates its level during the cell cycle (Garcia-Jove Navarro et al., 2013). Different phases of the cell cycle of non-small cell lung carcinoma cells showed differential Api5 protein expression (Garcia-Jove Navarro et al., 2013). Keeping its levels high during the G1 and S phases of the cell cycle was correlated with the

transition of cells from G1 to S by regulating the transcription activity of E2F1. However, the mechanism of its post-translational degradation during G2-M was not known. We identified that the degradation of Api5 begins from the prophase and continues in metaphase. The degradation of Api5 completes in the anaphase of the cells. In our study, we also identified that Api5 undergoes post-translational degradation through the proteasomal pathway. Before the post-translational degradation, Api5 undergoes ubiquitination, a well-known post-translational modification for cell cycle regulators like p53, E2F1, and almost all the cyclins (Venuto and Merla, 2019). We also identified that FBXW2 is the critical regulator of Api5 during the G2-M phase of the cell cycle. FBXW2 degrades Api5 in the G2-M phase. p300 was found to be reduced during the G2-M phase of the cell cycle that could also be the reason for a partial reduction in Api5 protein levels in the G2-M phase of the cell cycle. Generally, cell cycle phase regulatory proteins are also regulated by different kinases (Roskoski, 2019). In our study, we also discovered Aurora kinase B as the key regulator of Api5. Aurora kinase B is one of the kinase families that govern the cell cycle transition from G2 to M (Figure 5.41).

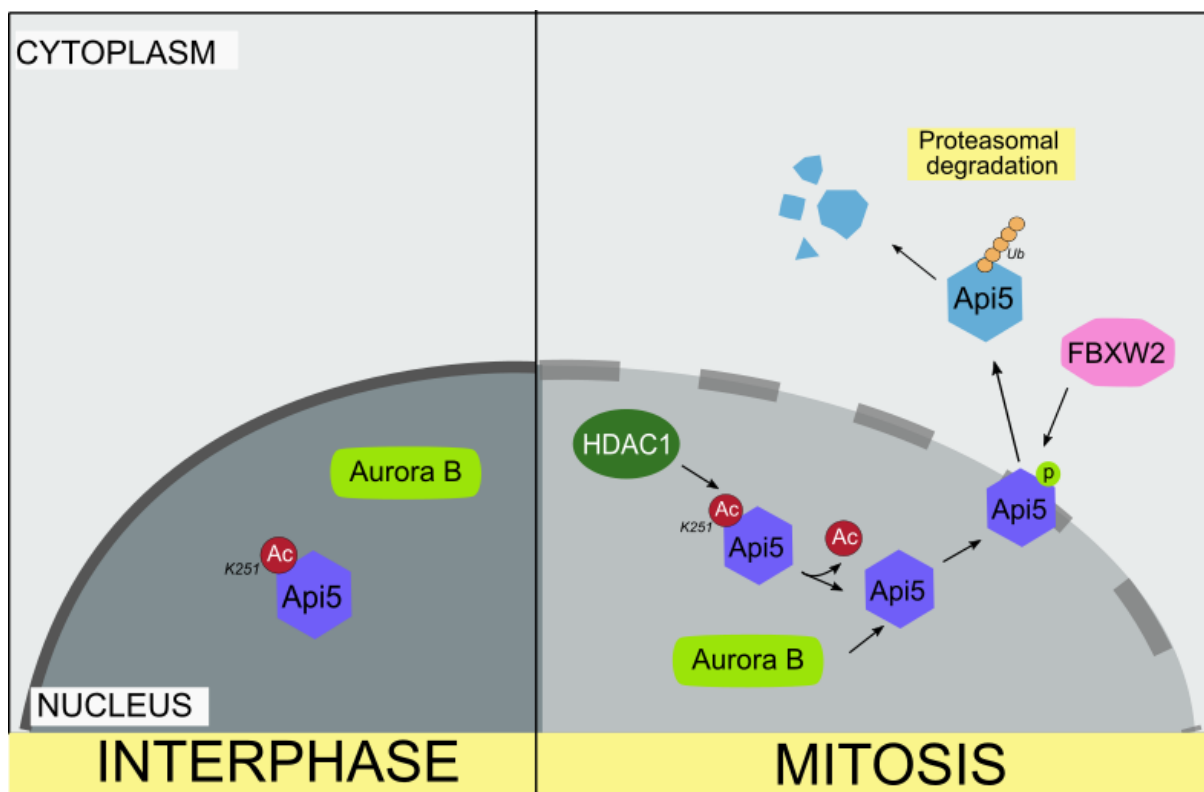


Figure 5.41: Schematic representation depicting the regulation of Api5 in G2-M phase of cell cycle

A proposed model deciphering the pathway of regulation of Api5 by its key regulators HDAC1, Aurora kinase B, and FBXW2 in the G2-M phase of cell cycle

As mentioned earlier, Api5 levels peak during the G1 and S phase of the cell cycle and are thereby able to play a role in the regulation of cell cycle progression and the proliferation of cells by possibly functioning as a transcription factor. During the G1 and S phases of the cell cycle, Api5 may be interacting with chromatin to transcribe those genes that regulate the progression of the cell cycle. Transcription of those genes may also regulate the proliferation of cells. Thus it may be inferred that acetylation of Api5 by p300 possibly provides additional functionality to Api5 that includes the transcription of cell cycle regulators. We also observed that Api5 is essential for the proliferation of the cells as depletion of Api5 leads to a reduction in the survival of the cells. This reduction of survival can also be linked to the defect in mitosis as depletion of Api5 leads to chromosomal defects like lagging, bridging, and acentric chromosomes. These lagging chromosomes can be enveloped with a membrane to form separate genetic entities like micronuclei (Crasta et al., 2012). Api5 was also found to be associated with the regulation of ploidy levels in the cells, as the Api5 knockdown cells showed multipolar structure. This suggested that Api5 depleted cells could not sense their ploidy levels. This also ensures that Api5 is required in the early phases of the cell cycle like G1, S, and G2 for the error-free completion of cell division. To achieve its molecular functions, Api5 regulates p53 protein expressions. We observed that the knockdown of Api5 leads to the accumulation of p53. It has been reported that p53 is involved in cellular processes proliferation by controlling G1- S transition, senescence by increasing p21 protein levels, nuclear architecture defects by regulating the levels of Lamin proteins, and finally, the proper mitotic division by regulating the proper positioning of chromosomes (Barnes et al., 2019; Meitinger et al., 2016; Yoon et al., 2019). Thus it confirms that Api5 being an anti-apoptotic protein, also regulates cell cycle progression (Figure 5.42).

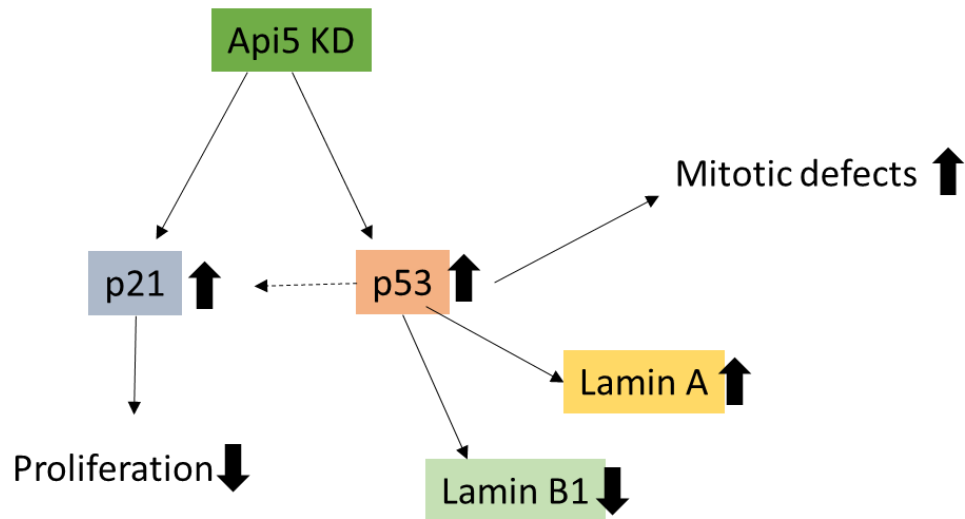


Figure 5.42: Plausible pathway for cell cycle regulation by Api5

A proposed pathway deciphering the regulation of proliferation, nuclear architecture, and mitotic defects by Api5.

Chapter 6

ATR facilitates the degradation of Api5 through the ubiquitin-proteasome pathway via FBXW2 to regulate apoptosis upon DNA damage

6.1 Background

It has been shown that Api5 inhibits DNA damage mediated apoptosis (Rigou et al., 2009a); however, the mechanism of inhibition is not known. Moreover, the regulation of Api5 upon DNA damage-induced apoptosis is also not clear. The focus of this chapter is to identify the regulation of Api5 upon DNA damage-induced apoptosis. For this purpose, both chemicals (Etoposide and camptothecin) and UV -irradiation were used as DNA damaging agents. Camptothecin and UV inhibit topoisomerase I (Subramanian et al., 1998; Yoshida et al., 1993) and Etoposide inhibits topoisomerase II (Baldwin and Osheroff, 2005), which induces DNA damage in the form of single and double-stranded breaks, respectively. ATR and ATM both are sensor kinases that sense the intensity of DNA damage and activate Chk1 and Chk2, respectively, through various mediator proteins like TopBP1, claspin and BRCA1 (Abraham, 2001; Cortez et al., 1999) (Harper and Elledge, 2007). Activation of checkpoint proteins leads to cell cycle arrest and DNA repair. If the damage is irreparable, the cell decides to undergo apoptosis. In this chapter, we identified the pathway and regulators of the post-translational degradation of Api5 upon DNA damage-induced apoptosis. Moreover, the function of Api5 in the regulation of DNA damage-induced apoptosis was also established.

6.2 Results

6.2.1 Api5 levels reduce upon DNA damage-induced apoptosis

Api5 has been earlier reported to play a significant role in DNA damage-induced apoptosis. It was important to investigate the mechanism of regulation of Api5 during DNA damage. To understand the regulation of Api5 upon DNA damage, cells were treated with different concentrations of both chemicals and radiation damage. Camptothecin and etoposide were used as chemical DNA damage causing agents, while UV was used for radiation damage. To understand the regulation of Api5 upon DNA damage-induced apoptosis, cells were treated with various concentrations of etoposide for 24 hours and camptothecin for 16 hours and western blotting analysis was performed. The blots were probed with pChk1 and pChk2. The presence of phosphorylated checkpoint proteins (pChk1 and pChk2) was observed in treated cells, indicating the induction of single-strand and double-strand breaks, respectively, by both the drugs (Figure 6.1A-C).

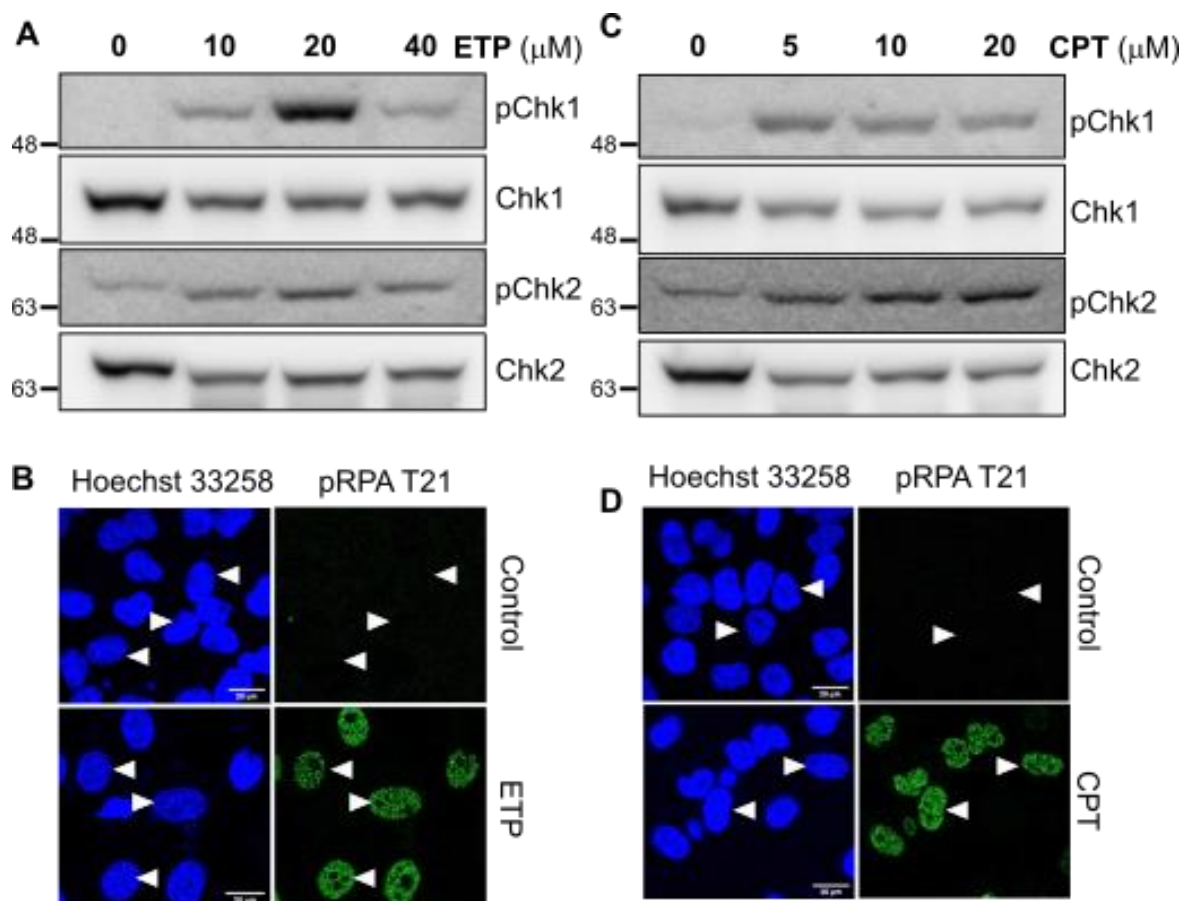


Figure 6.1: Etoposide and camptothecin cause DNA damage in MCF7 cells.

MCF7 cells were treated with varying doses of A) etoposide and B) camptothecin were lysed, and immunoblotting was performed to analyse the levels of pChk1, Chk1, pChk2, and Chk2. Immunofluorescence was performed to analyse RPA activation after B) etoposide, D) camptothecin treatment. Data is representative of three independent experiments.

5 μ M of camptothecin and 20 μ M of etoposide showed adequate activation of Chk1 and Chk2, suggesting the induction of DNA damage. These results were further corroborated by immunofluorescence assay, where RPA activation was used as a marker of DNA damage (Figure 6.1B-D).

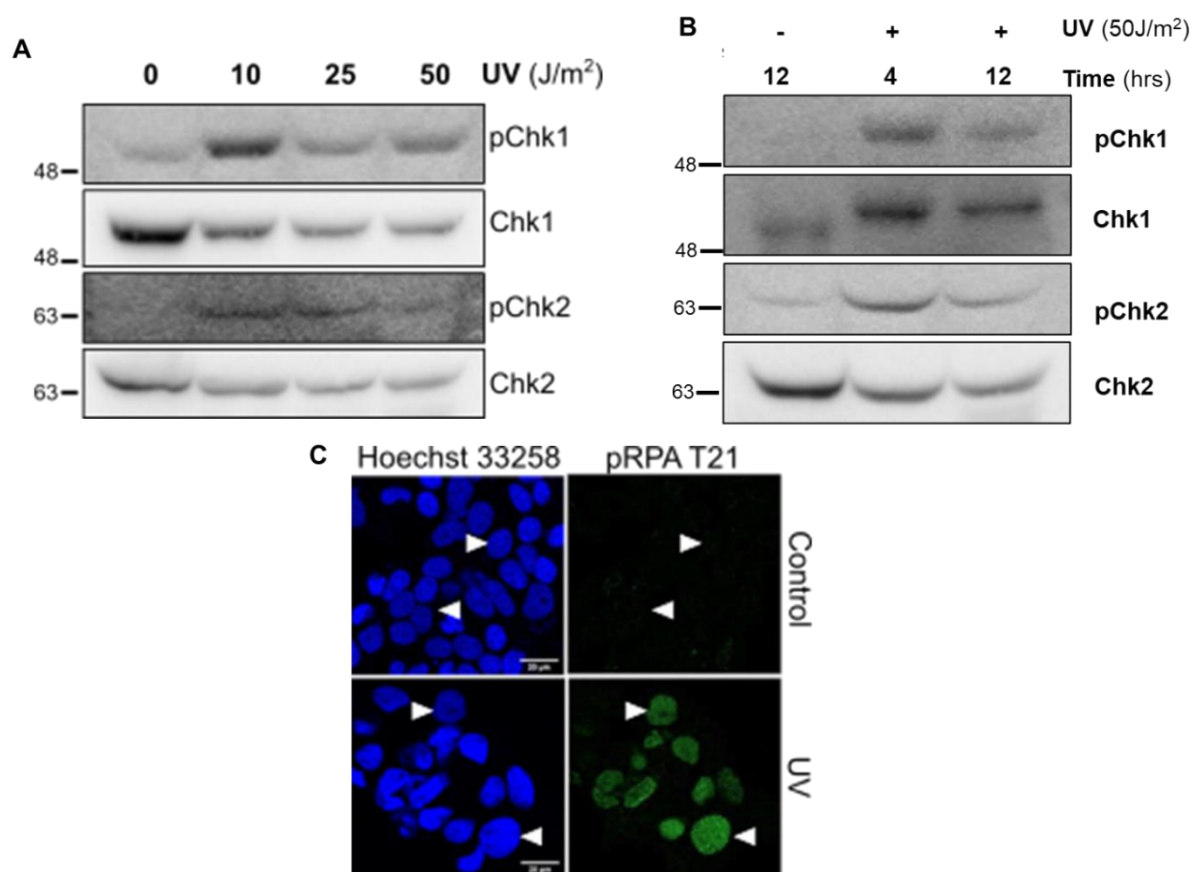


Figure 6.2: UV irradiation causes DNA damage in MCF7 cells.

MCF7 cells were treated with A) varying doses of UV or B) 50J/m² UV for varying time points were lysed, and immunoblotting was performed to analyse the levels of pChk1, Chk1, pChk2, Chk2. C) Immunofluorescence was performed to analyse RPA activation after UV treatment (50J/m² for 12 hrs). Data is representative of three independent experiments.

To find the effect of radiation damage on Api5 levels, cells were irradiated with different doses of UV, and lysates were collected 12 hours post-treatment and western blotting was performed. The presence of phosphorylated checkpoint proteins (pChk1 and pChk2) was observed in treated samples, confirming the induction of single-strand and double-strand breaks, respectively (Figure 6.2A). Cells treated with 50J/m² of UV for different time points like 4 and 12 hrs also showed Chk1, Chk2 and RPA activation suggesting that the dose is sufficient to cause DNA damage (Figure 6.2B-C).

From these results, it was clear that all these doses of camptothecin, etoposide and UV were able to cause DNA damage. Next, it was decided to check whether these

drug doses causing DNA damage were also sufficient to initiate the process of apoptosis. For that, cells were treated with different concentrations of etoposide, camptothecin and UV. Cells were lysed, and western blotting was performed to check the presence of different apoptotic markers. The presence of both active initiator (Caspase2) and executioner (caspase 3 and caspase 9) caspases along with cleaved PARP 1 showed that these concentrations were able to induce apoptosis (Figure 6.3A-C). It was observed that 10 μM of camptothecin and 20 μM of etoposide, and 50J/m² of UV could initiate apoptosis after inducing the DNA damage in MCF7 cells.

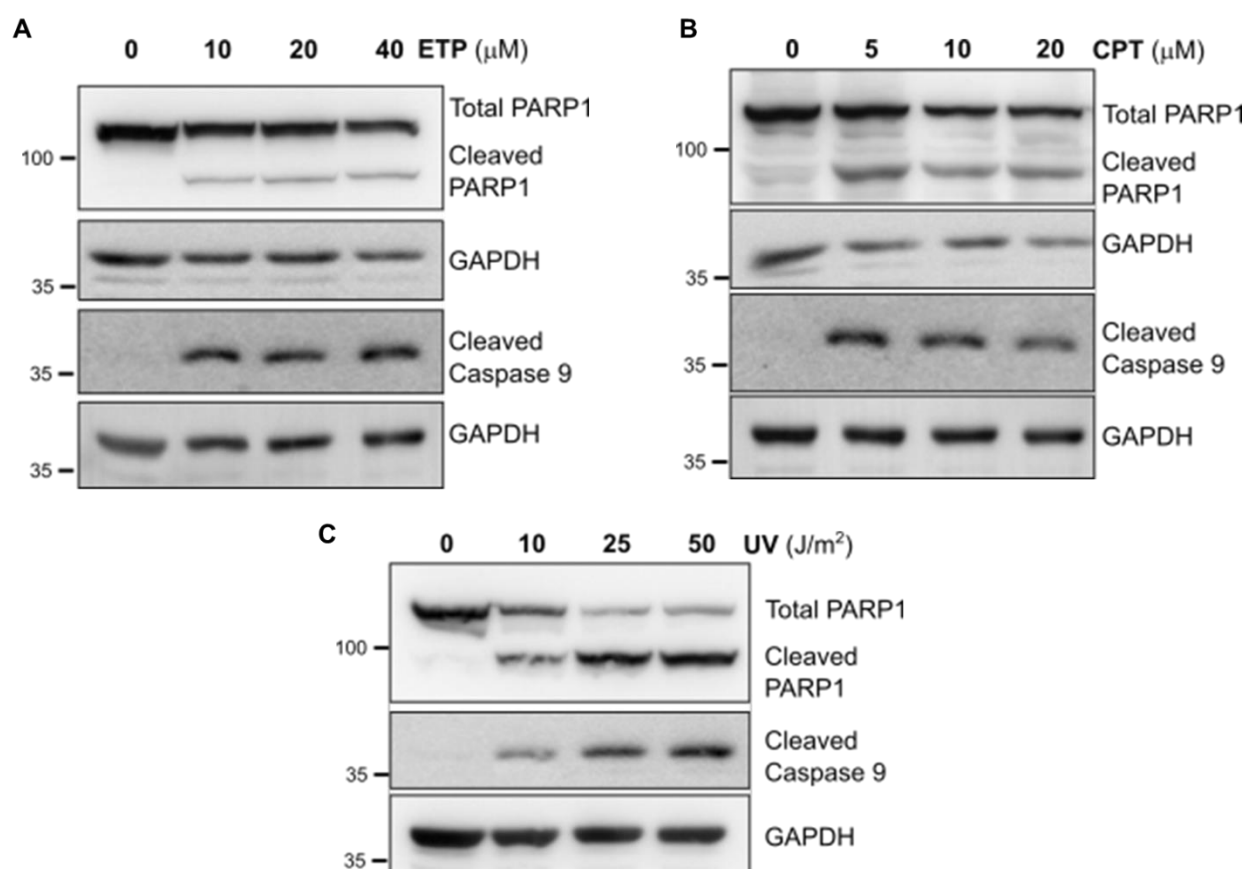


Figure 6.3: Etoposide, camptothecin, and UV irradiation cause DNA damage-induced apoptosis in MCF7 cells.

MCF7 cells were treated with varying doses of A) etoposide, B) camptothecin and C) UV were lysed, and immunoblotting was performed to analyse the levels of PARP and Caspase 9. Data is representative of three independent experiments.

After standardising the dose and time for each DNA damaging agent, the level of Api5 was analysed in the treated cells. Reduction of Api5 protein levels was also

observed in a dose-dependent study using etoposide, confirming that Api5 levels decrease upon DNA damage-induced apoptosis (Figure 6.4A-B). It was observed that protein levels of Api5 were reduced in a dose-dependent as well as time-dependent manner upon treatment of camptothecin and UV (Figure 6.4C-F, 6.5A-B, and 6.6A-B).

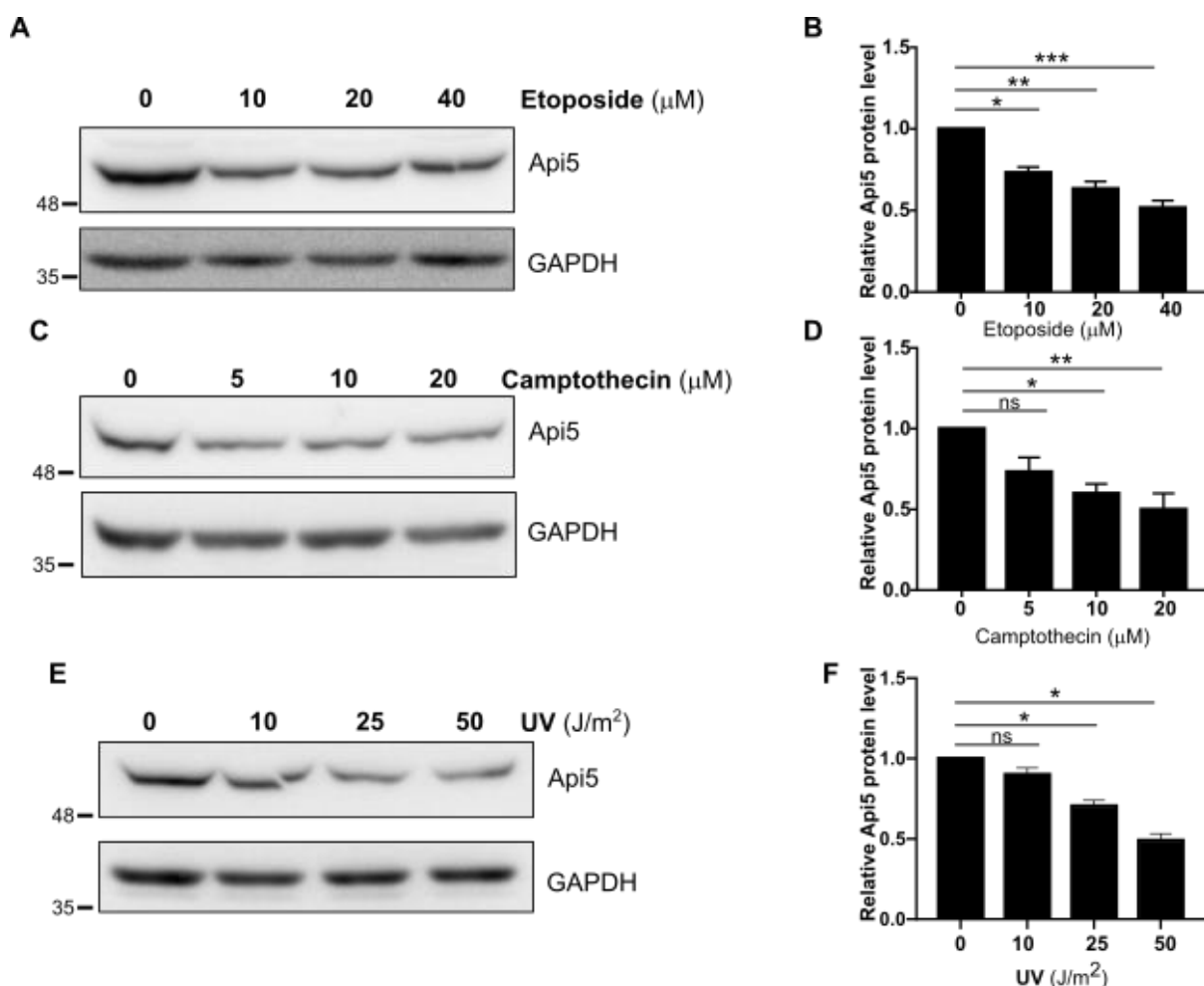


Figure 6.4: Api5 level decreases upon DNA damage

MCF7 cells were treated with varying doses of A) etoposide, C) camptothecin and E) UV were lysed, and Api5 levels were analysed using immunoblotting and B, D, F) quantified after normalising with GAPDH. Data is representative of three independent experiments and statistical analysis used was One way Anova; ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

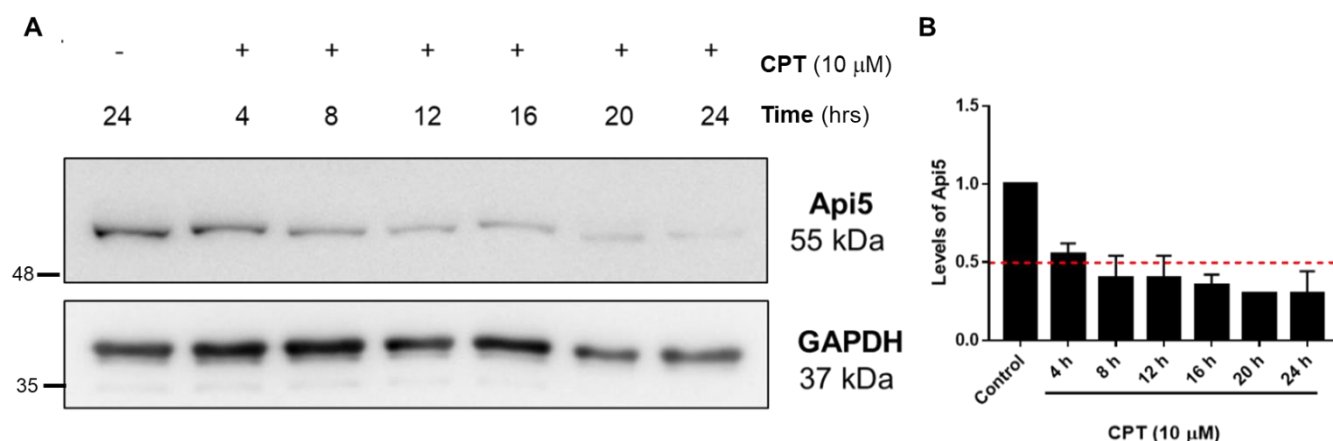


Figure 6.5: Api5 levels reduces upon camptothecin in a time-dependent manner

A) MCF7 cells were treated with 10 μ M camptothecin for varying time points were lysed, and Api5 levels were analysed using immunoblotting and B) quantified after normalising with GAPDH. Data is representative of three independent experiments.

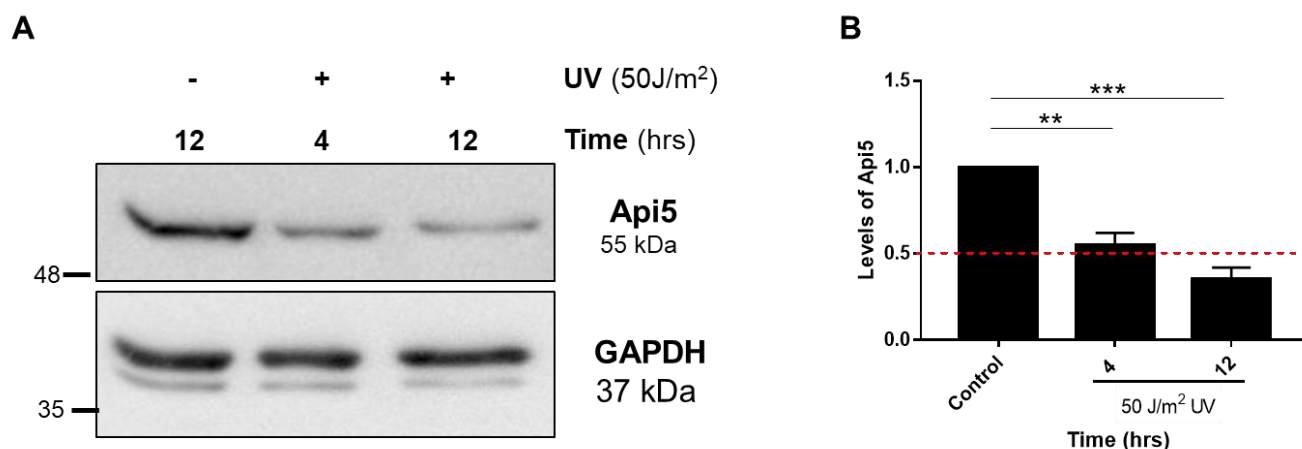


Figure 6.6: Api5 levels reduces upon UV irradiation in a time-dependent manner

A) MCF7 cells were treated with 50 J/m² UV for varying time points were lysed, and Api5 levels were analysed using immunoblotting and B) quantified after normalising with GAPDH. Data is representative of three independent experiments and statistical analysis used was One way Anova; **p<0.01, ***p<0.001.

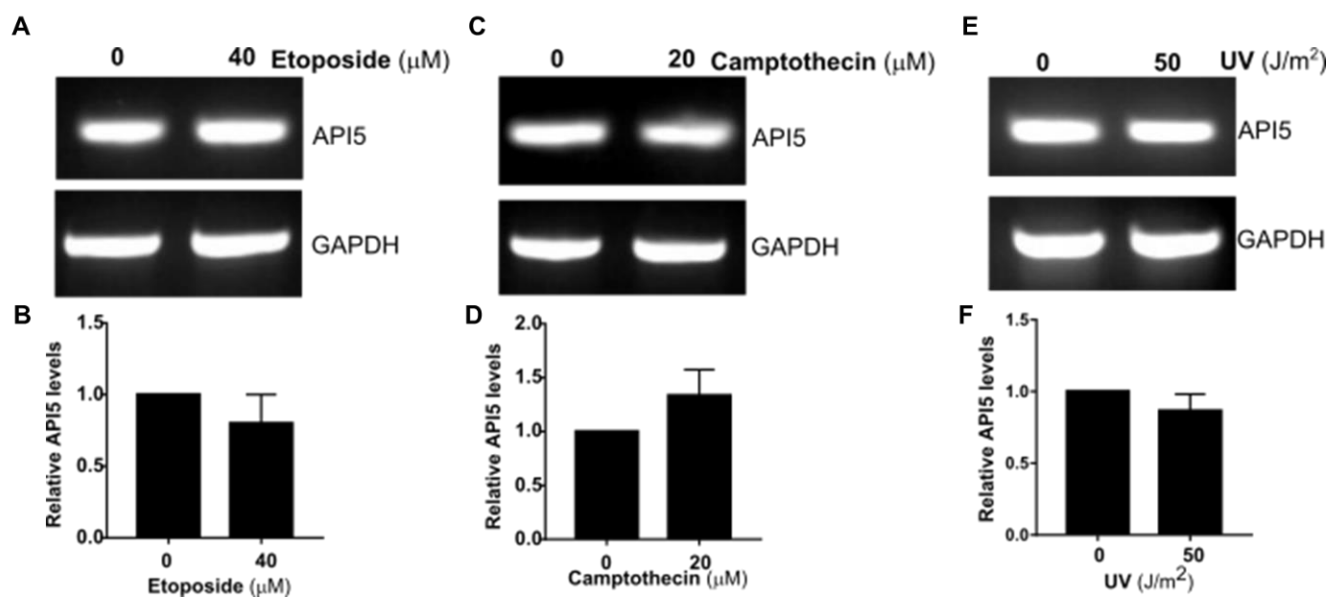


Figure 6.7: *API5* transcript level do not change upon DNA damage

API5 transcript levels upon A) etoposide, C) camptothecin, and E) UV treatment were analyzed using semi-quantitative PCR and B, D, F) quantified after normalising with GAPDH. Data is representative of three independent experiments.

This reduction in Api5 levels may be due to a decrease in the transcription of Api5. To investigate whether the Api5 transcript levels also reduce upon DNA damage-induced apoptosis, cells were treated with etoposide, camptothecin and UV for 24 hrs, 16 hrs and 12 hrs, respectively. Total RNA was extracted by trizol extraction method, cDNA was synthesized by reverse transcriptase PCR method. Semi-quantitative PCR was performed to check the transcript levels of *API5*. It was observed that *API5* transcript was unaltered upon DNA damage-induced apoptosis (Figure 6.7), thereby indicating Api5 to undergo post-translational degradation following DNA damage.

6.2.2. Api5 undergoes DNA damage-mediated degradation through the ubiquitin-proteasomal pathway by FBXW2

Previous experiments suggested that Api5 undergoes degradation via the proteasomal pathway during physiological conditions, and Api5 levels reduce upon DNA damage. It was hypothesised that Api5 undergoes proteasomal mediated degradation upon DNA damage. As proteasome complex is localised in the cytoplasm while Api5 is a nuclear protein, we first investigated the localisation of Api5 following UV damage. In control cells, Api5 was observed to be present in the nucleus, while upon UV treatment, most cells showed nuclear and cytoplasmic

localisation of Api5 (Figure 6.8A-B). Interestingly, the increase in the percentage of cells showing the only cytoplasmic presence of Api5 upon UV-irradiation in the 8 and 12 hrs post-treatment suggests that Api5 compartmentalisation changes upon UV damage (Figure 6.8B).

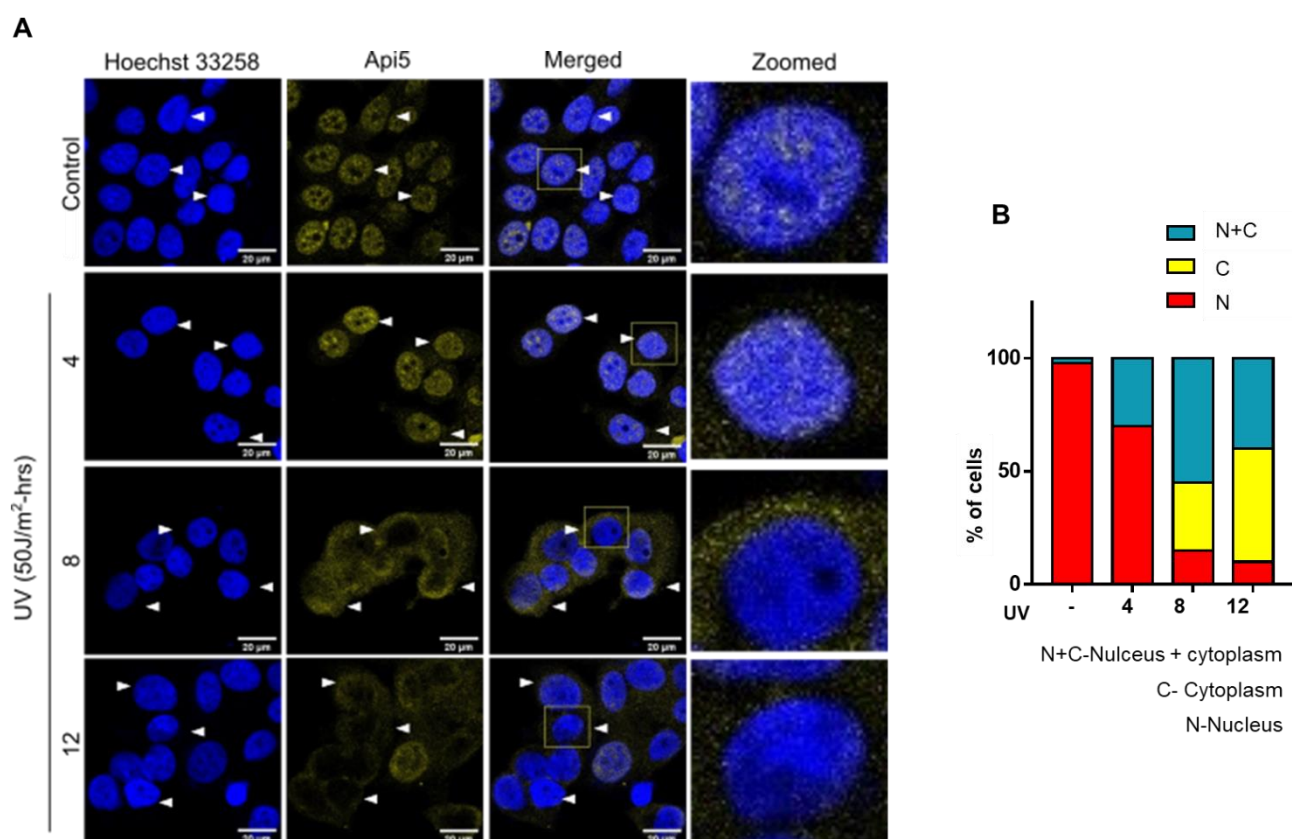


Figure 6.8: Api5 localises to the cytoplasm upon DNA damage

A) MCF7 cells treated with 50 J/m² UV and fixed post 4, 8, and 12 hrs of irradiation. Immunofluorescence was performed using Api5 specific antibody and imaged in Leca sp8 microscope at 63X oil immersion objective (scale bar: 20 μ m) and B) cells with different Api5 staining patterns were manually counted and analyzed. Data is representative of three independent experiments.

To investigate whether Api5 undergo proteasomal pathway-mediated degradation upon UV damage, Api5 levels were analysed upon inhibition of proteasomal pathway following UV damage using MG132. Restored levels of Api5 were observed upon inhibition of proteasomal pathway following UV damage using immunoblotting suggesting that DNA damage mediated degradation of Api5 was also through the proteasomal pathway (Figure 6.9A-B). Proteasome pathway-mediated degradation of Api5 upon DNA damage was confirmed using immunofluorescence assay that

showed that Api5 protein levels remained unaffected upon inhibition of proteasome pathway while only UV treated showed a reduction of Api5 protein levels (Figure 6.10 A-B).

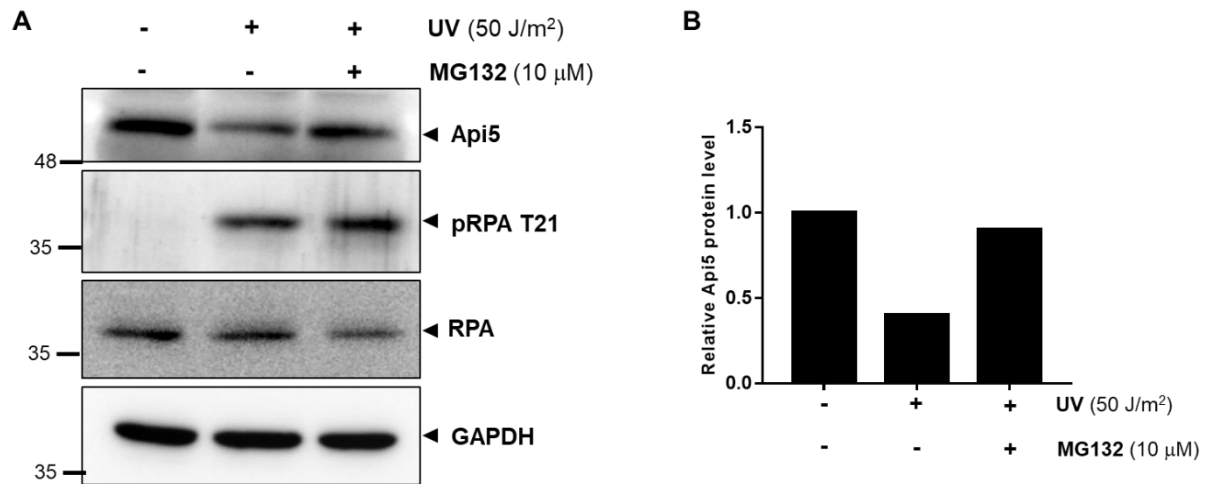


Figure 6.9: DNA damage-induced degradation of Api5 is through proteasomal pathway

A) MCF7 cells treated with 50 J/m² UV and 10 μM MG132 for 4hrs were lysed after 12 hrs post UV treatment, and Api5 levels were analysed using western blotting. B) The fold change of Api5 was quantified after normalisation with GAPDH. Data is representative of three independent experiments.

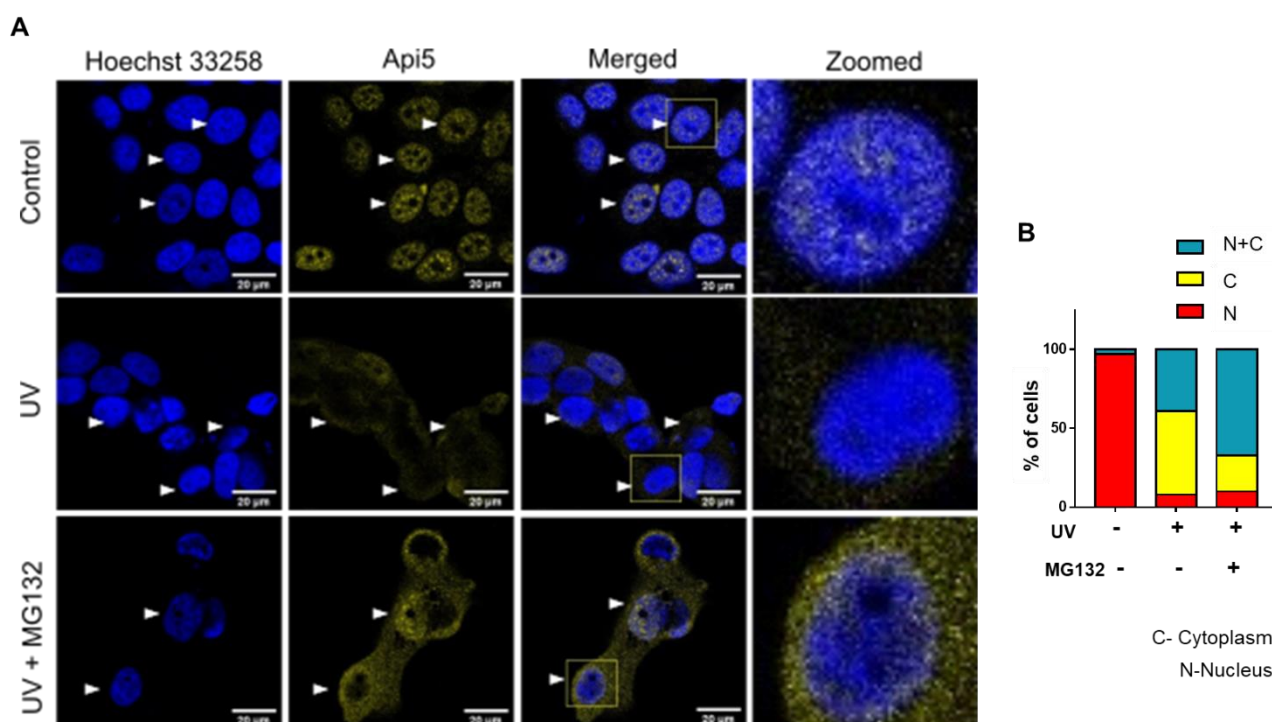


Figure 6.10: DNA damage-induced degradation of Api5 is through proteasomal pathway

A) Immunofluorescence was performed on MCF7 cells irradiated with 50 J/m² UV with and without 10 μM MG132 imaged in Leica sp8 confocal microscope (scale bar- 20 μm). B) Api5 localisation changes were manually analysed. Data is representative of three independent experiments.

To illustrate whether Api5 undergoes ubiquitination before undergoing degradation via the proteasomal pathway upon DNA damage, mCherry-tagged Api5 over-expressing stable cells were treated with and without UV for 4 hrs. These cells were lysed, and whole-cell lysates were immune-precipitated using GFP-specific antibody, and western blotting was performed using a ubiquitin-specific antibody. It was observed that Api5 undergoes poly-ubiquitination, and the poly-ubiquitination enhances upon DNA damage (Figure 6.11).

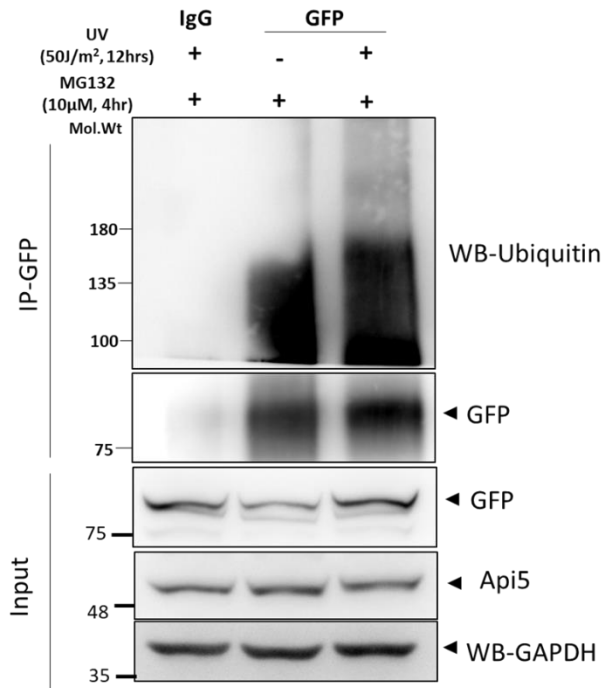


Figure 6.11: Api5 undergo poly-ubiquitination upon DNA damage-induced apoptosis

mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV and 10 μM MG132 for 4 hrs were lysed 12 hrs post UV treatment, and immunoprecipitation was performed using GFP specific antibody. Western blotting analysis was performed to analyse the ubiquitination status of Api5 using ubiquitin-specific antibody

To prove that DNA damage-mediated degradation of Api5 was also through FBXW2, Api5 levels were analyzed in FBXW2 knockdown stable cells upon UV damage. Api5 levels did not reduce UV damage in FBXW2 knockdown cells (Figure 6.12A-B), suggesting that Api5 degradation requires FBXW2 E3 ubiquitin ligase activity.

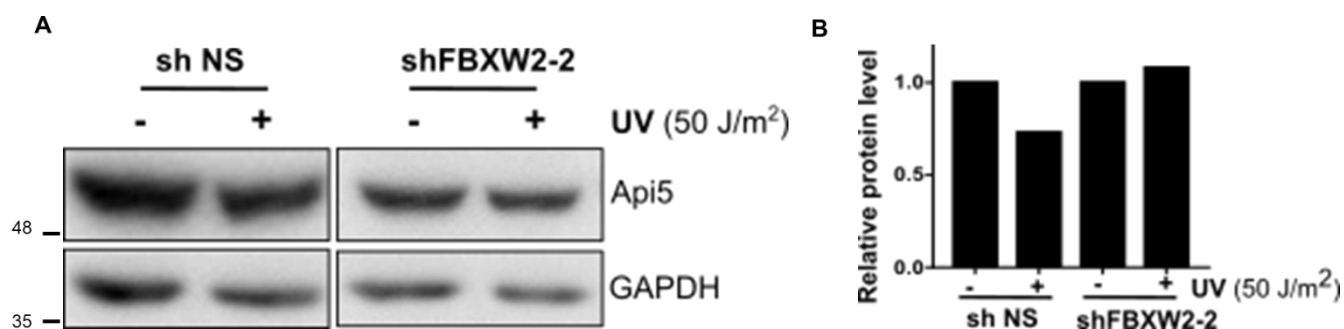


Figure 6.12: DNA damage-induced degradation of Api5 is by FBXW2

A) HeLa cells stably expressing shNS and shFBXW2#2 were lysed 12 hrs post UV (50 J/m²) treatment, and immunoblotting was performed to analyse Api5 levels. B) Api5 levels were quantified after normalising with GAPDH. Data is representative of three independent experiments.

6.2.3 ATR regulates localisation and degradation of Api5 post DNA damage

Since it has been demonstrated that Api5 undergoes ubiquitin-proteasomal mediated degradation upon DNA damage, the possibility that Api5 may get phosphorylated by specific kinases that play critical roles during DNA damage response cannot be ignored. ATR is one of the most common and studied sensor kinases that helps repair DNA damage, cell cycle arrest, and apoptosis if the cell is not able to repair the DNA damage. To perform its functions, ATR interacts and phosphorylates BRCA1, CHEK1, MCM2, RAD17, RPA2, SMC1 and p53/TP53, which collectively inhibit DNA replication and mitosis and promote DNA repair, recombination and apoptosis. After confirming the post-translational reduction of Api5 upon DNA damage, it was decided to find the regulator behind this reduction. Irradiation by UV is a very well-known and effective method of inducing DNA damage that causes significant DNA damage in the form of both single-strand and double-strand DNA breaks. Thus UV was preferred and selected as DNA damaging agent for further studies. ATR is also the main sensor protein that helps regulate DNA damage response and repair upon UV irradiation. To investigate the role of ATR in the regulation of the degradation of Api5 upon DNA damage, the ATR kinase activity was blocked using a small chemical inhibitor, cells were irradiated with UV, and the levels of Api5 were analysed. Api5 expression levels were observed to decrease upon UV damage; however, Api5 levels remained unaltered in cells where the ATR kinase activity was inhibited (Figure 6.13A-B). These results were further

corroborated using immunofluorescence studies where the cytoplasmic localisation of Api5 upon UV damage reduced upon inhibition of ATR (Figure 6.14A-B).

Interestingly Api5 was also found to form speckles inside the nucleus after blocking the ATR kinase activity in UV-treated cells. These data prove that ATR regulates DNA damage-mediated degradation of Api5.

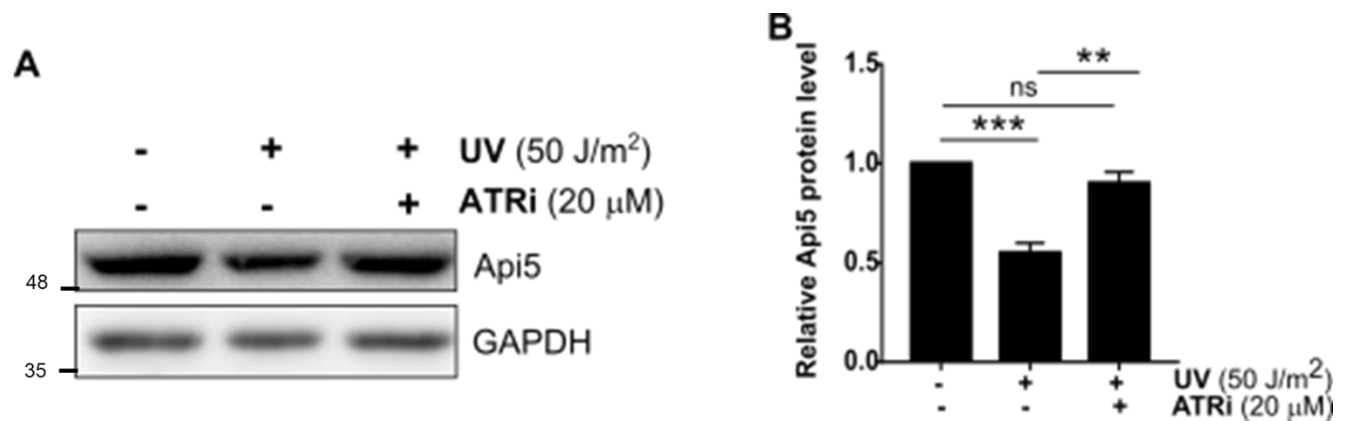


Figure 6.13: ATR regulates Api5 upon UV induced DNA damage

A) MCF7 cells were treated with UV (50 J/m² for 12 hrs) and ATRi (VE 821- 20μM for 12 hrs) were lysed, and Api5 levels were analysed using western blotting. B) The fold change of Api5 was quantified after normalisation with GAPDH. Data is representative of three independent experiments and statistical analysis used was One way Anova; ns p>0.05, **p<0.01, ***p<0.001.

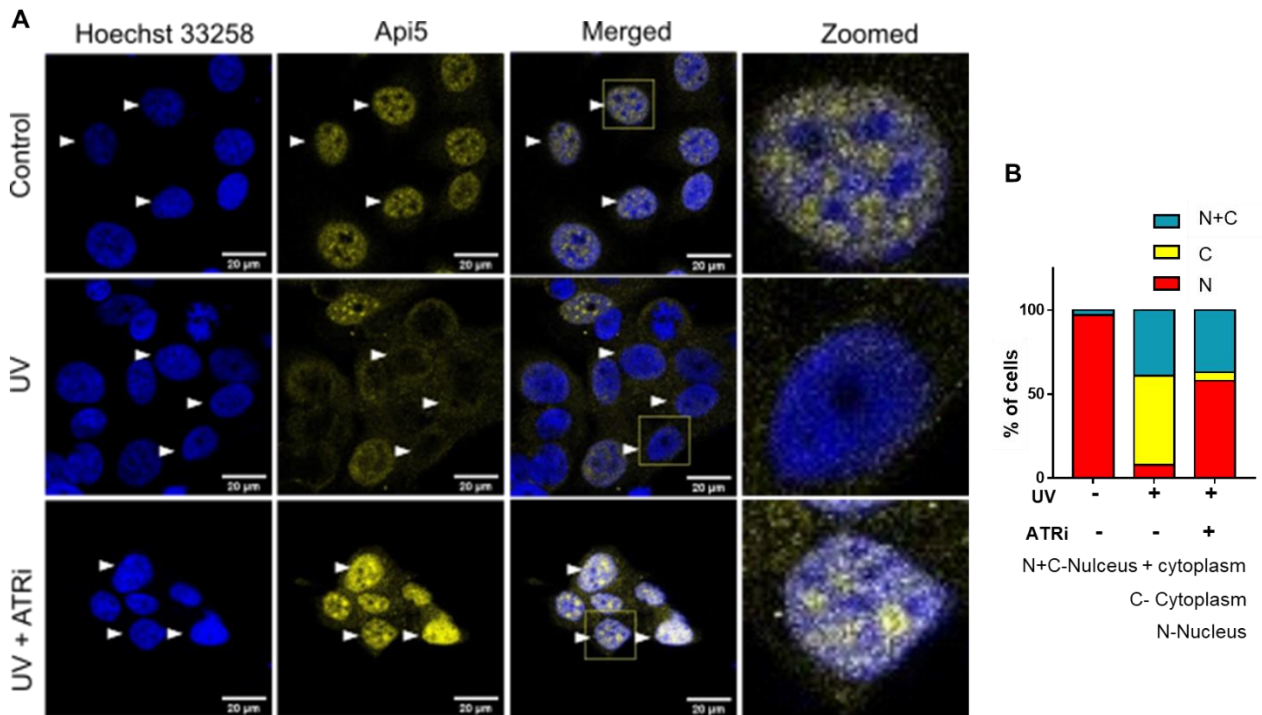


Figure 6.14: ATR regulates Api5 localisation upon UV induced DNA damage

A) Immunofluorescence assay was performed on MCF7 cells treated with UV (50 J/m² for 12 hrs) and ATRi (VE 821- 20μM for 12 hrs) using Api5 specific antibody B) and Api5 localisation was manually analysed. Data is representative of three independent experiments.

After confirming the role of ATR in the regulation of Api5, it was hypothesized that ATR might be the kinase that is phosphorylating Api5. To carry out the function of phosphorylation, ATR should interact physically with Api5. To demonstrate the interaction between ATR and Api5, immunoprecipitation studies were carried out in stable cells expressing mCherry-tagged Api5 using GFP-specific antibody following UV damage. ATR was observed to interact with Api5 (Figure 6.15A). This interaction was further confirmed by reverse immunoprecipitation, where the pulldown was performed using an ATR-specific antibody (Figure 6.15B). The presence of Api5 in the ATR pull-down lysates confirmed the interaction between Api5 and ATR.

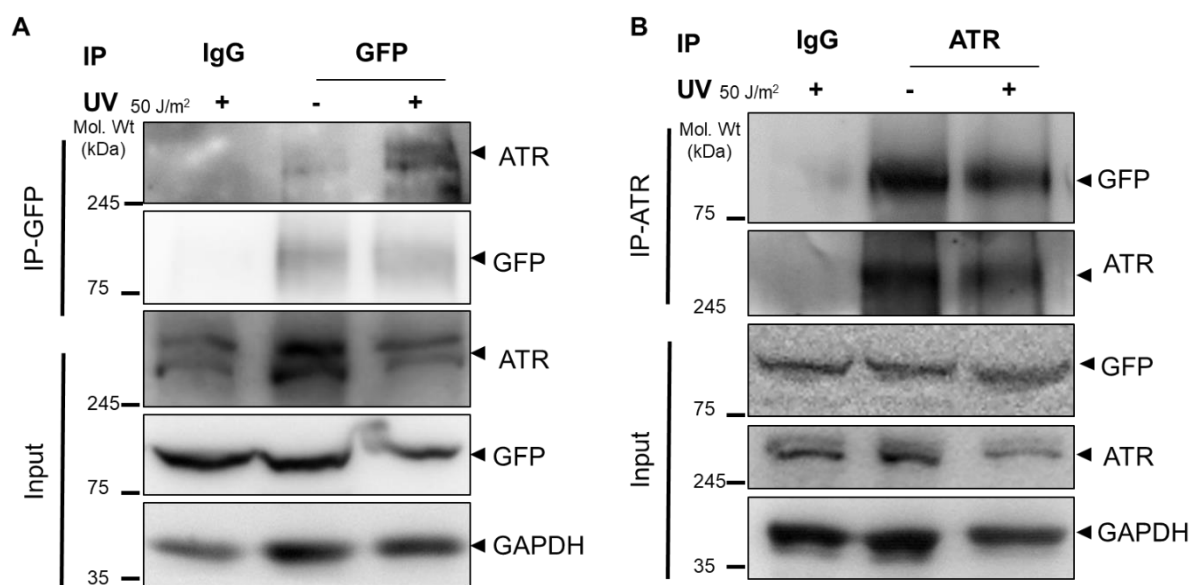


Figure 6.15: ATR interacts with Api5 upon UV induced DNA damage

mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV were lysed 12 hrs post-treatment, and immunoprecipitation was performed using A) GFP and B) ATR specific antibody. Western blotting analysis was performed to analyse the presence of ATR and Api5, respectively.

6.2.4 ATR phosphorylates Api5 at serine 138 and regulates apoptosis upon DNA damage

In silico studies predict Api5 to get phosphorylated by many different kinases, including Akt, Aurora kinase B, ATM and ATR (data not shown). As ATR was found to be an interactor and regulator of Api5 upon DNA damage, it was interesting to investigate whether ATR can phosphorylate Api5 upon DNA damage. ATR is a serine-threonine kinase that phosphorylates its substrates at either serine or threonine residues followed by glutamine. Phospho predicts site analysis showed that ATR could phosphorylate Api5 at serine 138 site (Figure 6.16A). To prove Api5 is one of the substrates of ATR phosphorylation, mCherry-tagged Api5 stable cells were lysed and pulled down using GFP- specific antibody, and immunoblotting was performed using a phospho-serine specific antibody. The presence of phospho-serine at the size of Api5 confirmed that Api5 undergoes phosphorylation upon DNA damage (Figure 6.16B). This phosphorylation could be a signal for Api5 to undergo ubiquitin-proteasomal mediated degradation. To understand the role of this phosphorylation on the stability of Api5, site-directed mutagenesis was performed to mutate serine 138 to alanine. mVenus tagged wild-type, and S138A mutant of Api5

was ectopically expressed, and their stability was analysed upon DNA damage using immunoblotting. Upon DNA damage, it was observed that protein levels of wild type Api5 reduced while Api5 S138A remained unaltered, suggesting that phosphorylation at serine 138 of Api5 is responsible for its degradation (Figure 6.16C-D).

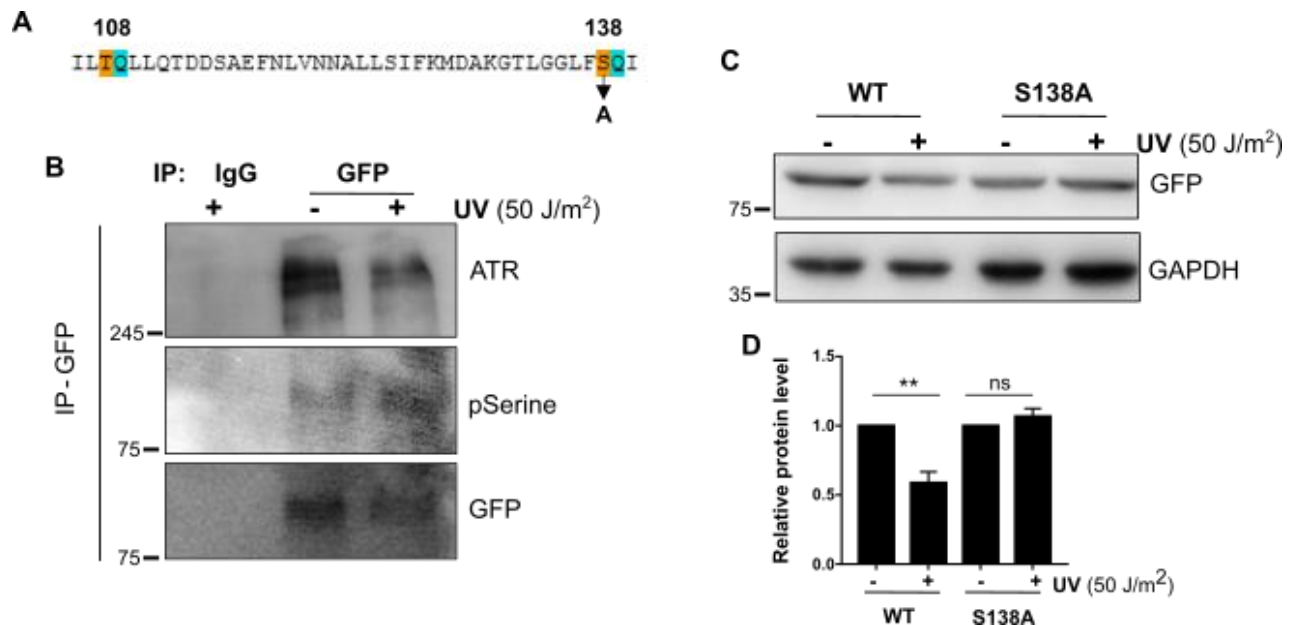


Figure 6.16: ATR leads to phosphorylation of Api5 at Serine 138 upon DNA damage

A) *In-silico* analysis predicts possible ATR phosphorylation sites in Api5 protein highlighting T108 and S138. B) mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV were lysed 12 hrs post-treatment, and immunoprecipitation was performed using GFP-specific antibody. Western blotting analysis was performed to analyse the presence of phospho-serine and ATR. Serine at 138 residues on Api5 was mutated to alanine by site-directed mutagenesis. MCF7 cells transfected with mVenus tagged-WT and S138A-Api5 mutant were UV irradiated (50 J/m²) post 24hrs of transfection and lysed post 12 hrs of UV treatment. MCF7 cells transfected with mVenus tagged WT and S138A-Api5 mutant were lysed, and Western blotting was performed to check the levels of C) Api5, and D) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments and statistical analysis used was T-test; ns p>0.05, **p<0.01.

To understand the function of Api5 S138A mutant upon UV damage, levels of various DNA damage repair and apoptotic markers were analysed after transfection

of wild-type and Api5 S138A. Levels of pro-apoptotic proteins like cleaved PARP and caspase 9 were found to be elevated in wild-type cells upon UV damage while no change in levels of cleaved PARP or caspase 9 were observed in cells expressing Api5 S138A mutant, suggesting that ATR-mediated degradation of Api5 plays a significant role in the regulation of DNA damage-induced apoptosis (Figure 6.17A-C).

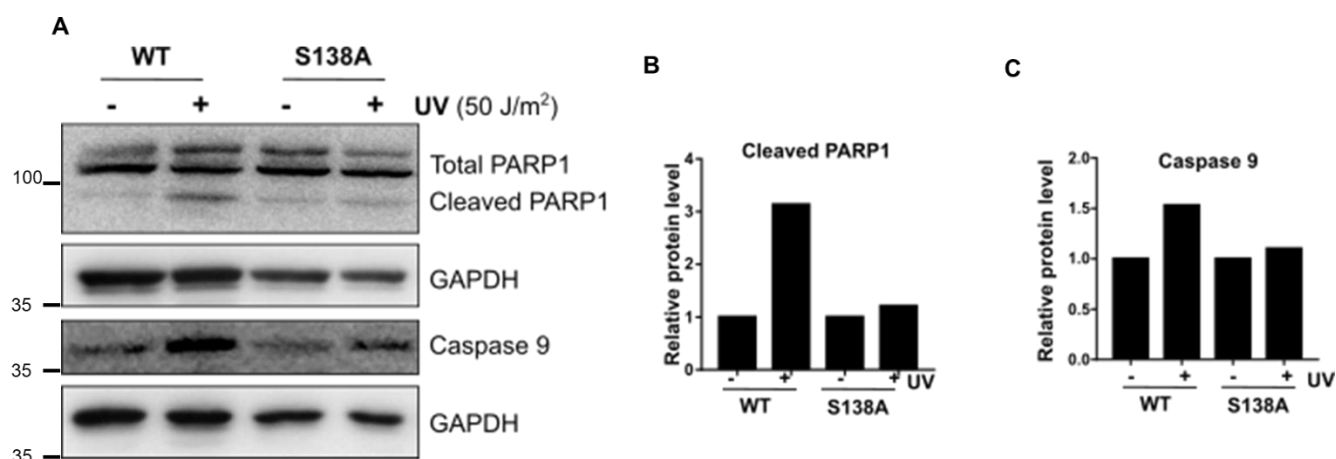


Figure 6.17: ATR mediated degradation of Api5 regulate DNA damage-induced apoptosis

MCF7 cells transfected with mVenus tagged-WT and S138A-Api5 mutant were UV irradiated (50 J/m²) post 24hrs of transfection and lysed post 12 hrs of UV treatment. MCF7 cells transfected with mVenus tagged WT, and S138A-Api5 mutant were lysed, and Western blotting was performed to check the levels of A) PARP and Caspase 9, and B-C) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

The reduction in levels of apoptotic markers in Api5 S138A mutant cells may be due to reduced sensing of UV damage. It was, therefore, interesting to look for other DNA damage markers. As expected, there was a significant difference in levels of total p53 and activation of p53 at serines 15, 37 and 46 upon DNA damage. Cells expressing Api5 S138A showed reduced activation of p-p53 S15, p-p53 S37, p-p53 S46 as well as total p53 as compared to cells expressing wild-type Api5 upon UV damage (Figure 6.18A-D), suggesting that phosphorylation of Api5 at serine 138 not only regulates the stability of the protein but also desensitise cells towards the DNA damage as well as DNA damage-induced apoptosis.

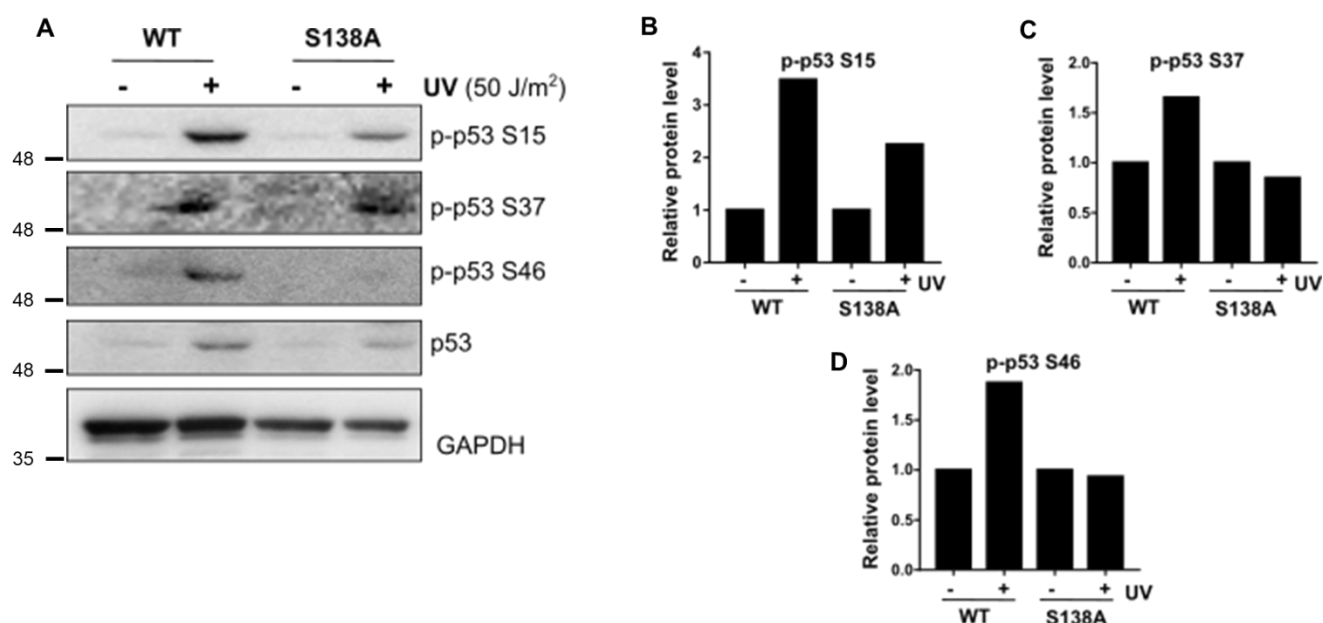


Figure 6.18: ATR mediated degradation of Api5 regulate DNA damage sensing
MCF7 cells transfected with mVenus tagged-WT and S138A-Api5 mutant were UV irradiated (50 J/m²) post 24hrs of transfection and lysed post 12 hrs of UV treatment. MCF7 cells transfected with mVenus tagged WT and S138A-Api5 mutant were lysed, and Western blotting was performed to check the levels of A) p53 S15, p53-S37, p53 S46, and total p53 and B-D) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

6.2.5 De-acetylated form of Api5 undergoes degradation during DNA damage

Our studies have demonstrated that p300 and HDAC1 are the enzymes involved in regulating the stability of Api5. p300 acetylates Api5 at lysine 251 (K251) and maintains its stability while the HDAC1 deacetylates and leads to the degradation of Api5 (Chapter 3). As our results also report Api5 to undergo ubiquitin-proteasome pathway mediated post-translational degradation, it was interesting to investigate the role of p300-mediated acetylation and HDAC1 deacetylation on the regulation of stability and anti-apoptotic function of Api5 following DNA damage. UV-irradiated cells were treated with romidepsin, a small chemical inhibitor of HDAC1 and levels of Api5 were analysed post UV damage. Api5 levels remained unaltered upon inhibition of HDAC1 in UV-irradiated cells suggesting that DNA damage-induced degradation of Api5 requires de-acetylation by HDAC1 (Figure 6.19A-B).

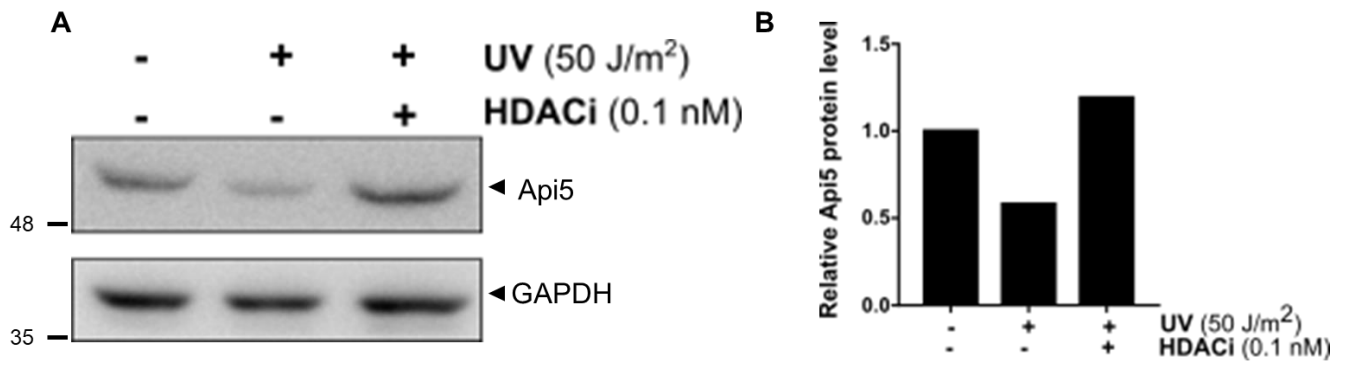


Figure 6.19: De-acetylated form of Api5 undergoes degradation upon DNA damage

A) MCF7 cells treated with HDACi (romidepsin-0.01nM for 12hrs) and UV (50 J/m²) were lysed post 4 hrs of UV irradiation, and Api5 levels were analysed using western blotting. B) The fold change of Api5 was quantified after normalisation with GAPDH. Data is representative of three independent experiments.

To check for other molecular players involved in acetylation and stability of Api5 upon UV damage, cells were treated with UV-irradiation, and protein levels of p300 and HDAC1 were analysed. p300 levels were reduced upon UV damage while HDAC1 levels remained unaffected (Figure 6.20A-F).

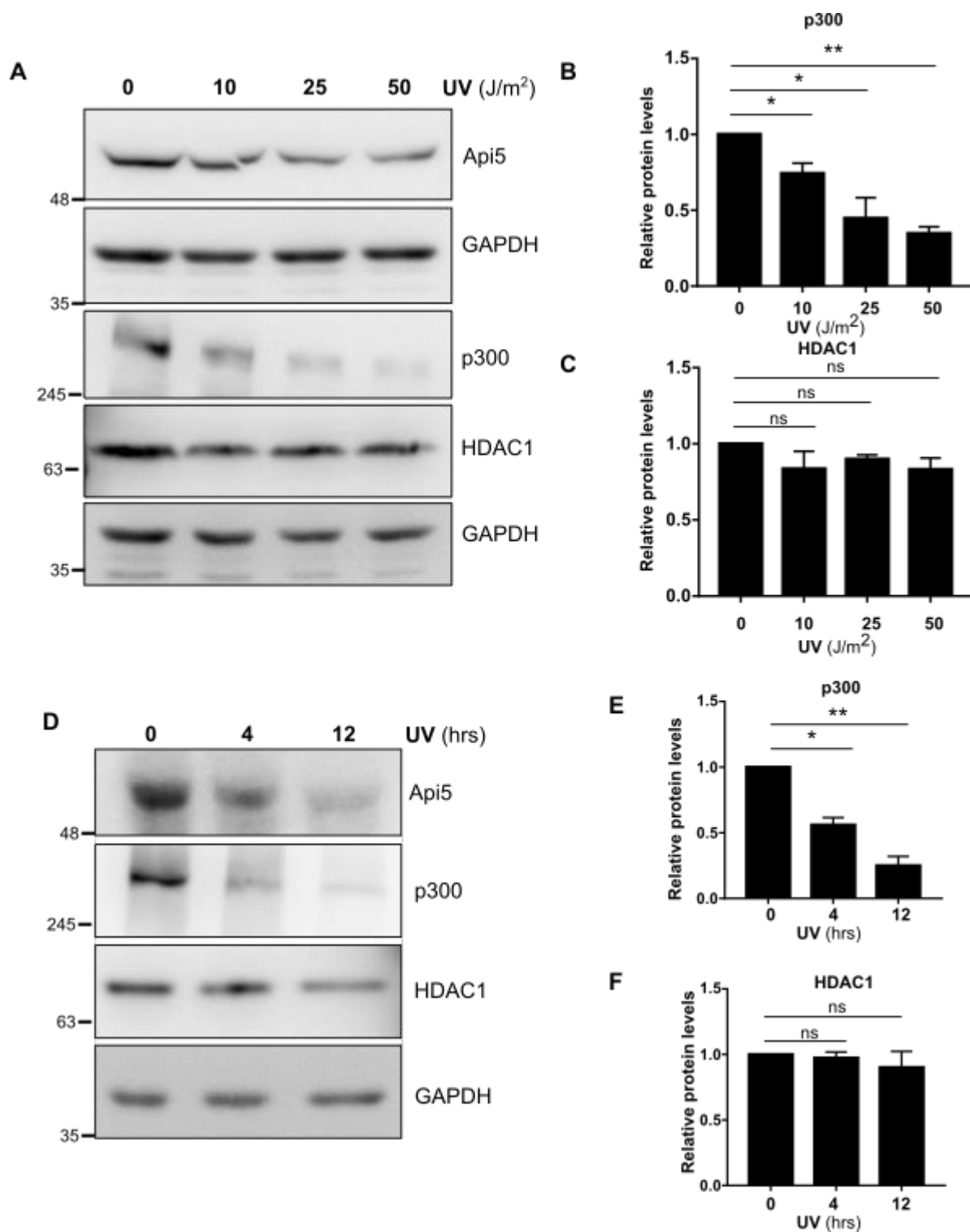


Figure 6.20: p300 decreases while HDAC1 remains unchanged upon DNA damage

MCF7 cells irradiated with A) varying UV doses for 12 hrs and D) 50 J/m² UV for different time points were lysed and immunoblotted to analyse for the levels of Api5, p300, and HDAC1. B, C, E and F) The protein levels were quantified after

normalising to GAPDH. Data is representative of three independent experiments and statistical analysis used was One way Anova; ns $p>0.05$, * $p<0.05$, ** $p<0.01$.

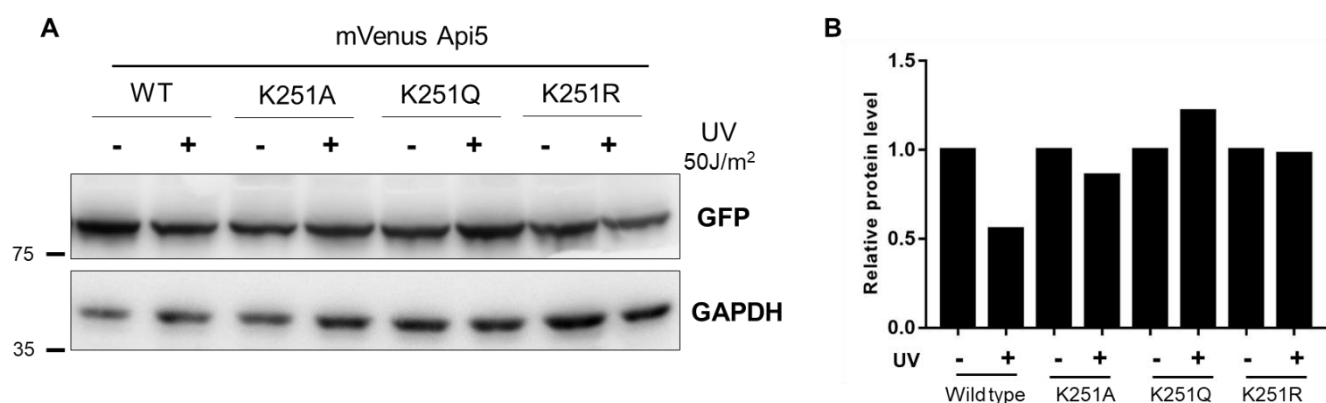


Figure 6.21: Acetylation status affects DNA damage-induced degradation of Api5

MCF7 cells were transfected with mVenus tagged K251 mutant constructs and treated with 50J/m² UV post 24 hrs of transfection and lysed 12 hrs post UV treatment. Western blotting was performed to check for the levels of A) GFP (mVenus Api5) and B) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

To demonstrate that acetylation of Api5 at K251 is required for maintaining the stability of Api5 following DNA damage, mVenus-tagged K251 mutants of Api5, (K251A: charge deficient mutant, K251R: charge mimic mutant, and K251Q: constitutive acetylation mimic mutant) were ectopically expressed along with wild-type Api5 followed by UV-irradiation and the levels of Api5 were analysed post DNA damage. Wild type Api5 levels were observed to reduce while other K251 mutant protein levels did not alter upon UV-irradiation, suggesting that acetylation at lysine 251 plays a crucial role in maintaining the stability of Api5 (Figure 6.21A-B).

The stability of proteins is generally considered to be direct evidence for their functions; therefore, levels of various DNA damage and apoptotic markers were analysed in Api5 wild-type and K251 mutant expressing cells upon UV damage. Wild-type Api5 showed increased levels of cleaved PARP (Figure 6.22A-C), caspase

2 and caspase 9 (Figure 6.23A-C), while the other Api5 acetylation mutants failed to show any difference in levels of the proteins.

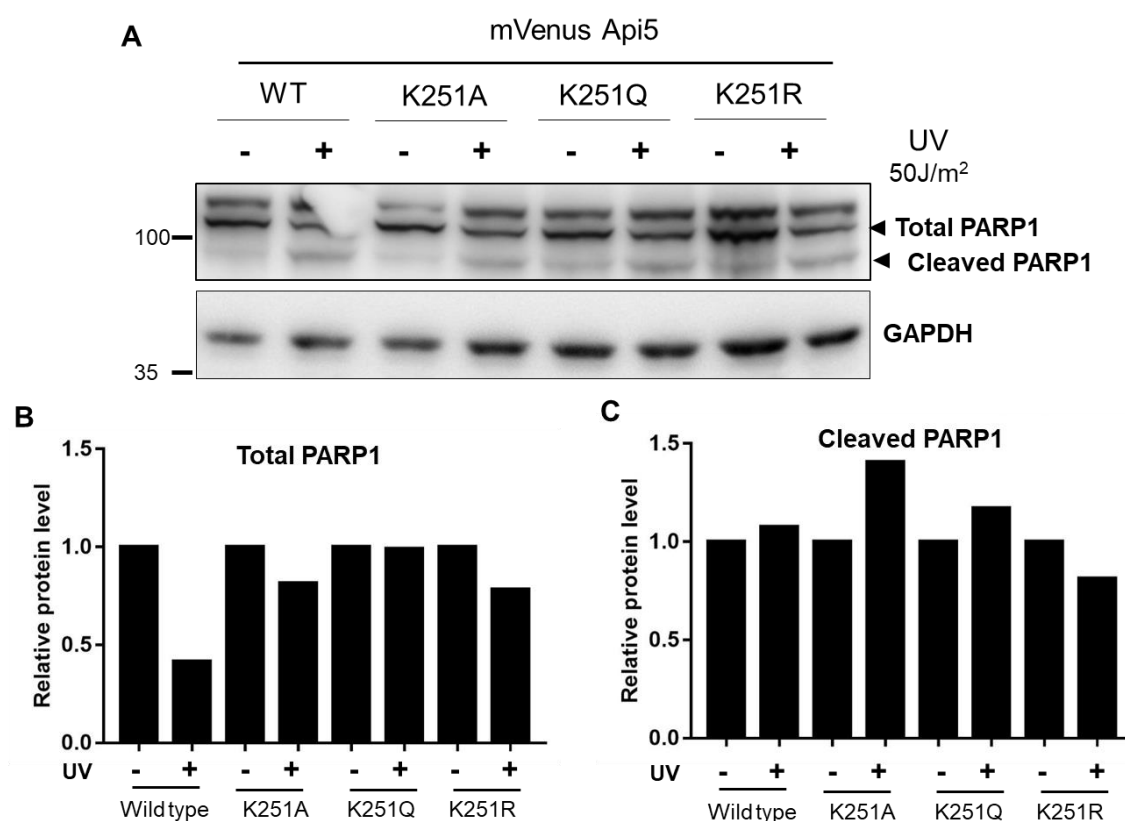


Figure 6.22: Acetylation status of Api5 affects DNA damage-induced apoptosis

MCF7 cells were transfected with mVenus tagged K251 mutant constructs and treated with 50J/m² UV post 24 hrs of transfection and lysed 12 hrs post UV treatment. Western blotting was performed to check for the levels of A) PARP1 and B-C) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

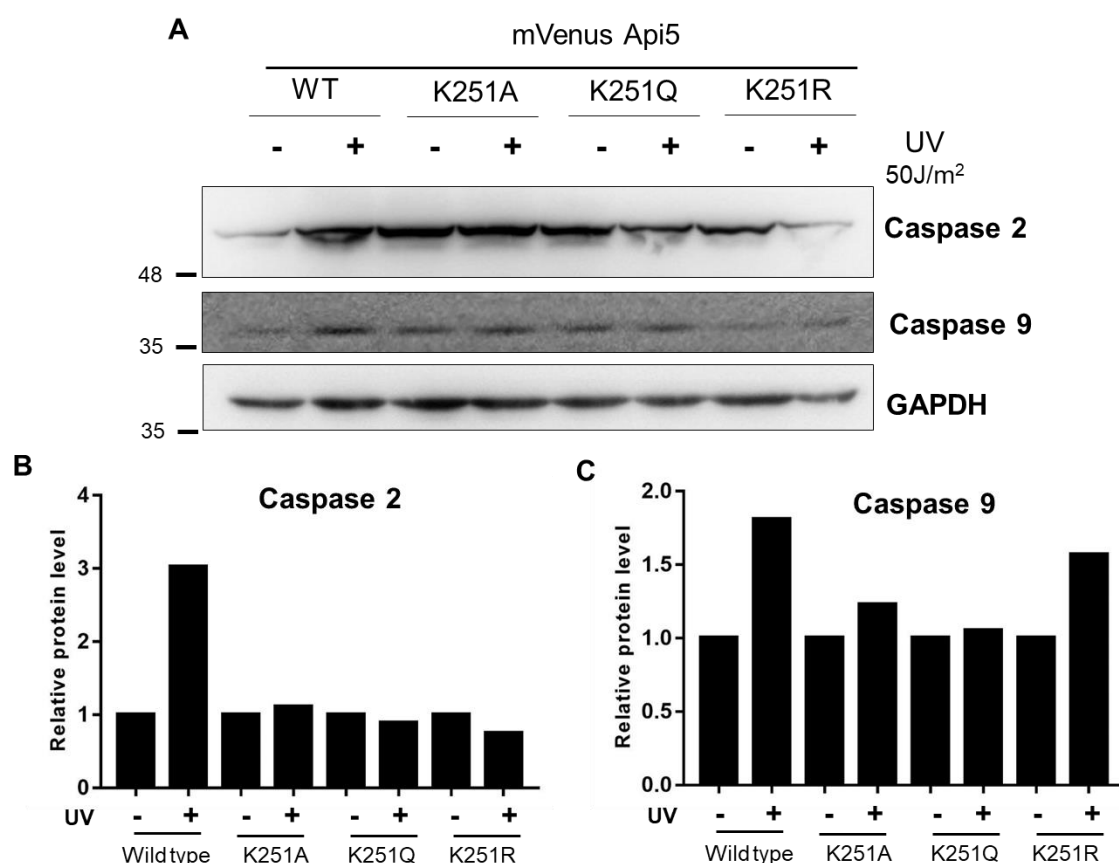


Figure 6.23: Acetylation status of Api5 affects caspase activation

MCF7 cells were transfected with mVenus tagged K251 mutant constructs and treated with 50J/m² UV post 24 hrs of transfection and lysed 12 hrs post UV treatment. Western blotting was performed to check for the levels of A) Caspase 2 and Caspase 9 and B-C) quantified after normalising to the loading control GAPDH. Data is representative of three independent experiments.

To understand the role of Api5 and K251 acetylation of Api5 in cell survival upon DNA damage, wild type and K251 mutants were stably expressed in cells with Api5 stable knockdown. Cells with Api5 knockdown showed reduced survival upon camptothecin and etoposide compared to the control cells, while upon rescue with wild type Api5, cell survival was restored (Figure 6.24A-B). K251Q mutant of Api5 also facilitated cell survival similar to wild type Api5; however, K251A mutant of Api5 could not rescue the cell survival upon DNA damage. These results suggest that Api5 acetylation at K251 plays a significant role in regulating DNA damage-induced apoptosis.

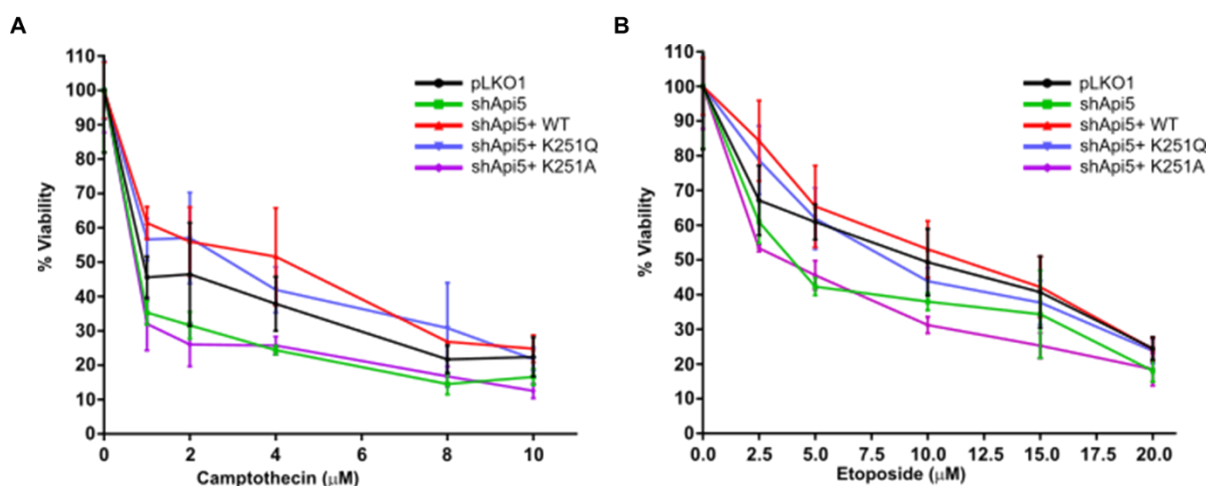


Figure 6.24: Acetylation status of effects chemoresistance

MCF7 cells stably expressing shApi5 and shApi5 resistant K251 mutants were treated with varying concentrations of A) campdothecin and B) etoposide for 24hrs. The viability of cells was measured using an MTT assay. Data is representative of three independent experiments.

6.3 Discussion

Api5 is an anti-apoptotic protein that helps survive cells during serum starvation conditions (Tewari et al., 1997). Later it was found that the anti-apoptotic function of Api5 was not only confined to serum starvation conditions but also could potentially inhibit apoptosis during infections and DNA damage conditions (Mayank et al., 2015) (Rigou et al., 2009b) (Imre and Rajalingam, 2017). However, the mechanism by which Api5 can regulate itself during stress conditions like serum starvation, infections and DNA damage is yet to be understood. In this study, we report that Api5 undergoes degradation upon induction of DNA damage-induced apoptosis. UV-irradiation and chemical damage resulted in a reduction in Api5 protein levels. Our results suggest that post-translational degradation of Api5 upon DNA damage-induced apoptosis is through the proteasomal pathway. Ubiquitination is the post-translational modification that targets Api5 towards the proteasome machinery. However, we are not dismissing the possibility that Api5 may be regulated via other post-translational modifications and/or other pathways involved in the post-translational degradation of Api5.

FBXW2 is an SCF type of E3 ubiquitin ligase involved in the ubiquitin-mediated degradation of potential cell cycle regulator SKP2 (Xu et al., 2017). It has already

been reported that in lung carcinomas, SKP2, a potential oncogenic protein, gets degraded by FBXW2, which thus acts as a tumour suppressor. Api5 is also upregulated in different types of cancers like cervical, oesophageal and lung cancers (Bousquet et al., 2019; Cho et al., 2014; Lawrenson et al., 2015; Xu et al., 2017). Multiple studies by various groups suggest that Api5 can also be a potential oncogenic factor. Its downregulation could potentially make cells vulnerable towards DNA damage mediated cell death (Rigou et al., 2009b). Our studies confirmed that FBXW2 interacts with Api5 and promotes its degradation through ubiquitination during physiological conditions and DNA damage-induced apoptosis.

Phosphorylation is a secondary but essential signal for the degradation of multiple proteins via the proteasomal pathway. ATR is one of the primary sensor kinases that, upon sensing DNA damage, activates a cell cycle checkpoint response by phosphorylating specific proteins like Chk1 and p53 (Abraham, 2001; Liu et al., 2000; Tibbetts et al., 1999). Our results indicate that ATR binds to Api5 and phosphorylates it at the serine 138 residue. Phosphorylation of Api5 at serine138 is required for its degradation during DNA damage-induced apoptosis. Upon DNA damage, Api5 first gets de-acetylated by HDAC1, and the de-acetylated Api5 undergoes post-translational degradation through the proteasomal pathway after getting phosphorylated by ATR. The phosphorylated form of Api5 localises to the cytoplasm and undergoes degradation post-DNA damage, while the nuclear-localised form of Api5 is acetylated and stabilised by p300 histone acetyltransferase. The acetylated form of Api5 that does not undergo degradation upon DNA damage may be playing a role in DNA repair and differential regulation of apoptosis upon DNA damage. In our studies, we identified ATR as a major regulator of Api5; however, we are also aware that other regulators like ATM and DNA-PKcs may also play a role that is yet to be investigated. Higher levels of Api5 are found to be associated with higher aggressiveness of cancers and poor cancer patient survival (Kim et al., 2000). Ectopic expression of Api5 can also promote properties like invasion, migration and metastasis in cancers (Basset et al., 2017). Depletion of Api5 induces apoptosis, while over-expression of Api5 provides chemo-resistance to certain cancers (Kim et al., 2000). These studies demonstrate Api5 to be a potential oncogene, and it is essential to understand the mechanism of regulation of stability and the function of Api5 upon DNA damage. By understanding the molecular

mechanism of regulation of Api5, multiple cancers can be targeted using techniques or molecules which specifically block the function of Api5. These molecules can be used as potential therapeutic agents to treat cancers where Api5 is over-expressed. Taken together, we report FBXW2 and ATR as the novel regulators of Api5 upon DNA damage. Upon DNA damage HDAC1 de-acetylates Api5 and de-acetylated Api5 undergo post-translational degradation. ATR phosphorylates Api5 at S138 upon UV irradiation and targets it towards proteasome complex for its degradation through FBXW2 E3 ubiquitin ligase (Figure 6.25).

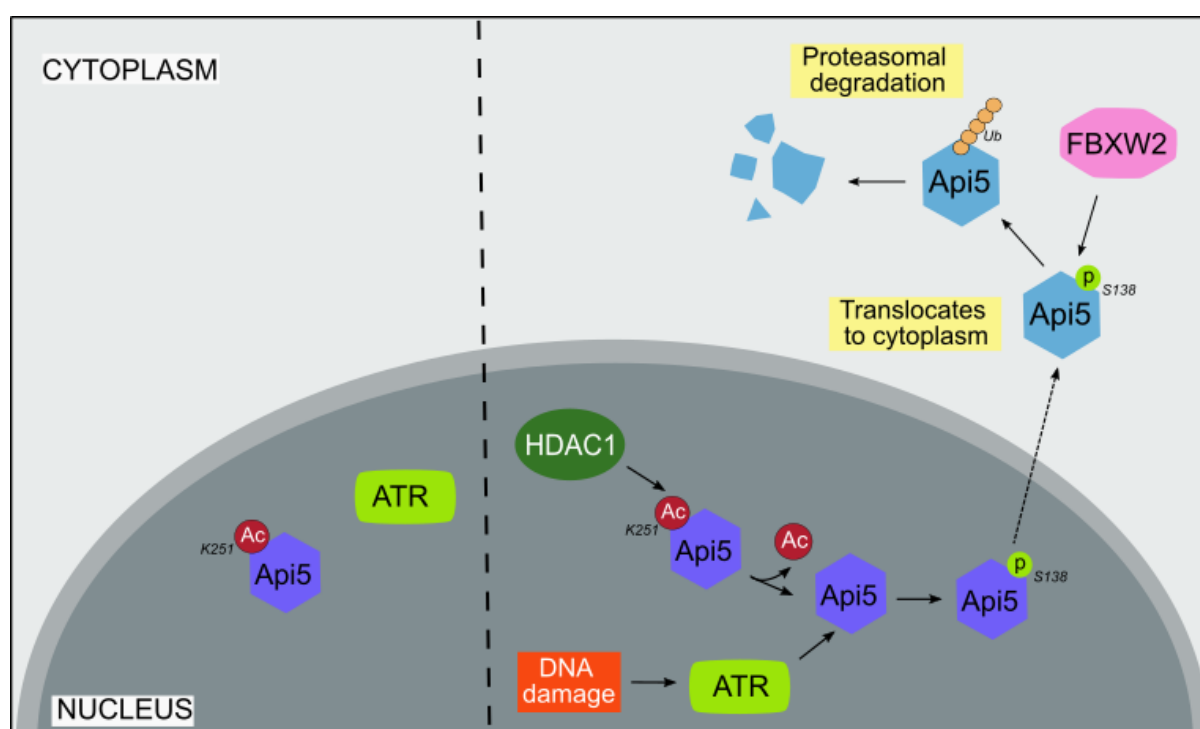


Figure 6.25: Schematic representation depicting the regulation of Api5 upon DNA damage

A proposed model deciphering the pathway of regulation of Api5 by its key regulators HDAC1, ATR, and FBXW2 upon DNA damage

Chapter 7

Discussion and future perspectives

7.1 Discussion

The biological functions of the cells are regulated by various bio-macromolecules such as proteins, carbohydrates, lipids, and nucleic acids. Proteins are the main macromolecules that provide various aspects to the cell. For a cell, proteins act as the structural unit as it is one of the main constituents of the plasma membrane along with lipids (Herrmann et al., 2021). Proteins also act as the mechanical support for a cell by being the essential part of the cytoskeleton (Herrmann et al., 2007). Proteins provide energy to the cell by being the central players of metabolic pathways (Zaidi et al., 2012). Proteins as receptors help to sense the intracellular as well as the extracellular environment (Jia et al., 2015). For cells, proteins act as both transporters as well as the mode of transport as they perform the roles of both cargo or carriers and vesicles (Cabukusta and Neefjes, 2018). Thus it can be considered that proteins are one of the major functional regulators of the cells. At the organism levels also, proteins make sure that the intact and error-free genetic material is being transferred to the coming generations (Yang and Gao, 2018). As this bio-macromolecule is involved in these many sensitive activities of the cells, for a cell, also it becomes mandatory to regulate the synthesis, stability, and functionality of the proteins. Maintaining the stability of the proteins is as important as synthesising them as the cell would prefer to maintain the protein levels to meet the adequate requirement of structural support, energy, ability to sense and motion. A half-life of a protein can also be considered the duration of its function. Sometimes for a cell, it becomes essential to shut the ongoing physiological response to initiate the new one. This shutting is achieved by either inactivating or degrading the protein. Most of the time, inactivation of the proteins also ends up with their degradation (Taguchi et al., 2012). The inability of a cell to degrade its protein leads to its accumulation beyond the threshold that eventually turns out as pathological conditions like disorders, syndromes, and diseases (Krisko and Radman, 2019). For example, the accumulation of amyloid-beta and Tau protein beyond its critical concentration inside the cell results in Alzheimer's disease (Bloom, 2014).

As mentioned earlier, that there are multiple studies where Api5 was found to be upregulated in multiple types of cancers (Bousquet et al., 2019; Cho et al., 2014; Lawrenson et al., 2015; Xu et al., 2017), and this upregulation causes induction of

hyper-proliferation (Cho et al., 2014). These upregulation-related pathophysiological conditions could also be because of the alteration in dynamics of synthesis and degradation. To avoid such conditions, it becomes mandatory and beneficial to understand the molecular mechanism of dynamics of stability and degradation.

In our third chapter, we discovered that p300, a histone acetyltransferase is an enzyme that acetylates Api5 at K251. This acetylation is very crucial for the stability and function of Api5 in physiological conditions. This p300 mediated acetylation enhances the stability of Api5. We also observed that acetylated Api5 remains inside the nucleus, which is its native compartment. Thus to conclude, p300 acts as a major regulator of Api5 stability. In this chapter, we also observed that Histone deacetylase 1 (HDAC1) is the enzyme that removes the acetyl group from Api5 and makes it susceptible to undergo post-translational degradation. Before going to the protein level degradation, Api5 switches its localisation from the nucleus to the cytoplasm. Although we found out the crucial regulators of Api5, some other various aspects and regulators are yet to be discovered. For example, upon stabilisation by p300, the function of Api5 inside the nucleus is not identified.

As reported, K251 acetylation of Api5 is conserved from protists to mammals (Han et al., 2012); thus, it also becomes more relevant to understand the physiological function of acetylated Api5. Our results and literature suggested that Api5 is present inside the nucleus and interacts with the chromatin content of the cells. This also draws attention to the possibility of Api5 being a transcription factor. This transcriptional activity might be regulating the cell cycle progression. One other aspect to be understood in the regulation of Api5 stability is the involvement of carrier and transporter proteins. As it is known that de-acetylated Api5 is translocated to the cytoplasm, there must be some transporters that help in its translocation. These are some aspects that can be relevant to study in normal physiological as well as pathophysiological conditions like cancer. The relevance of studying the regulation of localisation also becomes more prominent as various types of cancers are the outcome of the mislocalisation of proteins (Lovelock et al., 2006; Santivasi et al., 2015).

As it was discovered in chapter 3 that deacetylation of Api5 leads to its post-translational degradation, we identified the pathway and mechanism of

posttranslational degradation of Api5 in chapter 4. We observed that ubiquitination is the posttranslational modification that targets Api5 towards the proteasome system for its degradation. This degradation is achieved and accomplished with the help of SCF-type E3 ubiquitin ligases. FBXO3, FBXO6, and FBXW2 are the E3 ubiquitin ligases that, upon overexpression, degrades Api5. Multiple reports have suggested that FBXW2 acts as a potential tumour suppressor and Api5 a potent oncogene; thus, FBXW2 could be a key player leading to degradation of Api5 (Basset et al., 2017; Xu et al., 2017; Yang and Sun, 2019; Yin et al., 2019). In our studies, we observed that FBXW2 interacts with Api5 at both endogenous well as exogenous levels. Accumulation of Api5 in FBXW2 knockdown cells confirmed that FBXW2 is the main E3 ubiquitin ligase involved in the degradation of Api5. FBOX are the newly discovered proteins that regulate essential physiological processes of the cells like cell division and DNA replication (Dutta et al., 2019; Zheng et al., 2016). Thus, FBOX proteins can be used as agents to treat diseases caused by the accumulation of causative proteins.

Api5 protein expression has been reported to fluctuate during the cell cycle phase (Garcia-Jove Navarro et al., 2013). Api5 levels are maximum during the G1 and S phases of the cell cycle, while in the G2-M phase, it is at its minimum (Garcia-Jove Navarro et al., 2013). It was interesting to know how Api5 was undergoing post-translational degradation in the G2-M phase as the API5 mRNA levels were not affected across the cell cycle. We observed that Api5 undergoes post-translational degradation via proteasomal pathway after ubiquitination (Chapter 5). FBXW2 knockdown studies confirmed that Api5 degradation in the G2-M phase is achieved by FBXW2. In this chapter, we also discovered Aurora kinase B as a major regulator of Api5 in the G2-M phase of the cell cycle. Aurora kinase B regulates the mitosis phase entry of the cells. We also speculated that Aurora kinase B can phosphorylate Api5 before ubiquitin-proteasome-mediated degradation.

We also identified the biological functions of Api5 when its levels are high during the G1 and S phases of the cell cycle. We observed that Api5 in G1 and S phase helps in maintaining the nuclear architecture. For the proteins whose levels are cell cycle phase-dependent, cells must regulate their levels tightly as any amount of change in their protein levels can affect the cell cycle. We observed that Api5 depleted cells tend to undergo senescence. Such cell cycle phase-dependent proteins are

essentially required to be maintained to the standard levels in their respective phases as these proteins are crucial for cell cycle progression (Martin et al., 1998). We also observed that depletion of Api5 causes drastic effects in the G2-M phase of the cycle. We observed phenotypes like chromosomal bridging, lagging, multipolar chromosomes, and micronuclei formation upon knockdown of Api5 during the G2-M phase of the cell cycle. Mechanistically we observed that p53 and p21 are the main regulators upregulated upon the knockdown of Api5 in the G1, S, G2, and M phases of the cell cycle. Multiple studies suggest the role of p53 and p21 in the proper completion of cell cycle phases (Fiscella et al., 1993; Xu et al., 2014). These proteins might also be playing a significant role in the regulation of mitotic defects in Api5 knockdown cells during the M phase of the cell cycle. This indicates that Api5, despite being an anti-apoptotic protein, also regulates the normal progression of the cell cycle.

During apoptosis, the cell is programmed to undergo certain morpho-physiological changes like cellular membrane blabbing (Bortner et al., 1995) and phosphatidylserine flipping to the outer leaflet of the membrane (Bratton et al., 1997). Inside the nucleus, chromatin undergoes heterochromatisation and fragmentation upon receiving the apoptotic cues from the surrounding micro-environment (Zhang and Xu, 2000). This apoptotic signal inside the cells can also be initiated upon experiencing high-intensity DNA damage. DNA damage beyond the extent of repair causes activation of apoptotic signalling cascade (Surova and Zhivotovsky, 2013). During the decision-making process to initiate apoptosis, cells regulate and activate a pool of proteins involved in the process of apoptosis. In the sixth chapter, we observed that Api5 at its endogenous levels inhibits apoptosis. Api5 protein expression reduces upon induction of DNA damage that leads to the initiation of apoptosis. The reduction of Api5 upon induction of DNA damage is through the ubiquitin-proteasome pathway that takes place inside the cytoplasm. Api5 being a nuclear protein, translocates to the cytoplasm upon DNA damage before its degradation through the proteasome system. FBXW2 is one of the major E3 ubiquitin ligases involved in the post-translational degradation of Api5 upon DNA damage. To proceed with ubiquitination-mediated degradation, Api5 is phosphorylated by ATR, a DNA damage sensor kinase at serine 138. This phosphorylation leads Api5 to undergo post-translational degradation via the proteasome pathway. Api5 mutant lacking this serine 138 site

does not undergo post-translational degradation upon DNA damage, and this non-degradable form of Api5 helps in inhibiting the apoptosis. This anti-apoptotic activity of Api5 S138A is through making the cells desensitise towards DNA damage. In this chapter, we also observe that acetylation of Api5 is also crucial for its degradation and regulation of apoptosis. K251Q, a constitutive acetylated mutant of Api5, does not show degradation and inhibits the activation of PARP1 and caspases, the key regulators of apoptosis. K251Q also shows significant chemo-resistance towards the chemical-induced DNA damage as the proliferation of cells is rescued upon ectopic expression of K251Q. Interestingly, it was observed that upon DNA damage, p300, the acetylase involved in the k251 acetylation and stability of Api5 also shows the reduction in its protein levels while HDAC1 levels remain unaltered. In this chapter, the role of Api5 in the regulation of DNA repair is being understood.

7.2 Future perspective

7.2.1 Post-translational modification(s) of Api5 upon serum starvation

While performing the cell cycle-related experiment, we observed that upon serum starvation, not only did Api5 levels increase, but there is also some retardation in the movement of gel. Api5 levels upon serum starvation show an upward shift after gel electrophoresis (Figure 7.1). This upshift is common amongst proteins that undergo post-translational modification in specific contexts. Thus it will be interesting to investigate whether the upward shift of Api5 is due to the post-translational modification(s) (PTM) and if it is through PTMs then to unravel the nature and kind of PTM(s). There are various PTMs like phosphorylation, acetylation, and sumoylation, to name a few, known in the literature to provide multiple functions to the proteins. These PTMs are regulated by various enzymes like kinases, acetylases, etc. It will be interesting to understand the molecular pathway in the regulation of post-translation of Api5 upon serum starvation. To identify the PTM(s) of Api5, mass spectrometry can be performed in serum-starved cells. Purified Api5 in Mass spectrometry will be showing a peak corresponding to the change in molecular weight of Api5 upon serum starvation. That change in molecular weight will be assigned for the particular kind of PTM. Once the nature of PTM is known for Api5, the player behind this PTM can also be found out by carrying out the inhibitor studies. Knockdown studies for that particular player can actually confirm the nature and pathway regulating the PTM of Api5 upon serum starvation.

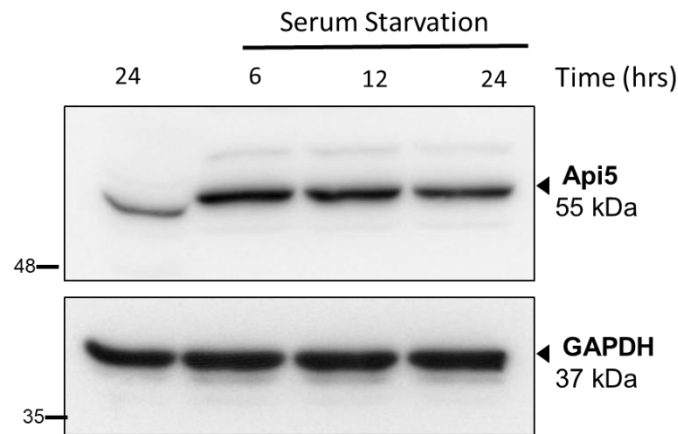


Figure 7.1: Api5 protein showed upward shift and accumulation upon serum starvation

MCF7 cells were serum-starved for various time points. Western blot analysis showing levels of Api5. GAPDH was used as a loading control for immunoblotting.

7.2.2 Cullin mediated degradation of Api5

We identified that FBXO3, FBXO6, and FBXW2 are the SCF E3 ubiquitin ligases involved in the degradation of Api5. Out of these three, FBXW2 has been shown to regulate and interact with Api5 at the endogenous level. But these FBOX proteins, to achieve their function of degrading the substrate proteins, require an additional protein subunit called Cullins. These Cullin proteins are also known to regulate the function of SCF type of E3 ubiquitin ligases (Xie et al., 2019). Henceforth it will be interesting to identify the Cullin subunit involved in the ubiquitin-mediated degradation of Api5. In order to identify the Cullin subunit of SCF involved in the degradation of Api5, knockdown studies can be performed. In these studies, each Cullin subunit can be depleted inside the cell by using either siRNA or shRNA method. Then the particular Cullin molecule(s) showing accumulation of Api5 upon its knockdown can be selected and used for further studies. Finally, the selected Cullin subunits can be immunoprecipitated using Api5 specific antibody to confirm its involvement in the degradation of Api5.

7.2.3 Role of Api5 as a transcription factor

We found that Api5 is localised in the euchromatin region of Api5, which corroborated with the findings of Garcia and the group. Recent studies suggested the interaction of Api5 with the RNA pol II (Bong et al., 2020). Taken these together,

it can be hypothesised that Api5 can act as a transcription factor or co-factor. It will be interesting to investigate the target genes for which Api5 can act as a transcription factor. The transcription regulatory function of Api5 can also be context-specific. It will also be interesting to determine the conditions in which Api5 will be transcribing those particular genes. To achieve this, systematic studies will be performed as follows. In order to achieve that, firstly, fractionation experiments can be performed. The presence of Api5 in the chromatin fraction of cells can indirectly suggest that Api5 can interact with chromatin. To further prove the transcription role of Api5 DNA binding, studies like Electrophoretic Mobility Shift Assay, Isothermal Calorimetry, and Dynamic Light Scattering can be performed. To identify the target genes for which Api5 can be a transcription factor, Chromatin Immunoprecipitation can be performed. By comparing the promoter region sequence similarity, the target genes can be screened. These target genes can finally be confirmed upon knockdown of Api5.

7.2.4 Role of Api5 in metabolic stress

We observed that Api5 regulates the DNA damage-induced apoptosis in the cells. However, apoptosis is not the only mechanism of cell death. Autophagy is also a cell death mechanism that helps maintain tissue homeostasis (Elshaer and Begun, 2017). Thus, it will also be interesting to understand the regulation of Api5 in autophagy and to elucidate the plausible role of Api5 in its regulation. As an ectopic expression of Api5 has been shown to inhibit apoptosis in serum starvation (Tewari et al., 1997), it will also be interesting to find its function in metabolic stress. To prove the induction of autophagy, various autophagy markers can be screened upon induction of metabolic stress in control as well as shApi5 cells. Beclin and LC3(II) are known autophagy markers. To understand the role of Api5 in metabolic stress control and Api5 depleted cells can be treated with known metabolic stress inducer and different metabolic stress markers like Reactive Oxygen species, inducible Nitric oxide synthase, and endothelial Nitric oxide synthase can be analysed. Api5 depleted cells can respond differently to metabolic stress as compared to the control cells. In such a scenario role of Api5 in regulating its metabolic stress can be understood. This variability in sensing the stress can also affect the process of cell death. Henceforth, it will be interesting to understand the mechanism of regulation of cell death by Api5 upon induction of metabolic stress.

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Appendix

50X TAE buffer (0.5 litre)

Glacial acetic acid	57.1ml
0.5M EDTA pH8.0	100ml
Tris base	242g

6X sample buffer (10ml)

1M Tris pH6.8	3.5 ml
DTT	0.93 g
Glycerol	3.6 ml
SDS	1.1 g
1% bromo-phenol blue	0.6 ml

10X SDS running buffer (2 litres)

Tris base	60.6 g
Glycine	288 g
SDS	20 g

20 X TBS (2 litres)

Tris base	120 g
NaCl	320 g
KCl	8 g
pH 7.6 with HCl	

10X Transfer buffer (2 litres)

Tris base	58g
Glycine	293g

Publications

List of publications

Research articles

Virender Kumar Sharma and Mayurika Lahiri, (2021) “Interplay between p300 and HDAC1 regulate acetylation and stability of Api5 to regulate cell proliferation”, *Sci Rep* **11**, 16427 (2021). DOI: <https://doi.org/10.1038/s41598-021-95941-4>

Virender Kumar Sharma, Sehbanul Islam, Janhavi Borkar, Sudiksha Mishra, Debiprasad Panda, Manas K Santra and Mayurika Lahiri (2021), “ATR and FBXW2 regulate proteasomal mediated degradation of Api5 to inhibit DNA damage induced apoptosis” bioRxiv doi:

<https://www.biorxiv.org/content/10.1101/2021.08.08.455545v1> (Under revision in JMB)

Virender Kumar Sharma, Rose Mary Roshan, Aishwarya Venkatravi, Rintu M Umesh, and Mayurika Lahiri, Aurora Kinase B regulate ubiquitin-proteasomal degradation of de-acetylated Api5 in G2-M phase to regulate cell cycle (*Manuscript under preparation*)

Collaborative research articles

Shalini Pandey, **Virender Kumar Sharma**, Ankur Biswas, Mayurika Lahiri and Sudipta Basu (2021), “Small Molecule-Mediated Induction of Endoplasmic Reticulum Stress in Cancer Cells”, *RSC Med. Chem.*, DOI: 10.1039/D1MD00095K

Javid Ahmad Malla, **Virender Kumar Sharma**, Mayurika Lahiri and Pinaki Talukdar (2020), “Esterase Activatable Synthetic M⁺/Cl⁻ Channel Induces Apoptosis and Disrupts Autophagy in Cancer Cells”, *Chemistry - A European Journal*, 05 12;59(20):7944-7952, **doi:** <https://doi.org/10.1002/chem.202002964>



OPEN

Interplay between p300 and HDAC1 regulate acetylation and stability of Api5 to regulate cell proliferation

Virender Kumar Sharma & Mayurika Lahiri

Api5, is a known anti-apoptotic and nuclear protein that is responsible for inhibiting cell death in serum-starved conditions. The only known post-translational modification of Api5 is acetylation at lysine 251 (K251). K251 acetylation of Api5 is responsible for maintaining its stability while the de-acetylated form of Api5 is unstable. This study aimed to find out the enzymes regulating acetylation and deacetylation of Api5 and the effect of acetylation on its function. Our studies suggest that acetylation of Api5 at lysine 251 is mediated by p300 histone acetyltransferase while de-acetylation is carried out by HDAC1. Inhibition of acetylation by p300 leads to a reduction in Api5 levels while inhibition of deacetylation by HDAC1 results in increased levels of Api5. This dynamic switch between acetylation and deacetylation regulates the localisation of Api5 in the cell. This study also demonstrates that the regulation of acetylation and deacetylation of Api5 is an essential factor for the progression of the cell cycle.

Apoptosis inhibitor 5 (Api5) is also known as anti-apoptotic clone 11, AAC-11. Api5 was discovered as a nuclear protein, which was responsible for inhibition of apoptosis under serum starved conditions¹. Api5 is a right-handed superhelix which is composed of all helical repeats. It has 19 α -helices and two 3_{10} helices². It has also been observed that all these helices interact with neighbouring helices to form an antiparallel helix pair. The N-terminal of Api5 comprises of α -helix from 1 to 11 which shares homology with the Huntingtin, Elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1 (HEAT) repeat structures of other proteins such as Importin β . The C-terminal of Api5 is made up of α -helices from residues 12–19 and share homology with armadillo (ARM)-like repeat structures of other proteins, for example, p120 catenin. The N-terminal of Api5 contains LxxLL motif which is an amphipathic α -helix that forms nuclear receptor co-factor interaction regions. This motif is present in the sixth α -helix and is required for maintaining stability of the protein by forming hydrophobic interactions with neighbouring α -helices and is also the region that interacts with FGF2³. The C-terminal region of Api5 has a nuclear localisation signal and a putative Leucine Zipper domain (LZD). LZD is present in the α 18 helix, between 371 and 391 amino acid residues. It interacts with Acinus, a protein which mediates fragmentation of chromatin during apoptosis⁴. Global mass spectrometric analysis of Api5 suggests the presence of one conserved acetylation site at lysine 251, which is present in the hinge region between α 13 and α 14².

It has been proven that Api5 inhibits the dE2F1-mediated apoptosis in *Drosophila* cells⁵. dE2F1 is a well-known pro-apoptotic gene responsible for apoptosis in the fruit flies. In the *Drosophila* embryonic cells, SL2, RNAi-mediated depletion of Api5 was found to be responsible for increased apoptotic cell death as compared to the dE2F1 over-expressed cells. Similar result was also observed in the human osteosarcoma cell line, Saos-2, where ectopic expression of Api5 decreased E2F1-mediated apoptosis in E2F1 over-expressing cells without affecting its transcriptional activity⁵.

According to the study by Rigou et al., AAC-11 binds to Acinus, a nuclear protein and plays a significant role in chromatin condensation during apoptosis⁴. Binding of AAC-11 to Acinus does not allow its cleavage by caspase-3, thus in turn inhibiting DNA fragmentation and apoptosis⁴.

Studies performed in melanoma cells, showed Api5 to modulate FGF2 and FGFR1 signalling which activates ERK. This activated ERK phosphorylates Bim, a pro-apoptotic protein. Phosphorylated Bim is the target

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for proteosomal degradation. Thus ubiquitin-mediated degradation of Bim is a means by which Api5 inhibits apoptosis in HeLa and 565mel cell lines⁶.

Mayank and group have reported Api5 to inhibit transcription of APAF1 gene. APAF1 is the main component of the apoptosome complex. Thus Api5 prevents the formation of the apoptosome, in turn inhibiting apoptosis⁷. A recent study suggests Api5 to physically interact with caspase 2, and prevent its activation, thus inhibiting apoptosis⁸.

Api5 has also been reported to be involved in the regulation of E2F1, a transcriptional activator of G1/S cell cycle transition genes, by enhancing the binding affinity of E2F1 to its target promoters. Knockdown of Api5 arrested H1299 cells at the G1 phase of the cell cycle, thus proving that apart from regulating apoptosis, Api5 also plays a critical role in maintaining normal cell cycle progression⁹.

Navarro et al. demonstrated that the levels of Api5 are regulated in a cyclic manner⁹. It was observed that the levels of Api5 was higher in the G1 phase and was stabilised during the G1/S transition. Interestingly, Api5 levels decreased as the cells proceeded further in the cell cycle, from G2 to G2/M phase⁹. This suggests that Api5 undergoes degradation during cell cycle progression. Knockdown of Api5 arrests H1299 non-small cell lung carcinoma cells at the G1 phase. This was further supported by Han and his group where they showed acetylation of Api5 at lysine 251 to be associated with its stability².

Api5 has been found to be overexpressed in different types of cancers like cervical, urinary bladder, lung, ovarian and oesophageal cancers^{6,10–13}. In cervical cancers, Api5 overexpression has been shown to promote invasion¹². Api5 has also been shown to promote the degradation of Bim, a pro-apoptotic protein⁶. In osteosarcomas, studies have shown Api5 to inhibit E2F1 as well as Acinus-mediated apoptosis^{4,5}. Api5 has been identified as a biomarker for cervical and ovarian cancers and a prognosis marker for non-small cell lung carcinomas^{11,13}. High levels of Api5 provide cancer cells the ability to evade immune response mediated cell death⁶. In breast cancers, Api5 interacts with the estrogen receptor to promote proliferation¹⁴. It has also been reported that Api5 promotes metastasis in breast cancers¹⁴. Higher levels of Api5 are associated with chemo-resistance¹⁵. It has been shown that tamoxifen-resistant breast cancer cells show an upregulation of Api5¹⁶, while cancer cells which are sensitive to anticancer agents like docetaxel show reduced levels of Api5¹⁷. Reduced and low levels of Api5 are associated with the increase in cell death in various cancers. Knockdown of Api5 resulted in the reduction in *in vivo* tumorigenicity in cancer cells¹⁴.

Api5 acetylation at lysine 251 is conserved from protists to mammals². De-acetylated form of Api5 is not stable and therefore undergoes post-translational degradation. However the mechanism of degradation and the enzymes involved in the process of acetylation and de-acetylation of Api5 is not yet known.

CBP/p300, GCN5/PCAF and TIP60/MYST1/2/3/4 are the major acetyltransferases involved in acetylation of most of the cellular proteins. Among this, p300 acetylates proteins involved in a number of diverse biological functions including proliferation, cell cycle regulation, apoptosis, differentiation and DNA damage response^{18–21}. p300 histone acetyl transferase was initially identified as a transcriptional activator that performs its function by acetylating histones in eukaryotic cells. p300 is capable of acetylating all the four histones^{22,23}. Later it was discovered that p300 also acetylates non-histone proteins like E2F1, p53, p73, Rb, E2F, myb, myoD, HMG(I) Y, GATA1 and α -importin^{24–33}. The role of p300 histone acetyl transferase in the regulation of the cell cycle is also known³⁴. Activity of p300 is required for normal transition from G1 to S phase of cell cycle³⁵. Mutations or abnormality in function of p300 leads to multiple disorders and cancer is most common amongst them^{36–39}.

Histone de-acetylases (HDACs) are the major de-acetylases involved in the de-acetylation of histones that regulate genetic expression of certain genes as well as non-histone proteins that regulate the processes responsible for maintaining cellular homeostasis^{40–43}. HDAC de-acetylates proteins involved in almost all the cellular events like cell cycle, replication, proliferation and apoptosis. It has also been shown that most of the proteins that get acetylated by p300 histone acetyltransferase also undergo de-acetylation by HDAC1, a member of class I HDACs, for example p53 and E2F1^{28,42}. Han and group in their studies have suggested the possibility of involvement of histone de-acetylases in the de-acetylation of Api5².

AMP-activated protein kinase (AMPK) is an energy sensor kinase that regulates almost all cellular processes like cell division, DNA replication, transcription activation and apoptosis^{44–46} by sensing AMP to ATP ratio and thereby regulating cellular homeostasis. It has also been reported that AMPK regulates G1/S transition of cells⁴⁷. Mutations in AMPK can lead to multiple disorders including cancer⁴⁸.

Protein kinase B (Akt) has various functions in cells that include proliferation, migration and transcription. Studies have shown PI3K/Akt pathway to also regulate cell cycle progression by phosphorylating the inhibitors of cyclin-dependent kinases (CKI) p21 and p27 Cip/Kip proteins^{49–51}. Akt has also been shown to play a role in regulating different pro-apoptotic molecules in order to enhance survival and proliferation of cancer cells⁵².

In this study, we report two novel and key regulators of Api5: p300 and HDAC1. We observed p300 to be the enzyme that interacts with and regulate Api5 levels in cells. p300 mediated acetylation of Api5 at lysine 251 provided stability to Api5, while the HDAC1-mediated deacetylation led to reduced levels of Api5 in the cells. Both these regulators also regulated the subcellular localisation of Api5 in cells. We observed that the de-acetylated protein was transported to the cytoplasm while the acetylated protein was present in the nucleus. The regulation of acetylation was also observed to regulate cell cycle progression. We also demonstrated AMPK and Akt to be the players regulating p300 activity, thereby leading to stability of Api5 in cells.

Results

In silico analysis predicts p300 histone acetyltransferase to acetylate Api5 at lysine 251. It has been reported in previous studies that Api5 undergoes acetylation at lysine 251, however, the enzyme that is required for the acetylation function has not yet been identified. Therefore, to identify the enzyme involved in the acetylation of Api5 we performed in silico analysis using the acetylation set enrichment based (ASEB) web

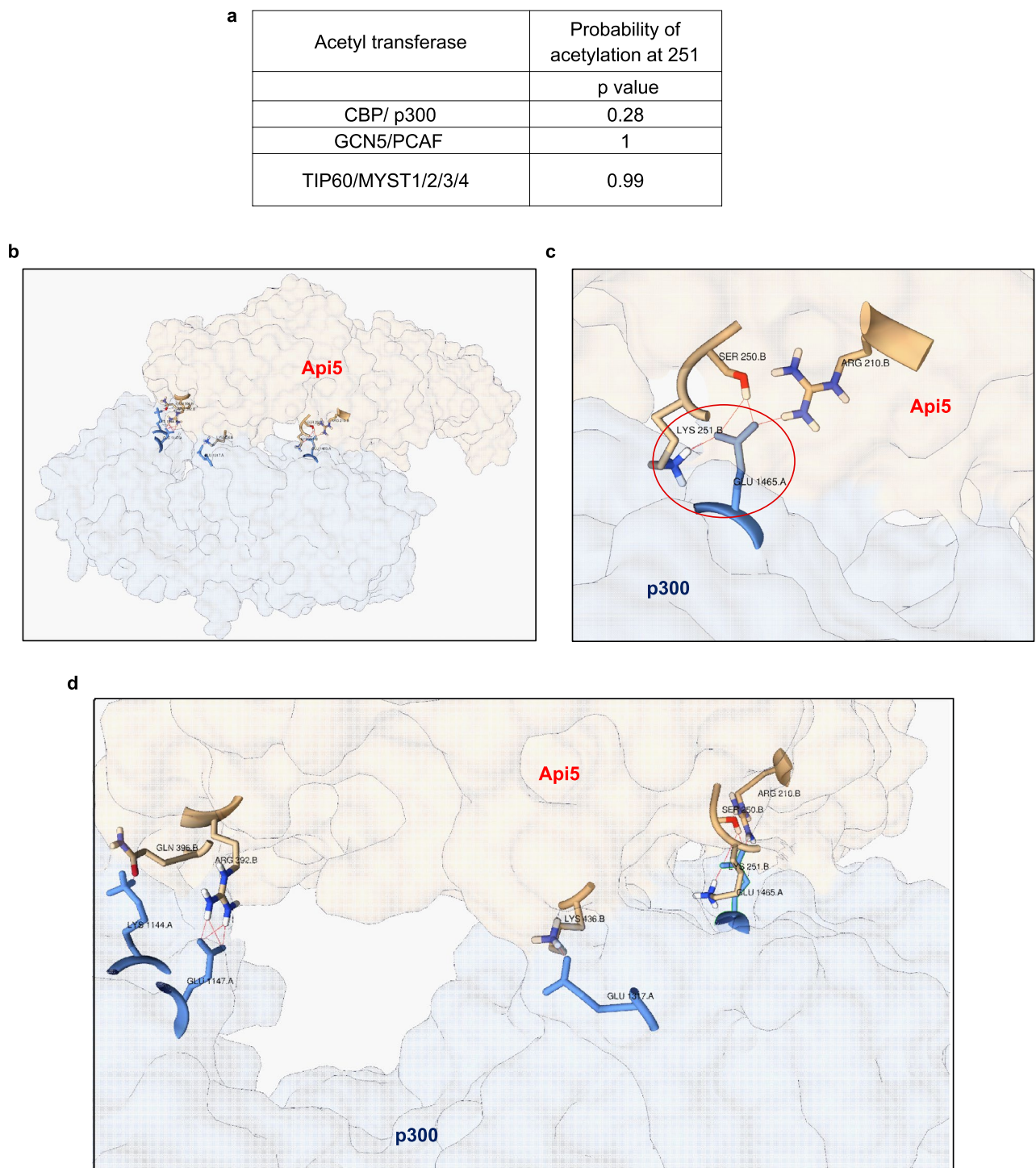


Figure 1. Api5 interacts with p300 in silico. **(a)** Various acetyl transferases and their probability to acetylate lysine 251 of Api5 represented as p value using ASEB webserver, where lower p value corresponds to a higher probability. **(b)** The lowest-energy docking position of Api5 and p300 analysed using HADDOCK webserver and output analysed using PIZSA represented as a cartoon diagram. **(c)** Interaction between Lysine 251 of Api5 with p300. **(d)** Various amino acid interactions between Api5 and p300.

server tool^{53,54} to predict the site as well as the enzyme which may be responsible for acetylation of Api5 at lysine 251. ASEB predicts the site of acetylation as well as the acetyl transferases involved in the acetylation of target proteins by analysing sequence similarity and protein–protein interaction related information of other known proteins. The prediction score is represented as p value wherein, lower p value corresponds to higher probability of the protein to be acetylated by the acetyl transferases⁵³. It was observed that amongst CBP/p300, GCN5/PCAF and TIP60/MYST1/2/3/4, CBP/ p300 had a lower p value suggesting a higher possibility of acetylating Api5 at

lysine 251 (Fig. 1a). Active site residues of p300 histone acetyltransferase were obtained from the literature⁵⁵. Docking was performed between p300 and Api5 using the High Ambiguity Driven protein–protein DOCKing (HADDOCK) webserver⁵⁶. HADDOCK server utilises the biochemical and biophysical properties of known proteins and predicts the interaction between two proteins along with the identification of amino acid residues involved in the interaction. The output was analysed using Protein Interaction Z Score Assessment (PIZSA)⁵⁷ and the best scoring model was chosen as shown in Fig. 1b–d. Interestingly the acetylating domain of p300 showed interaction with three amino acid residues of Api5 including lysine 251 (Fig. 1c,d).

p300 interacts with and regulates Api5. Acetylation of Api5 at K251 is responsible for maintaining its stability². To prove whether p300 histone acetyltransferase can regulate Api5 acetylation and thus stability, cells were treated with different concentrations of a small molecule chemical inhibitor of CBP/p300 (C646) and levels of Api5 were analysed. E2F1 and p53 are proteins which are stabilised upon acetylation by p300. Reduced levels of E2F1 and acetyl p53 confirmed the inhibition of CBP/p300 activity (Fig. 2a,c,d). Api5 protein expression was reduced upon p300 inhibition (Fig. 2a,b) without affecting the transcript expression of Api5 (Fig. 2e,f), thus demonstrating that CBP/p300 might be regulating Api5 at the post-translational level. In order to acetylate Api5, the interaction between CBP/p300 and Api5 is essential. Thus, to find out whether p300 and Api5 physically interact or not, stable cells expressing mCherry-tagged Api5 were immunoprecipitated using GFP-specific antibody (Fig. 2g). The presence of p300 histone acetyltransferase in the GFP pull down lysate confirmed the interaction between Api5 with p300. This interaction was further corroborated by reverse immunoprecipitation using p300 antibody and the presence of Api5 was confirmed using GFP specific antibody (Fig. 2h). However, immunoprecipitation of Api5 with GFP specific antibody did not show interaction with CBP (data not shown). These results conclusively demonstrate p300 to interact with Api5 and thus regulate Api5 protein expression without affecting the transcript levels.

HDAC1 interacts with and regulates levels of Api5. From the previous results, we discovered p300 to be the histone acetyltransferase to regulate Api5 protein levels. Next, we were interested in identifying the de-acetylase enzyme which might be involved in regulating the stability of Api5.

HDAC1 is one of the member of the large family of histone de-acetylases. HDAC1 belongs to class 1 HDAC proteins along with HDAC2. De-acetylation activity of HDAC1 is coupled with the acetylation activity of p300. Interestingly, HDAC1 has also been reported to be a part of the Api5 interactome⁵⁸. To investigate whether HDAC1 regulates Api5, cells were treated with different concentrations of romidepsin, a HDAC class 1 inhibitor and Api5 protein levels were analysed. Api5 protein levels increased upon treatment with romidepsin suggesting that HDAC1 may be involved in regulating the stability of Api5 (Fig. 3a,b). Levels of p21, a protein known to be degraded upon de-acetylation by HDAC1, also accumulated confirming the inhibition of HDAC1 activity (Fig. 3a and c). The accumulation of Api5 upon inhibition of HDAC1 could also be the result of increased transcription of Api5. To rule out this possibility, Api5 transcript levels were analysed upon HDAC1 inhibition. Api5 transcript levels remained unaltered upon romidepsin treatment as shown in Fig. 3d,e. These results suggest HDAC1 to play a role in regulating the stability of Api5 at the post translational level. In order to decipher whether HDAC1 interacted with Api5 to bring about the regulation, immunoprecipitation studies were conducted. To demonstrate the interaction of Api5 with HDAC1, whole cell lysates of mCherry-Api5 overexpressing stable cells were immunoprecipitated using GFP-specific antibody (Fig. 3f). Presence of HDAC1 in the GFP pull down lysates confirmed the interaction between HDAC1 and Api5. This interaction was further corroborated by reverse immuno-precipitation and pull down was performed using HDAC1 specific antibody. Api5 was found to be present in the HDAC1 pull down lysates (Fig. 3g). These studies thus validate that HDAC1 interacts with and regulates Api5 at the protein level. However, since romidepsin has been shown to inhibit both HDAC1 and HDAC2, we do not rule out the possibility of HDAC2 in the regulation of Api5.

Api5 is acetylated by p300 and de-acetylated by HDAC1 at lysine 251 residue. Previous experiments suggested p300 histone acetyltransferase and HDAC1 as the acetylating and de-acetylating enzymes respectively that regulate the levels of Api5. It was hypothesised that these two enzymes might also regulate the dynamics of acetylation and de-acetylation of Api5. Therefore, the acetylation status of Api5 was investigated upon inhibition of both p300 and HDAC1.

To investigate whether Api5 is acetylated by p300, mCherry-Api5 overexpressing stable cells were treated with and without C646 and immunoprecipitation studies were performed. Acetylated Api5 was detected using L-acetyl lysine specific antibody. Api5 acetylation was observed to be reduced upon inhibition of p300 when compared to the untreated control as shown in Fig. 4a. To check for the effect of HDAC1 inhibition on the acetylation status of Api5 similar immunoprecipitation experiments were performed with and without romidepsin. Acetylated levels of Api5 increased upon inhibition of HDAC1 (Fig. 4b). This, thus confirmed that Api5 undergoes acetylation and de-acetylation by p300 and HDAC1, respectively. Earlier studies by Han and group have shown that Api5 is acetylated at lysine 251². To identify whether Api5 acetylation and deacetylation by p300 and HDAC1 respectively occurs at the lysine 251 residue, K251 mutants of Api5 were generated using site-directed mutagenesis. mVenus-tagged wild type, K251A (acetylation-deficient mutant), K251R (charge-mimic mutant) and K251Q (constitutive acetylation mimic) mutants of Api5 were ectopically expressed in cells. The expression levels of mVenus, which is a proxy for ectopic expression of Api5 K251 mutants were analysed in the presence and absence of C646 and romidepsin using GFP-specific antibody. We expected K251A mutant of Api5 to be unstable and thus observed its expression to be low. However, to investigate the molecular function of p300 and HDAC1 during acetylation and deacetylation of Api5, cells were transfected with a higher amount of K251A plasmid in order to obtain comparable protein expression to that of the other mutants. It was observed that levels

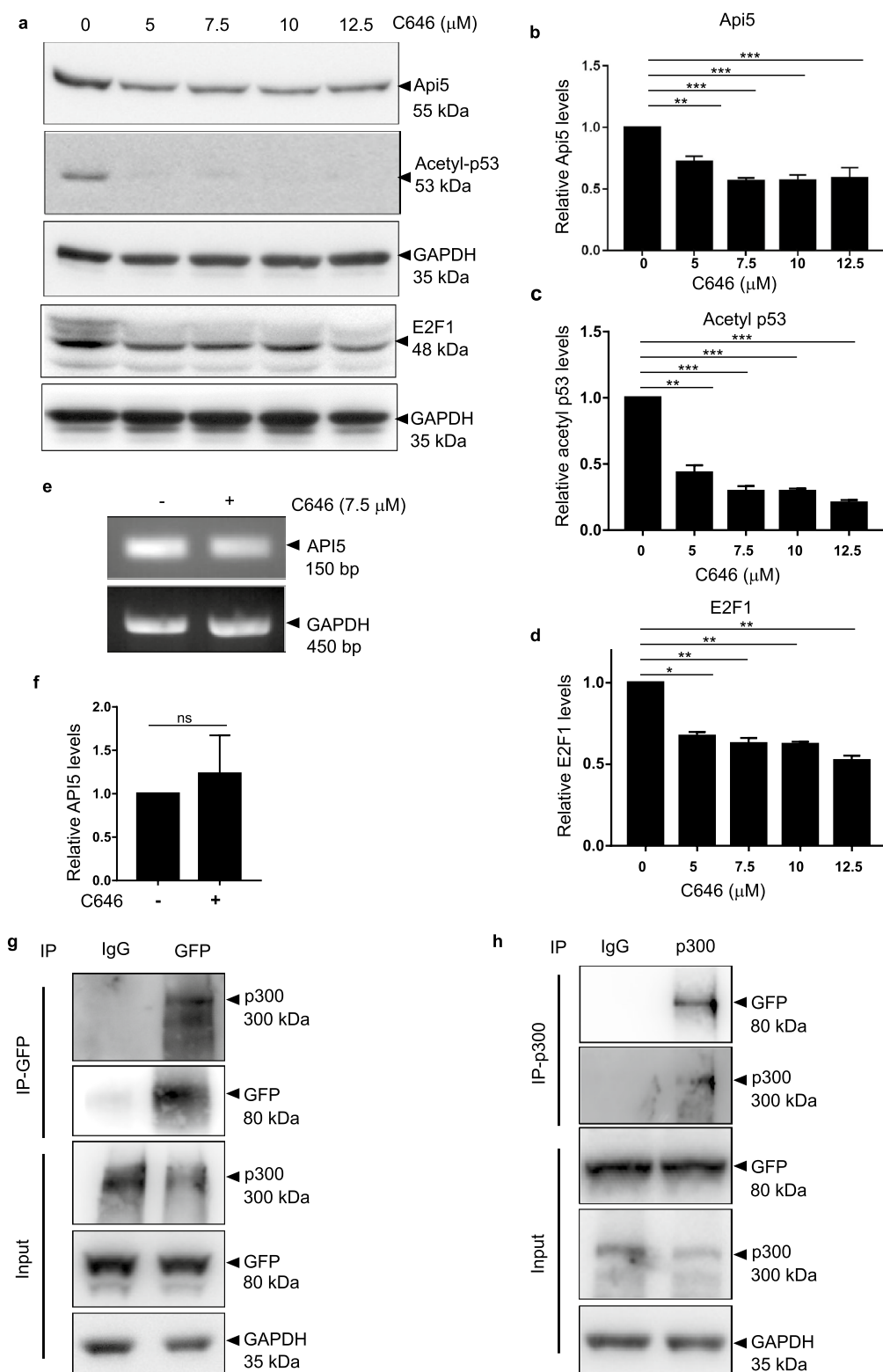


Figure 2. Interaction between p300 and Api5 stabilises Api5. **(a)** MCF7 cells treated with varying concentrations of CBP/p300 inhibitor C646 for 4 h were lysed and Api5 levels were analysed using western blotting. **(b)** Quantification showing the fold change in Api5 protein levels after normalisation with GAPDH. **(a–d)** Acetyl p53 and E2F1 were used as positive control for p300 inhibition. **(e)** API5 transcript levels upon C646 treatment was analysed using semi-quantitative PCR. **(f)** Quantification of transcript levels after normalising to GAPDH. MCF7 cells stably expressing mCherry-Api5 were lysed and immunoprecipitations were performed using **(g)** GFP and **(h)** p300-specific antibodies.

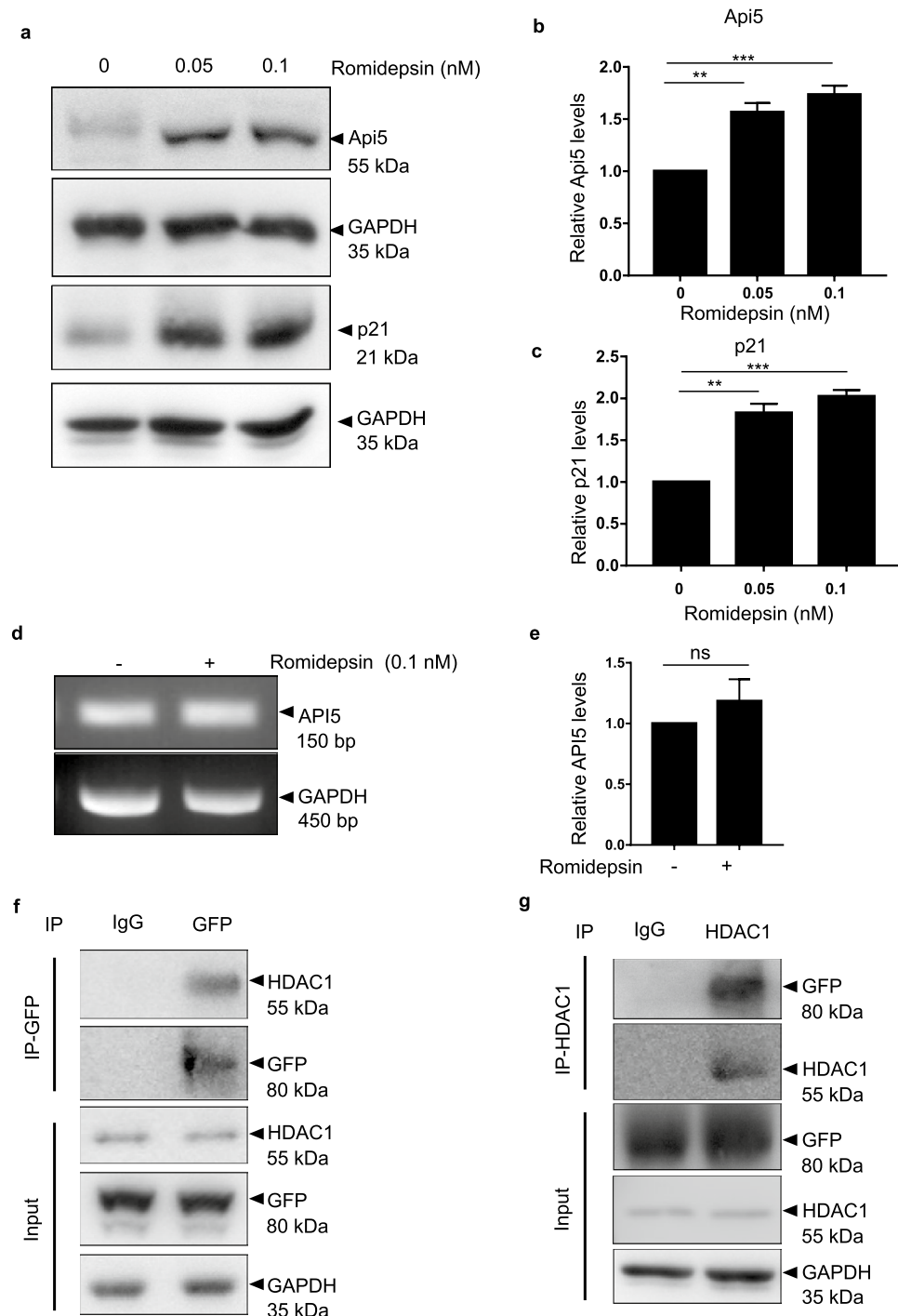


Figure 3. HDAC1 interaction destabilises Api5. **(a)** MCF7 cells treated with varying concentrations of the HDAC class 1 inhibitor, romidepsin for 16 h were lysed and Api5 levels were analysed using western blotting. **(b)** Quantification showing the fold change in Api5 protein levels after normalisation with GAPDH. **(a,c)** p21 levels were analysed to confirm the HDAC1/2 inhibition. **(d)** API5 transcript levels upon romidepsin treatment was analysed using semi-quantitative PCR and **(e)** quantified after normalising with GAPDH. MCF7 cells stably expressing mCherry-Api5 were lysed and immunoprecipitation was carried out using **(f)** GFP and **(g)** HDAC1-specific antibodies.

of wild-type Api5 were reduced while the levels of K251A, K251Q and K251R mutants of Api5 remained unaffected upon p300 inhibition (Fig. 4c,d). However, levels of wild-type Api5 increased upon inhibition of HDAC1

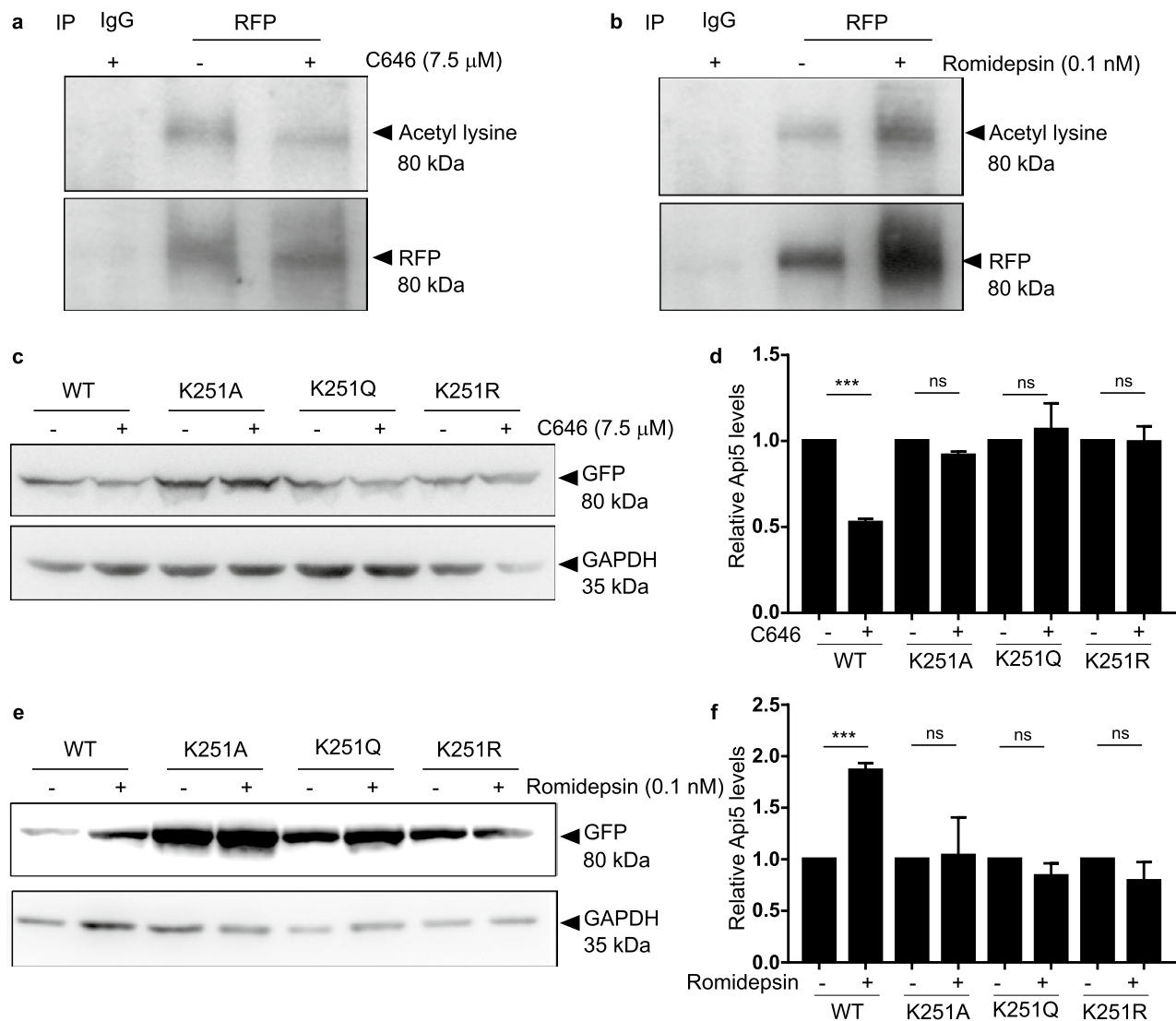


Figure 4. p300 and HDAC1 acetylate and deacetylate Api5 at K251, respectively. mCherry-tagged Api5 over-expressing MCF7 cells treated with (a) 7.5 μ M of C646 for 4 h and (b) 0.1 nM of romidepsin for 16 h were lysed and immunoprecipitation was performed using RFP-specific antibody. Western blotting analysis was performed to ascertain the acetylation status of Api5 using acetyl lysine specific antibody. MCF7 cells were transfected with 2 μ g each of WT, K251Q and K251R and 4 μ g of K251A plasmids and treated with (c) 7.5 μ M of C646 and (e) 0.1 nM of romidepsin post 24 h of transfection for 4 and 16 h, respectively. Western blotting was performed to check for the levels of mutant Api5 using GFP specific antibody. (d,f) Quantification showing the fold change in Api5 protein levels after GAPDH normalisation and represented as mean $\pm$ SEM.

by romidepsin whereas that of the other mutants remained unchanged (Fig. 4e,f). Therefore, it was established that p300 acetylates while HDAC1 de-acetylates Api5 at the lysine 251 residue.

Acetylation of Api5 by p300 is the signal for its nuclear localisation while de-acetylation leads to the translocation of Api5 to the cytoplasm.. From the previous results, it can be inferred that p300 acetylates Api5 at lysine 251, thereby maintaining its stability. It has earlier been reported that Api5 is a nuclear protein. To investigate the role of acetylation on Api5 function, cells were treated with the acetylation and de-acetylation inhibitors and immunofluorescence was performed. In control cells, Api5 was observed to be present in the nucleus while upon inhibition of p300, the percentage of cells showing Api5 to be present in the nucleus was negligible. Moreover, most cells showed nuclear and cytoplasmic localisation of Api5 (Fig. 5a,b). Interestingly, 24% of cells showed only cytoplasmic presence of Api5 upon p300 inhibition which was not observed in the control cells (Fig. 5b). Api5 localisation was also investigated upon inhibition of HDAC1. We found that, upon HDAC1 inhibition, majority of the cells (67%) showed both nuclear and cytoplasmic localisation of Api5, whereas 29% of cells showed only nuclear localisation of Api5 (Fig. 5c,d). Only 4% of cells showed cytoplasmic localisation of Api5 which is negligible. To follow the compartmentalisation switch of Api5, both p300 and HDAC1 were inhibited. The cytoplasmic localisation of Api5 that was observed upon p300 inhibition as well

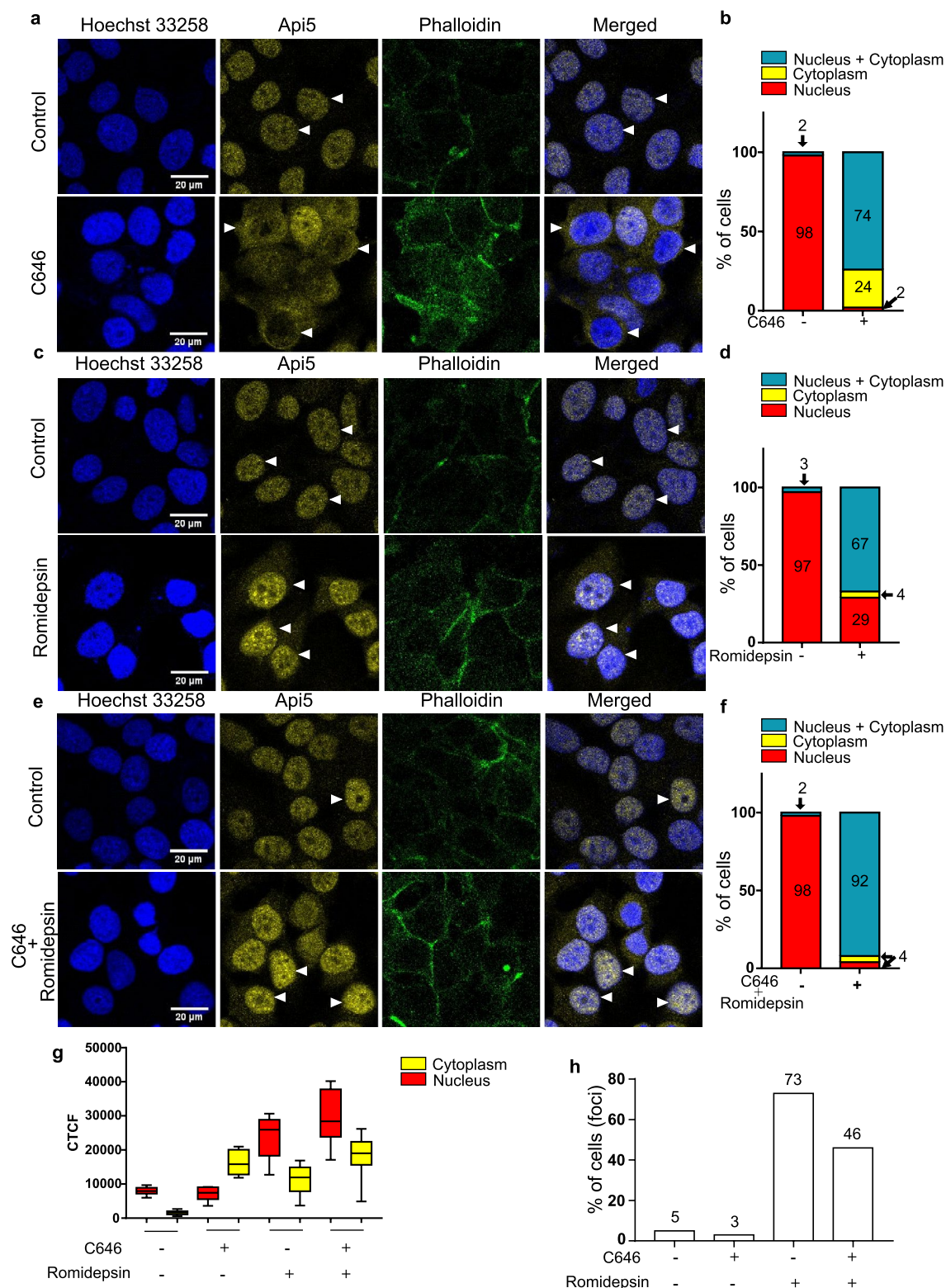


Figure 5. p300 and HDAC1 regulate Api5 localisation. MCF7 cells were treated with (a,b) 7.5 μ M C646, (c,d) 0.1 nM romidepsin and (e,f) 7.5 μ M C646 and 0.1 nM romidepsin for 4 h and 16 h, respectively. Immunofluorescence assay was performed using Api5 specific antibody and cells with different Api5 staining pattern were manually counted and analysed. The bar graph represents the percentage of cells with Api5 staining only in the nucleus (red), only in the cytoplasm (yellow) or in both (blue). (g) CTCF value of Api5 intensity in the nucleus and the cytoplasm was measured in the same experimental set up and the values are represented as box plots. Here, red corresponds to nuclear intensity and yellow corresponds to the cytoplasmic intensity of Api5. (h) Cells exhibiting Api5 nuclear foci following different inhibitor treatments were manually counted and represented as bar graphs showing percentage of cells with nuclear foci.

as the nuclear localisation observed upon HDAC1 inhibition was reduced upon inhibition of both p300 and HDAC1. 92% of cells showed both nuclear and cytoplasmic localisation of Api5 (Fig. 5e,f). To further confirm the role of p300 and HDAC1 in the translocation of Api5 from the nucleus to cytoplasm, the intensity of Api5 was measured in the nucleus and the cytoplasm. We observed the intensity of Api5 to increase in the cytoplasm upon treatment with C646 while the nuclear intensity increased upon romidepsin treatment when compared to control cells (Fig. 5g). Interestingly it was observed that upon inhibition of HDAC1, 73% of cells showed Api5 nuclear foci while the foci were reduced to 46% when both p300 and HDAC1 inhibitors were added in concert (Fig. 5h). These results were further confirmed in mCherry-tagged Api5 over-expressing cells (Supplementary Fig. S1 online). Therefore, it was concluded that Api5 acetylation at lysine 251 by p300 stabilises Api5 inside the nucleus while de-acetylation by HDAC1 at the same lysine residue directs Api5 to locate to the cytoplasm.

Acetylation of Api5 is critical for normal cell cycle progression. Lacazette and group have reported Api5 protein levels to peak during the G1 and S phases of the cell cycle while the transcript levels remained unaltered⁹. Our data as well as data from Han's group² have shown acetylation to be responsible for maintaining the stability of Api5. It may be hypothesised that the stability of Api5 during the G1 and S phases of the cell cycle may be due to acetylation of Api5. To demonstrate whether Api5 acetylation affected cell cycle profile, cells were transfected with the K251 mutants of Api5 in an Api5 knock down background and then analysed. During our studies we observed that cells with ectopic expression of K251A in an Api5 knockdown background were not able to survive in the culture. Hence, we conducted our further studies with WT, K251Q and K251R constructs. Interestingly, cells transfected with the K251R mutant of Api5 were observed to be arrested at the S phase of the cell cycle (Fig. 6a). PCNA, a S-phase cell cycle marker was also found to be upregulated in the Api5-K251R transfected cells (Fig. 6b,d) while the other cell cycle markers remained unaffected (Fig. 6b,e,f). This result was further corroborated with the observation that the Api5-K251R mutant expressing cells showed higher proliferation when compared to the other Api5 acetylation mutants (Fig. 6g). This increase in proliferation may possibly be due to an alteration in the cell cycle functioning resulting from the change in acetylation status of Api5. Therefore it was confirmed that the acetylation status of Api5 also regulated proliferation and cell cycle progression.

AMPK and Akt regulate the p300 mediated stability of Api5. It has already been reported that AMPK can either directly or indirectly regulate G1/S cell cycle transition regulators such as p53 and p21⁴⁷. AMPK requires the activity of AKT to regulate other cell cycle regulators⁵⁹. It has been previously reported that activated Akt can activate p300 by phosphorylating it at serine 1834 residue⁶⁰. Therefore to discern whether this signalling cascade led to the stability of Api5, both AMPK and Akt were inhibited using small molecule inhibitors. Api5 levels were observed to be reduced upon inhibition of both AMPK (Fig. 7a,b) and Akt (Fig. 7c,d) independently. It was also observed that upon inhibition of AMPK, Akt phosphorylation at serine 473 decreased which is known to be required to activate p300 (Fig. 7e,f). Our experiments suggest that inhibition of AMPK inhibits the activation of Akt. Akt is the kinase that has been reported to phosphorylate and activate p300 histone acetyl transferase. E2F1, a transcription factor is known to be acetylated by p300. This p300-mediated acetylation of E2F1 has been shown to regulate stability of protein. Reduced levels of E2F1 upon inhibition of AMPK and Akt separately confirms that both AMPK and Akt are involved in regulating the histone acetyl transferase activity of p300 (Fig. 7e–i). We propose a model where inhibition of activation of Akt and reduction in Api5 protein levels upon inhibition of AMPK confirms that p300-mediated stability of Api5 is achieved through the AMPK-Akt pathway as illustrated in the schematic in Fig. 8a.

Discussion

In this study we have shown that p300, a histone acetyltransferase to be responsible for acetylating and maintaining the stability of Api5. p300 is one of the most studied acetyltransferases which not only acetylates histone proteins but also other non-histone proteins too²³. E2F1 is another known protein that is stabilised upon acetylation by p300²⁸. E2F1 is a transcription factor that plays a critical role during the G1/S transition of the cell cycle²⁹. E2F1 levels vary during the different cell cycle phases, similar to that of Api5⁹. E2F1 protein levels peak during the G1 phase of cell cycle and starts to degrade as the cell crosses the S phase. p53, the master regulator of the cell is also acetylated by the p300²⁶. There are a number of studies that show that upon acetylation, p53 stabilises and thus, is able to perform its different functions like activation of DNA damage response and repair pathway, cell cycle arrest and apoptosis⁴².

Histone deacetylases are one of the members of the deacetylase family of enzymes that remove the acetylation from targeted proteins. Initially HDACs were identified and reported to de-acetylate histone proteins⁴⁰. But extensive studies have shown that HDACs can also de-acetylate non-histone proteins. Both E2F1 and p53, which are acetylated by p300, are also de-acetylated by Class I HDACs^{29,61}. E2F1 and p53 upon deacetylation become unstable and lose their activity. HDAC1 mediated de-acetylation of E2F1 also aids in its localisation shift from nucleus to cytoplasm.

Earlier studies have shown Api5 acetylation at K251 residue to stabilise Api5 inside the nucleus². However, the enzymes involved in the acetylation process were unknown. Our study, is the first to identify p300 and HDAC1 as the acetylating and de-acetylating enzymes of Api5 respectively, that work in concert, thus leading to the stability or instability of the protein during normal cell functioning. K251A mutant of Api5 has been reported to be unstable². This was further corroborated in our study where we were not able to observe optimum expression of K251A comparable to that of wild type Api5. In this study, we report that Api5, a nuclear protein under normal physiological conditions is acetylated by histone acetyltransferase p300. p300 is an essential regulator of Api5 as the acetylated protein is localised in the nucleus while the de-acetylated protein switches its localisation to the cytoplasm. As Api5 levels reduce upon inhibition of p300, it suggests that Api5 might be undergoing

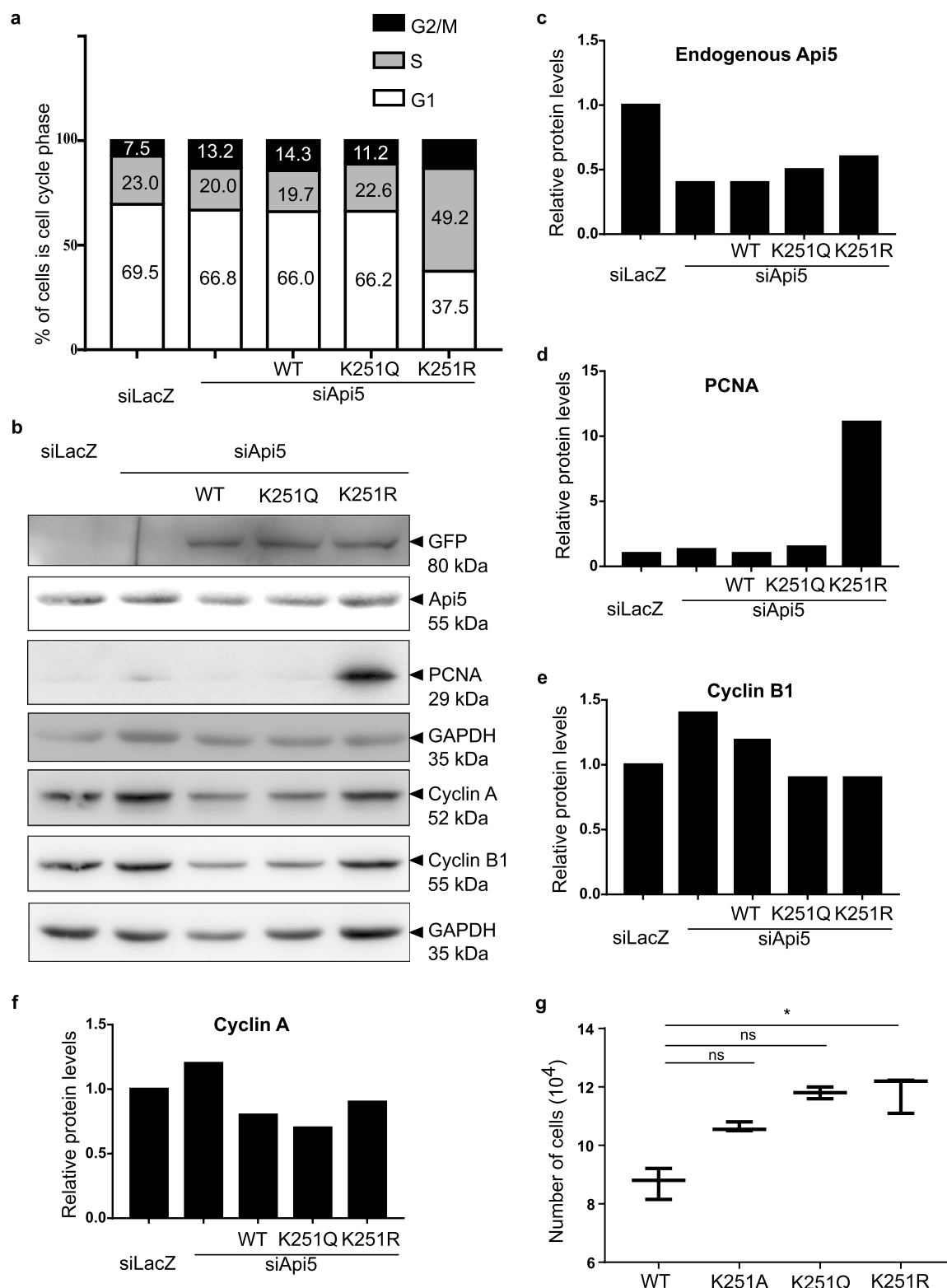


Figure 6. Acetylation status of Api5 affects cell cycle. MCF7 cells transfected with siRNA-resistant Api5-K251 mutants 24 h post Api5 knock down. Cells were fixed in ethanol and stained with propidium iodide and cell cycle profile was monitored. (a) Percentage of cells in the different cell cycle phases were analysed in a flow cytometer and plotted as box plots. (b) Western blotting was performed to check the levels of (b) Api5, PCNA, Cyclin A and Cyclin B1 and (c–f) quantified after normalising to the loading control GAPDH. (g) MCF7 cells transfected with mVenus-tagged Api5 WT and K251 mutants were counted using Sceptar™ to check for cell proliferation.

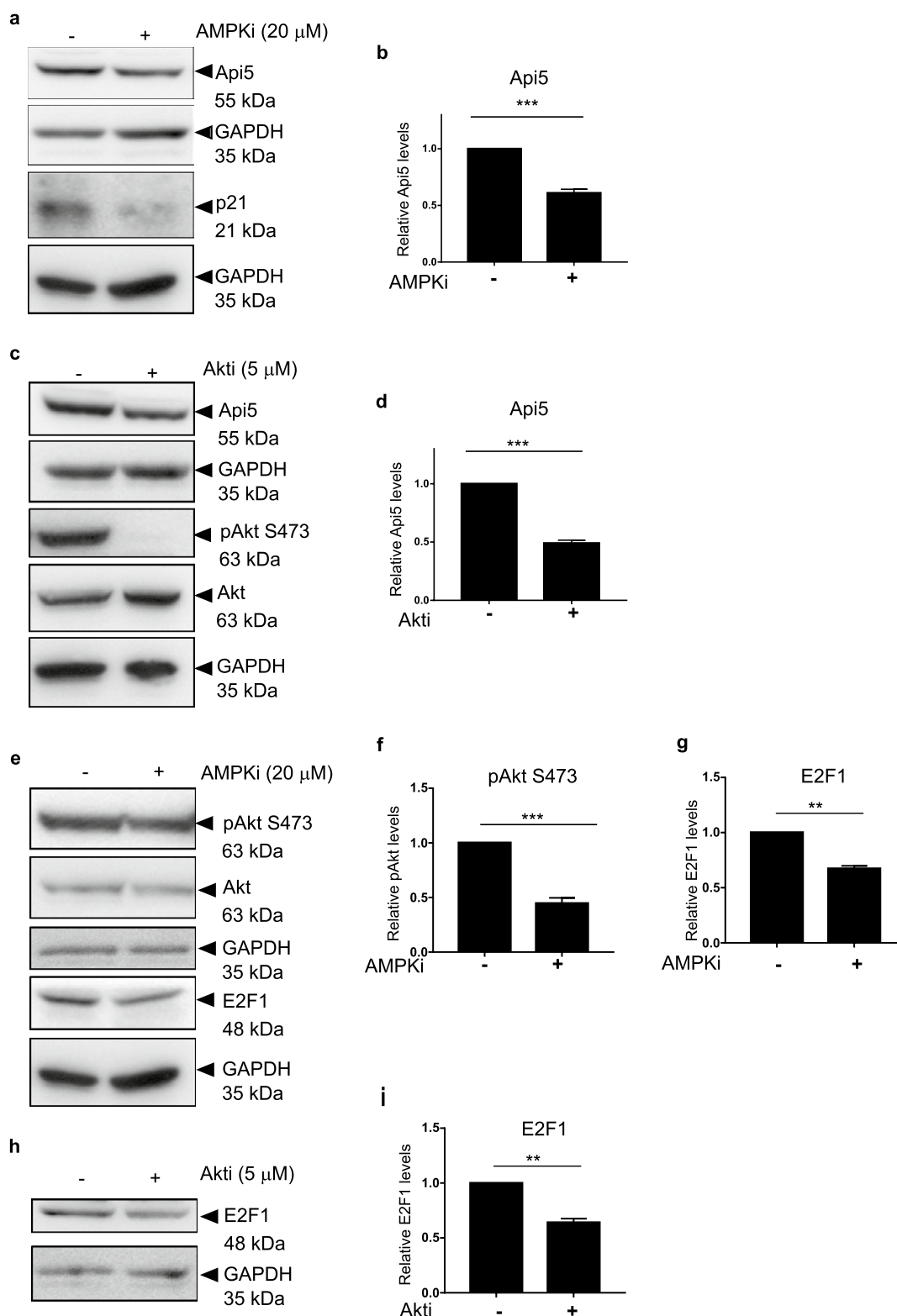


Figure 7. AMPK and AKT regulate Api5 stability. MCF7 cells were treated with (a) 20 μ M AMPKi, and (c) 5 μ M AKTi for 4 h and 24 h, respectively. Immunoblotting analysis was performed using Api5 specific antibody to check for Api5 expression levels. GAPDH was used as loading control. (b,d) Quantification showing the fold change in Api5 levels after normalisation to GAPDH. (a) p21 and (c) pAkt S473 were used as positive control to confirm AMPK and Akt inhibition, respectively. (e) pAkt S473 activation upon AMPK inhibition. pAkt, Akt and GAPDH blots were cropped and the full blot is provided in Supplementary figure S12. (f) Quantification showing the fold change in pAkt S473 activation after normalisation to GAPDH. (e) E2F1 levels upon AMPK inhibition and (g) Quantification of E2F1 levels after normalising to GAPDH. (h,i) E2F1 protein levels were analysed following Akt inhibition using immunoblotting.

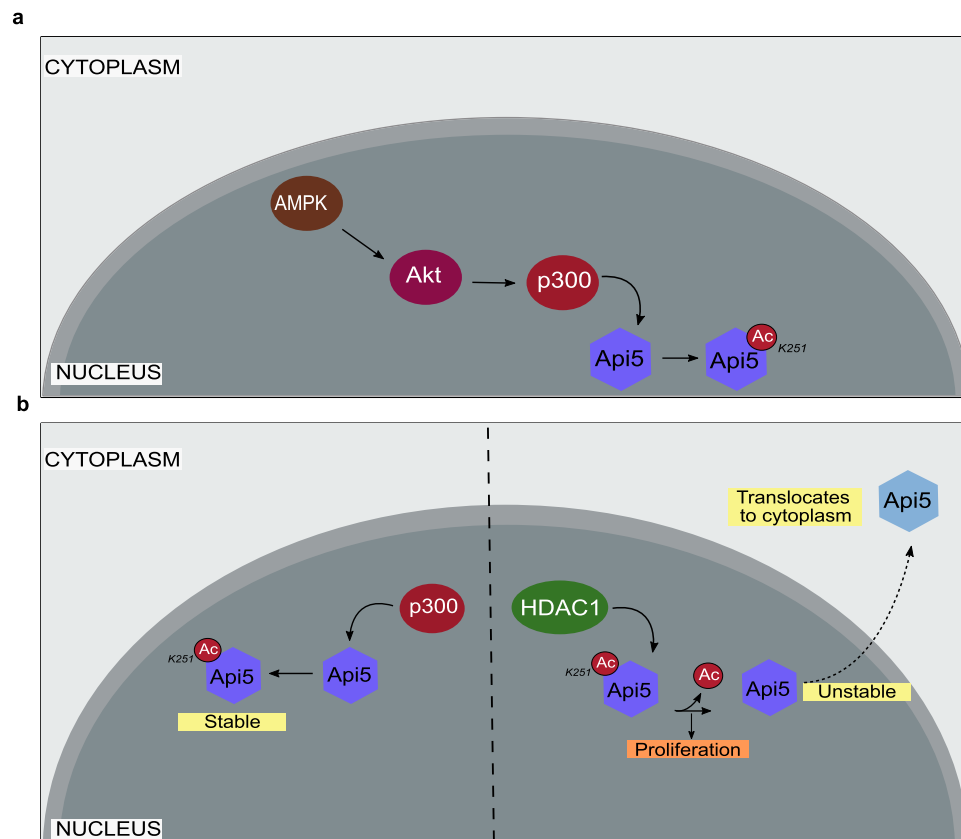


Figure 8. Schematic representation depicting the regulation of Api5 following acetylation and deacetylation. (a) Model predicting the regulation of Api5 via the AMPK, Akt and p300 signalling cascade. (b) p300 acetylation of Api5 at lysine residue 251 stabilises the protein in the nucleus; whereas de-acetylation of Api5 at K251 by HDAC1 de-stabilises the protein which enables it to get translocated to the cytoplasm. Overall, this leads to proliferation of the cells.

post-translational degradation in the cytoplasm. As the acetylated Api5 is stable in the nucleus and is not transported to the cytoplasm, it may also be inferred that acetylation prevents Api5 from binding to translocator/ transporter proteins that are responsible for its translocation/ transportation from the nuclear compartment to the cytoplasm. However, de-acetylation might be exposing Api5 binding site to transporter protein(s) that enables the protein to translocate to the cytoplasm.

As mentioned earlier, Api5 levels peak during G1 and S phase of cell cycle and is thereby able to play a role in the regulation of cell cycle progression as well as proliferation of cells by possibly functioning as a transcription factor. During G1 and S phases of the cell cycle, Api5 may possibly be interacting with chromatin to transcribe those genes that regulate progression of the cell cycle. Transcription of those genes may also regulate the proliferation of cells. Thus it may be inferred that acetylation of Api5 by p300 possibly provides additional functionality to Api5 that includes transcription of cell cycle regulators.

It has also been reported that Api5 is overexpressed in many cancers. This overexpression of Api5 promotes angiogenesis and metastasis in cancerous cells. Oncogenic properties of tumor promoting proteins can also be considered as an outcome of their stability. In this case, Api5 is being stabilised by the action of p300 histone acetyltransferase. Inhibition of p300 and induction of HDAC1 can reduce the oncogenic potential of Api5. Thus inhibitors of p300 and inducers of HDAC1 have therapeutic implications in the treatment of cancers that have high expression of Api5.

As K251 acetylation of Api5 is the only known post-translational modification which is conserved from protists to mammals², it suggests that conservation of this acetylation may play a key role during normal development. AMPK and Akt are important regulators of various cellular processes like cell division, transcription regulation and apoptosis^{44–46,49,50}. Reduced levels of Api5 upon inhibition of these key regulators also indicates its role in normal physiological and developmental processes. Although our study suggests that p300 is the acetylating and stabilising regulator of Api5 while HDAC1 is the de-acetylating and negative regulator of stability, one cannot ignore the possibility of other enzymes playing a role in the regulation of Api5 stability. Also, the inhibitor Romidepsin, used in our study has also been reported to inhibit HDAC2. Hence, we do not neglect the plausible role of HDAC2 playing a role in deacetylating Api5.

Thus taken together, our data is the first to demonstrate that p300 and HDAC1 are the novel regulators of Api5 which not only interact but also mediate the localisation and activity of Api5 by acetylating and de-acetylating

at lysine 251 respectively, leading to cellular proliferation thereby regulating cell cycle progression as illustrated in Fig. 8b.

Materials and methods

Chemicals and antibodies. Histone Acetyltransferase p300 inhibitor, C646 (382113), protease inhibitor cocktail (P2714), dimethyl sulfoxide (DMSO, D8418) and propidium iodide (PI, P4170) were purchased from Sigma-Aldrich, USA and romidepsin (FK228, Depsipeptide) from Selleck Biochem, USA. Lipofectamine 2000 (11668), Alexa fluor 488 goat anti-rabbit (A11034), Phalloidin 568 (A12380), Phalloidin 633 (A22284) and Hoechst 33258 (H3569) were purchased from Thermo Fisher Scientific, USA. GFP (ab290), RFP (ab62341) Cyclin B1 (ab32053), p21 (ab109520) and p300 (ab10485) antibodies were obtained from Abcam, USA, HDAC1 (sc-8410), E2F1 (C-20- sc-193) and Cyclin A (sc-271682) from Santa Cruz Biotechnology Inc, USA. PCNA (2586), pAkt Ser437 (9271), Akt (9272) and acetyl p53 Lys382 (2828) from Cell Signaling Technology, USA. Api5 (PAB7951) from Abnova, Taiwan, acetyl lysine (05-515) from Merck Millipore, USA, Hoechst 3,258 (H3569) was purchased from Molecular Probes, Thermo Fisher Scientific, USA and GAPDH (G9545) from Sigma-Aldrich, USA.

Plasmids. CSII-EF-MCS plasmid was a gift from Dr. Sourav Banerjee, NBRC, Manesar, India. pCAG-HIVgp and pCMV-VSV-G-RSV-Rev plasmids were purchased from RIKEN BioResource Centre, Japan. mVenusC1 was gifted by Jennifer Lippincott-Schwartz, NIH, USA in which Api5 was cloned. The Api5 was made siRNA-resistant and K251 mutants were generated using site directed mutagenesis Details of plasmids used and cloning protocols are provided in the supplementary information.

Cell culture and drug treatments. MCF7 cells were obtained from the European Collection of Cell Cultures (ECACC). HEK293T cells were a gift from Dr. Jomon Joseph (NCCS, Pune). The cells were maintained in 100 mm dishes (Thermo Fisher Scientific, USA 172931) and were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM; Lonza Group AG, Switzerland, 12-604F) containing 10% heat inactivated FBS (Thermo Fisher Scientific, USA, 10270106) and 100 units/mL penicillin-streptomycin (Lonza Group AG, Switzerland, 17-602E) and incubated at 37 °C humidified 5% CO₂ incubators (Eppendorf, GmbH or Thermo Scientific, USA).

For inhibitor studies, 7×10^5 cell were seeded on 35 mm dishes (Thermo Fisher Scientific, USA, 153066) 16 h prior to treatment of C646 or Romidepsin and incubated for 4 h and 12 h, respectively.

MCF7 cells stably expressing mCherry-tagged Api5 were generated using lentiviral-mediated transduction. Briefly, 7.5×10^5 HEK293T cells were seeded on 35 mm dish and transfected with 1 µg of mCherry-CSII-EF-MCS or mCherry-CSII-EF-MCS-Api5 plasmid along with 1 µg of pCAG-HIVgp and 0.5 µg pCMV-VSV-G-RSV-Rev packaging plasmids using Lipofectamine 2000. 1 mL DMEM containing 30% FBS was added to the cells 24 h post transfection. 5×10^5 MCF7 cells were seeded on a 35 mm dish for transduction. Viral supernatant was collected 48 h post transfection and filtered through a 0.45 µm filter to get rid of the cell debris. Filtered viral supernatant containing media along with 1 ml fresh media was added to the MCF7 cells. 4 µg polybrene was added to the cells to increase the transduction efficiency. Cells were replenished with fresh medium 48 h post transduction. Transduced MCF7 cells were passaged and used for further experiments.

For transfections, 6×10^5 cells were seeded in 35 mm dishes and incubated at 37 °C overnight. siRNA duplexes targeting Api5 and LacZ were purchased from Dharmacon (Thermo Fisher Scientific, USA). Sense sequences of the siRNA are: Api5, 5'-GACCUAGAACAGACCUCAUU-3', LacZ, 5'-CGUACGCGGAUA CUUCGA dTdT-3'. siRNA against LacZ and Api5 were transfected as described earlier⁶². Api5 K251 mutants which were generated by site directed mutagenesis were transfected 24 h post knockdown of Api5.

Immunoblotting and immunoprecipitation. mCherry-tagged Api5 over-expressing stable cells with or without treatments were lysed in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na₃VO₄ and protease inhibitors. Immunoprecipitations were performed as described⁶³ using GFP, RFP, p300, or HDAC1-specific antibodies. 2 µg of antibody (IgG or GFP/RFP/p300/HDAC1) was used to pull down 800 µg of protein for performing the pull down and 3% of whole cell extract have been loaded as input. Western blotting was performed with Api5, GFP, acetyl lysine and GAPDH antibodies as previously mentioned⁶². Immunoblots include all the experimental lanes of a said experiment and there are no composite images where two or more blots were merged together. The blots were processed as horizontal cut strips in the respective antibody dilutions and imaged as individual strips in ImageQuant LAS4000 (GE Healthcare, now Cytiva, USA).

Semi-quantitative PCR. RNA extraction and cDNA synthesis of C646 and Romidepsin treated cells were performed as described previously⁶⁴. Semi-quantitative PCR was performed using API5 specific forward (5'-CGAGTGGCAGATATACTAACGC-3') and reverse (5'-TCCTCTCCTTGAAGTATTTGGC-3') primers. GAPDH was used as endogenous control and was amplified using forward (5'-ACCACAGTCCATGCCATCAC-3') and reverse (5'-TCCACACCCTGTTGCTGTA-3') primers. The following PCR cycle was used for the amplification: 95 °C for 60 s, 58 °C for 45 s, 72 °C for 60 s and final extension for 3 min. The experiments were repeated three times to confirm API5 levels.

Immunofluorescence. For immunofluorescence, 6×10^5 cells were seeded on coverslips placed in 35 mm dishes. After treatment of C646 or romidepsin, cells were fixed in 4% paraformaldehyde and permeabilised using 0.5% Triton X after PBS washes. After PBS-Glycine wash, cells were blocked using 10% FBS for 1 h and incubated overnight with Api5 antibody at 4 °C. Cells were incubated with secondary antibodies conjugated with Alexa

Fluor for 1 h at room temperature along with phalloidin. Nuclei were stained with Hoechst 33258 for 5 min and washed twice using PBS. The cells were mounted using mounting media and imaged under 63× oil immersion objective of the SP8 confocal microscope (Leica, Germany). Corrected total cell fluorescence (CTCF) was calculated to measure the intensity of Api5 fluorescence in nucleus and cytoplasm of the cells. Briefly, the nuclear and cytoplasmic boundary was marked manually and measure the area, integrated density and mean grey value of Api5 fluorescence. Also measure the mean intensity of background to calculate corrected fluorescence. CTCF was calculated with the formula $CTCF = \text{integrated density} - (\text{area of selected area} \times \text{mean fluorescence of background readings})$. CTCF of at least 200 cells were calculated per experiment set.

Cell proliferation assay. For cell proliferation assay, 6×10^5 MCF7 cells were seeded in 35 mm dishes and incubated at 37 °C overnight. mVenus-tagged WT and K251 mutants of Api5 plasmids were transfected as mentioned before. 48 h post transfection, cells were trypsinised and pelleted down by centrifugation at 1000 rpm for 5 min. Cell pellet was suspended in 1× PBS. 1:10 dilution of the suspended cell pellet was prepared in 1X PBS and the cell count was measured using 60 µm sensor connected to Sceptar™ hand-held automated cell counter (Millipore, Sigma-Aldrich, USA).

Flow cytometry. MCF7 cells after transfection were trypsinised using 1× trypsin and collected in media. After pelleting, the cells were washed with cold PBS twice to remove excess media. The cells were then fixed in 70% ethanol overnight at 4 °C and stained with 1 mg/ml propidium iodide in the presence of RNase A (20 mg/ml) at 37 °C for 1 h. Cell cycle profile was analysed on BD Accuri™ C6 flow cytometer (BD Biosciences, USA).

Statistical analysis. All experiments were performed with a minimum of 3 independent biological replicates. Densitometry analysis of western blots were performed using ImageJ software. Results were analysed from a minimum of three independent experiments and plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) represented in bar graphs as Mean ± SEM. Student's t test and Mann Whitney U test was used to test the significance in difference of Api5 levels between two samples and One-way Anova and Dunnett's multiple comparisons test for more than two samples. p value > 0.05 was considered non-significant. *, ** and *** correspond to $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

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Author contributions

V.K.S. and M.L. conceived and conceptualised the project. V.K.S. designed, performed and analysed the experiments. V.K.S. and M.L. wrote the paper.

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Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials

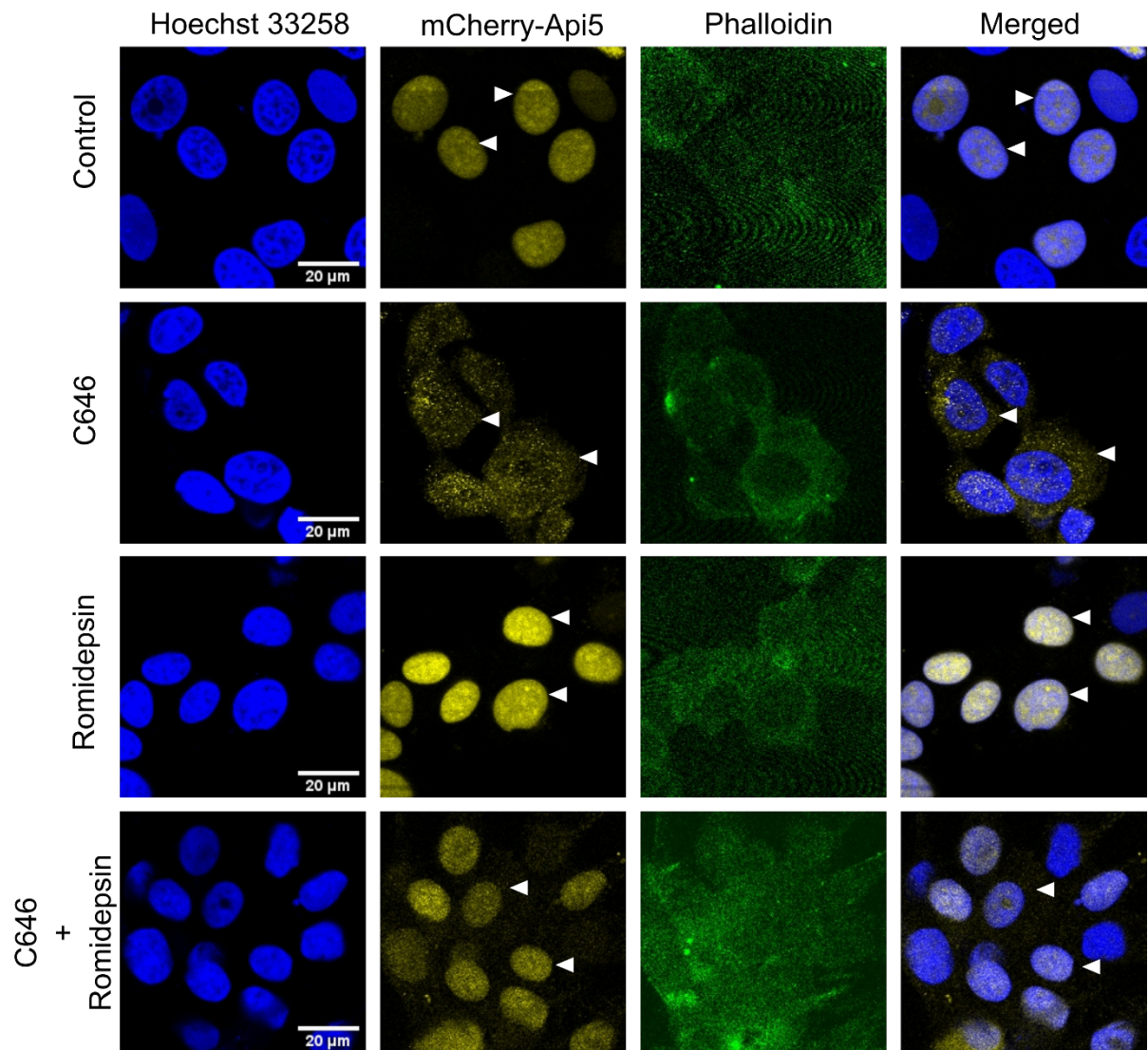
Interplay between p300 and HDAC1 regulate acetylation and stability of Api5 to regulate cell proliferation

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Supplementary Figure S1

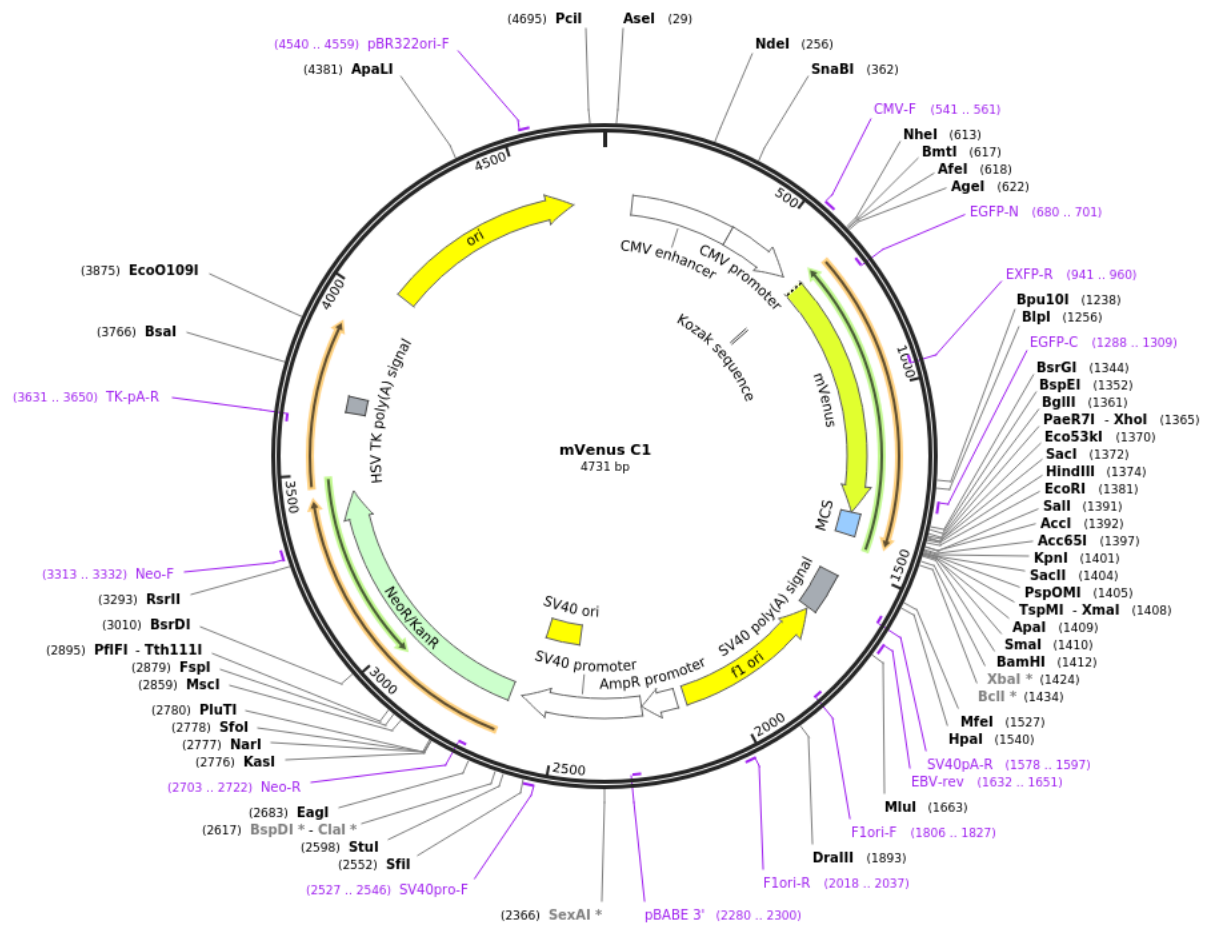


Supplementary Figure S1: p300 and HDAC1 regulate Api5 localisation

mCherry tagged Api5 over-expressing MCF7 cells were treated with 7.5 μM C646, 0.1 nM romidepsin for 4 hrs and 16 hrs respectively. mCherry fluorescence was visualised using confocal microscopy (scale bar: 20 μm).

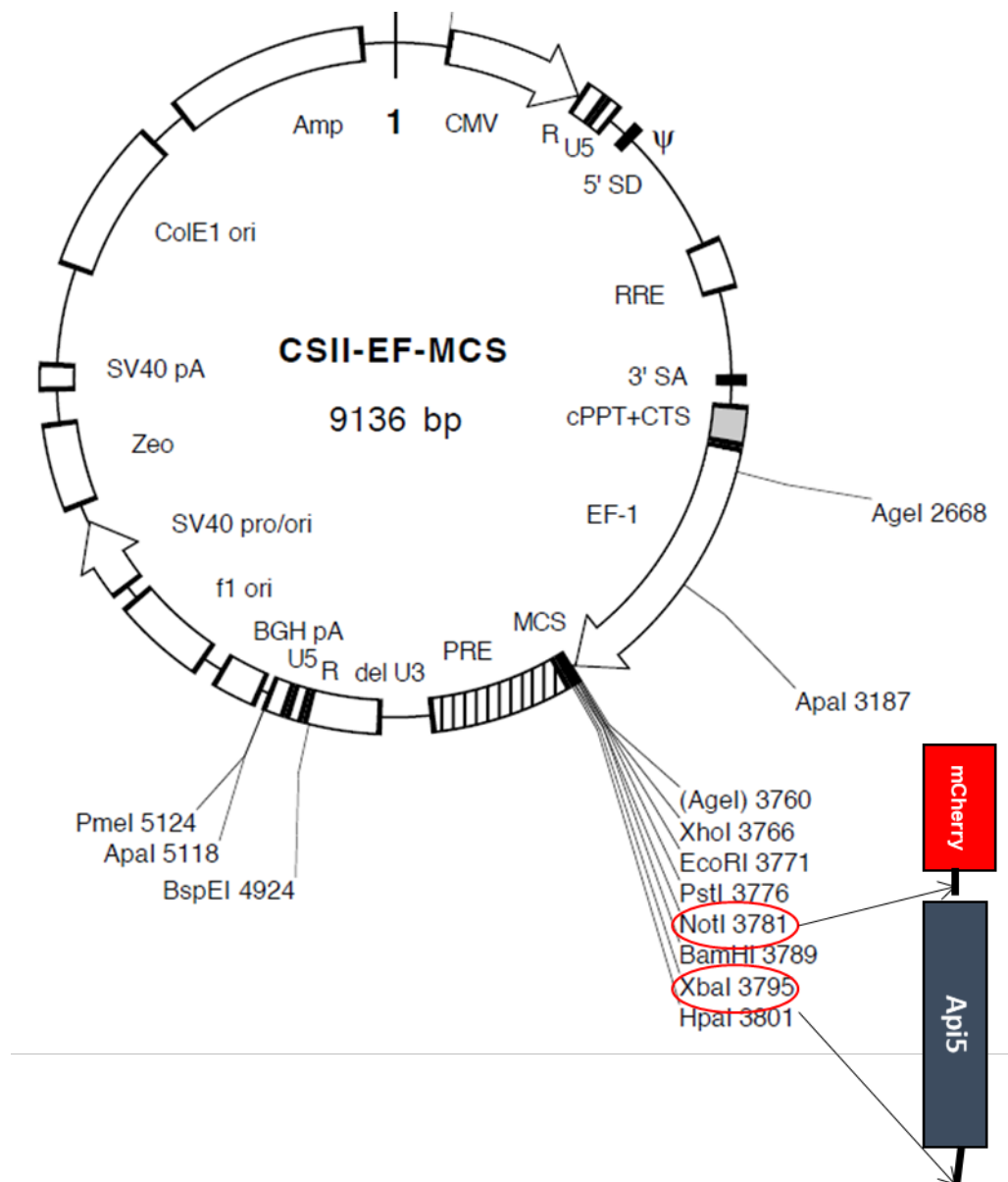
Supplementary Figure S2

Created with SnapGene®



mVenusC1 was gifted by Jennifer Lippincott-Schwartz, NIH, USA in which Api5 was cloned. The vector map was obtained from Addgene web site.

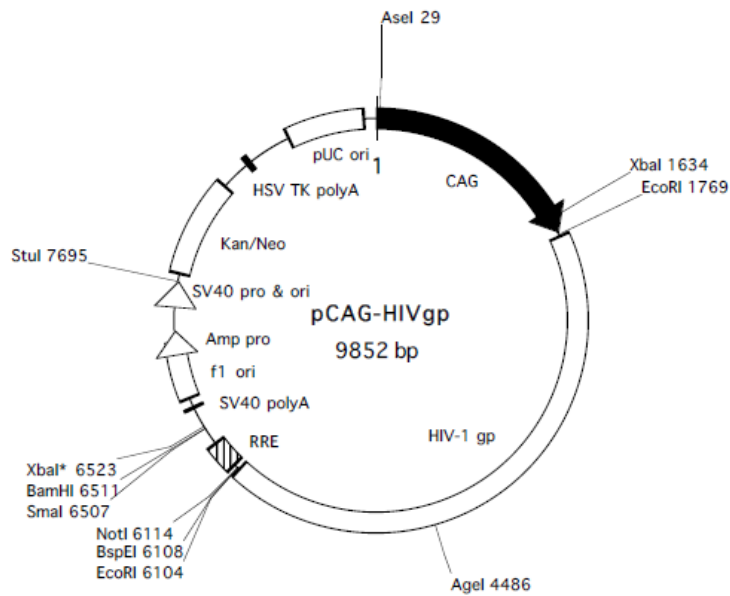
Supplementary Figure S3



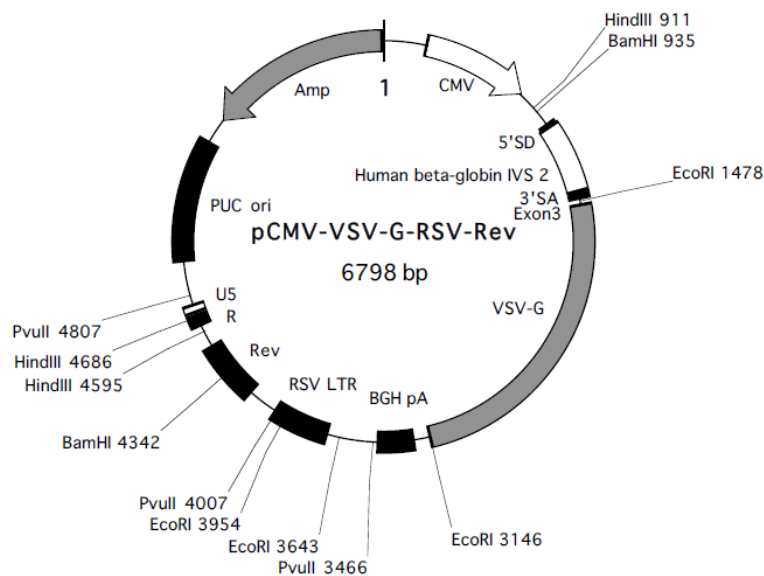
CSII-EF-MCS plasmid was a gift from Dr. Sourav Banerjee, NBRC, Manesar, India. Api5 was cloned into CSII-EF-MCS vector with a mCherry tag between NotI and XbaI sites. The vector map of CSII-EF-MCS plasmid was provided by Riken BioResource Centre, Japan.

Supplementary Figure S4

a.

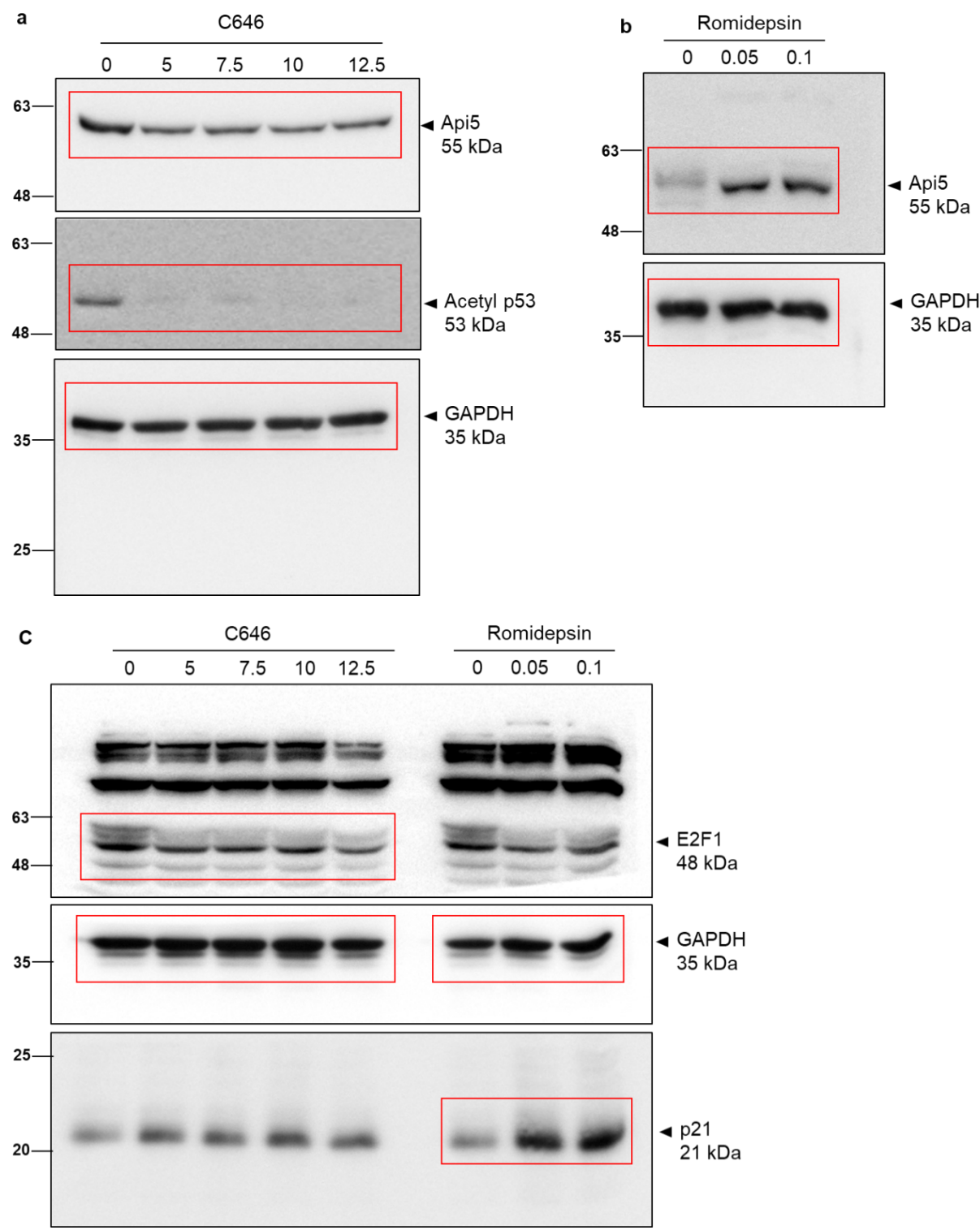


b.



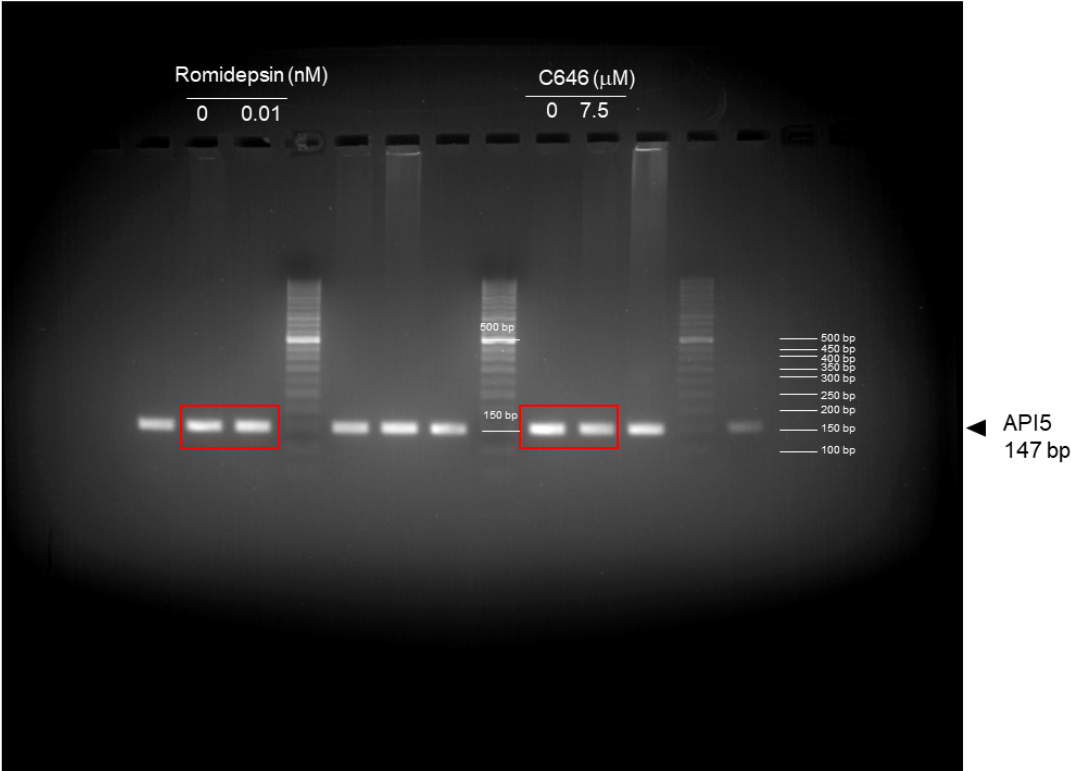
a) Plasmid details of expression vector of HIV-1 gag and pol for lentivirus vector packaging and b) envelope VSVG protein and Rev for producing lentiviral particles to generate mCherry-Api5 stable cell lines. The plasmids was purchased from RIKEN BioResource Centre, Japan and the vector map was provided by them.

Supplementary Figure S5: Full blots for Figures 2a and 3a.

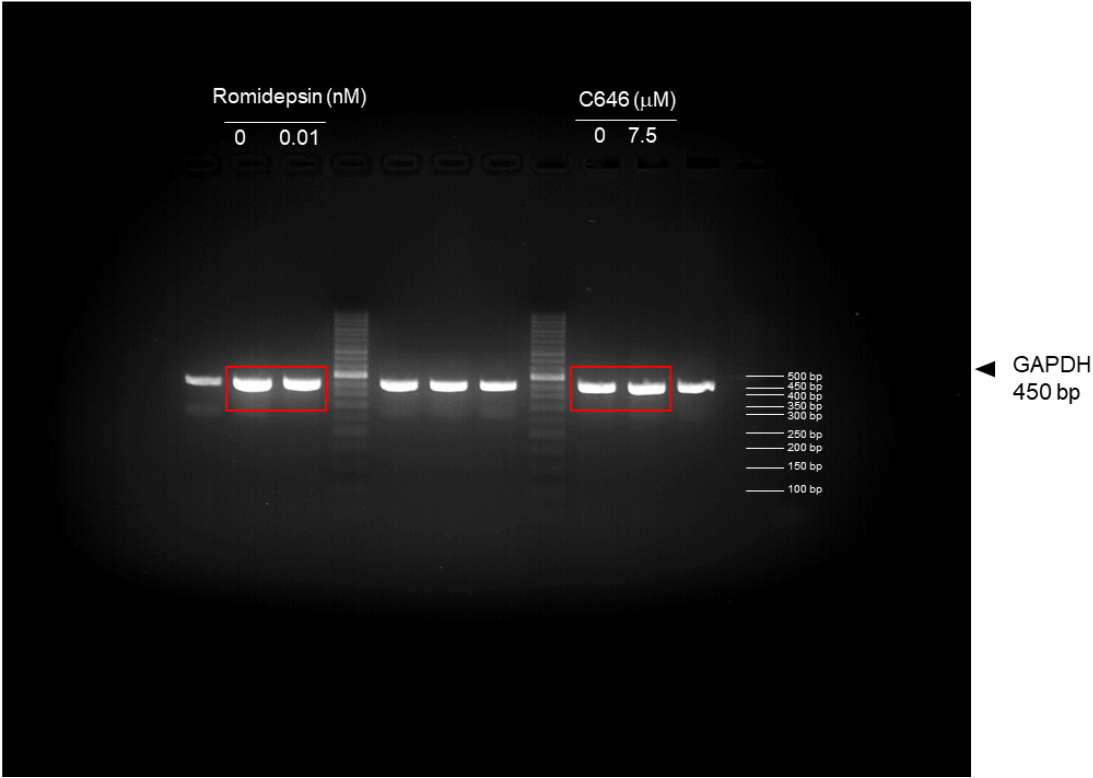


Supplementary Figure S6: Agarose gels for Figures 2e and 3d

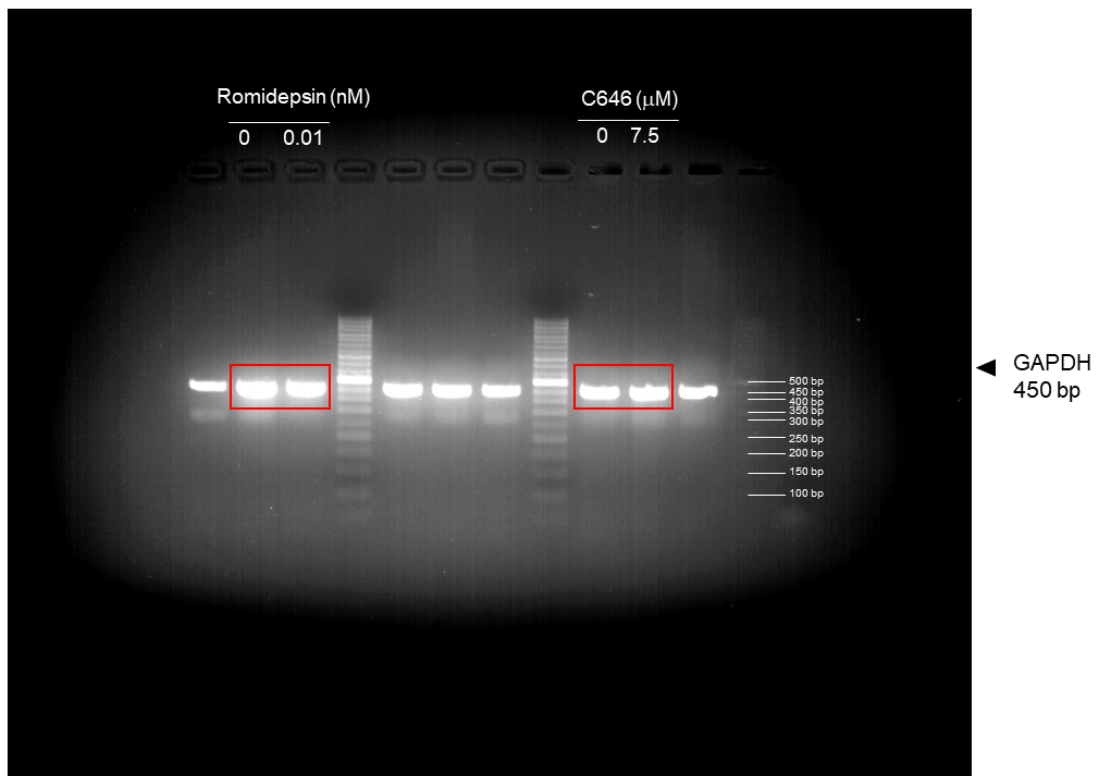
a



b

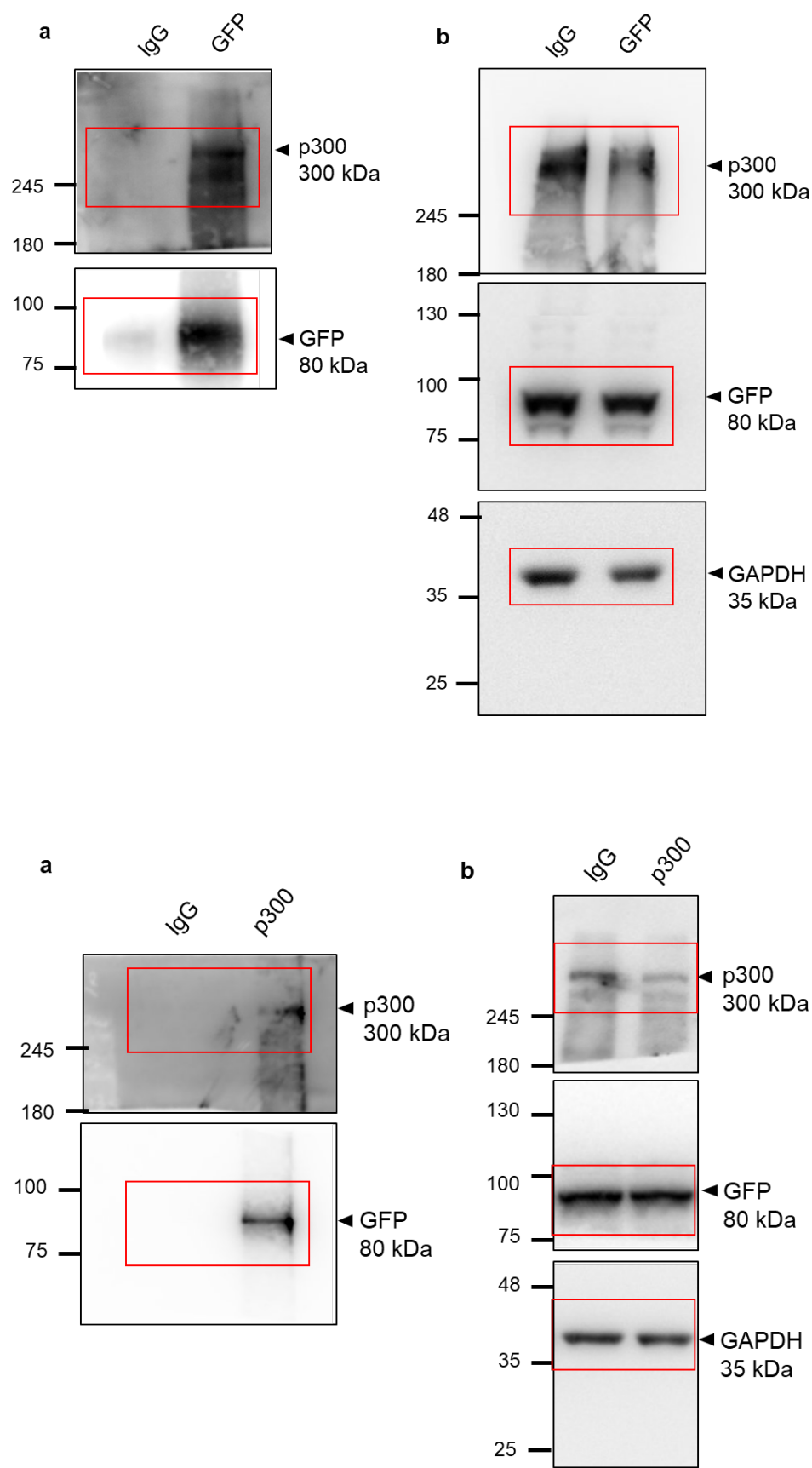


c

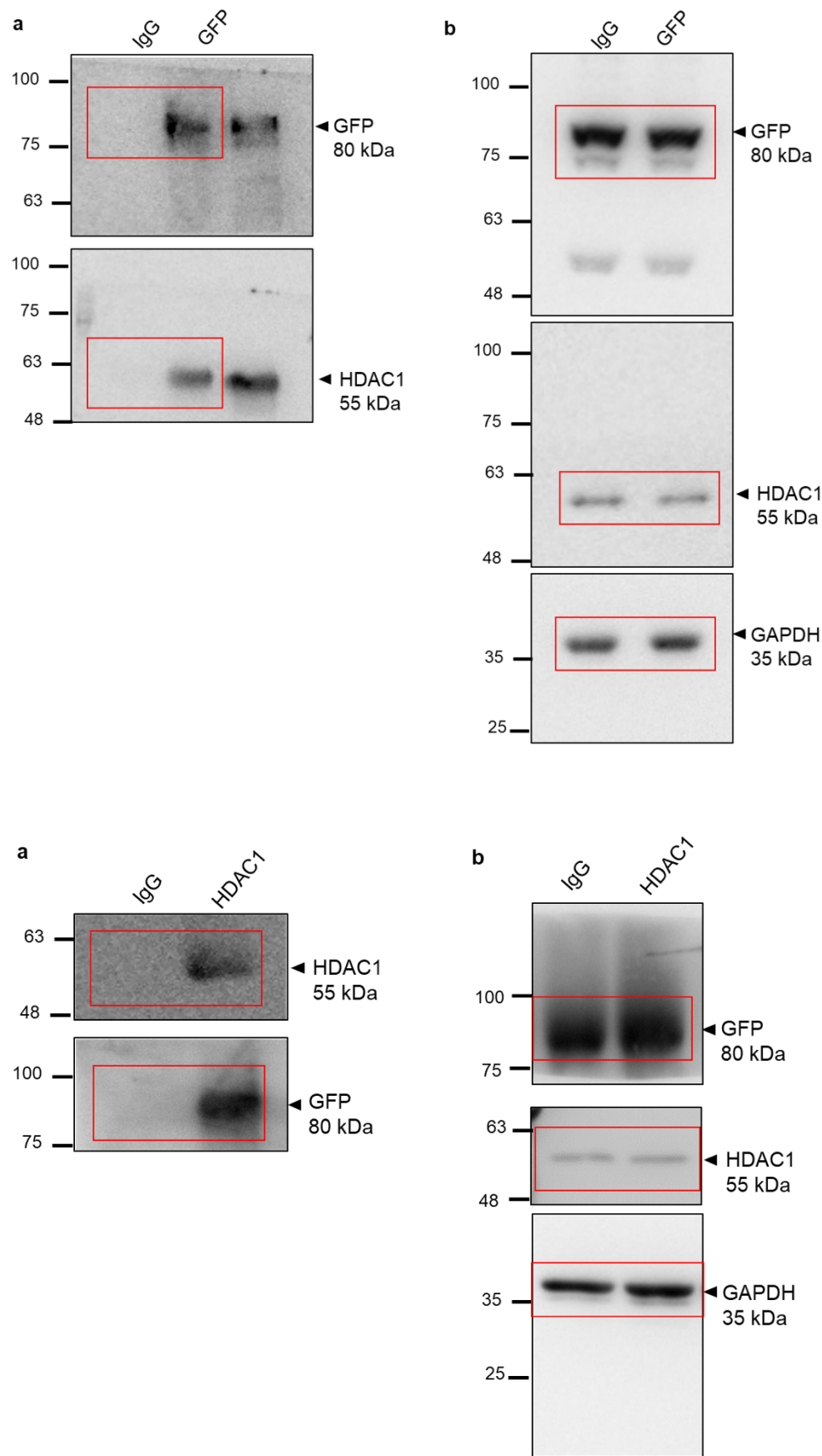


The PCR was set up as mentioned in materials and methods. We prepared cDNA master mix and aliquoted 10 μ L of reaction mixture into separate tubes and added the API5 and GAPDH primers respectively. The PCR reaction was carried out in the same PCR machine with gradient temperature. The electrophoresis of PCR products was carried out in two separate gels for a) API5 and b) GAPDH low exposure image and c) GAPDH high exposure image.

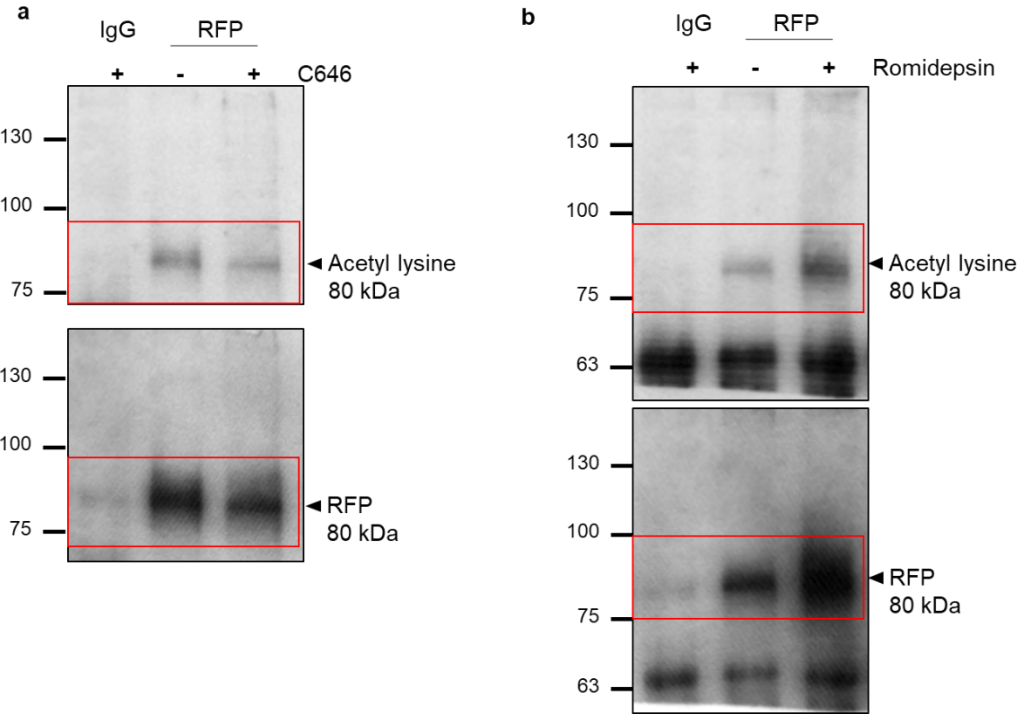
Supplementary Figure S7: Full blots for Figure 2g and 2h.



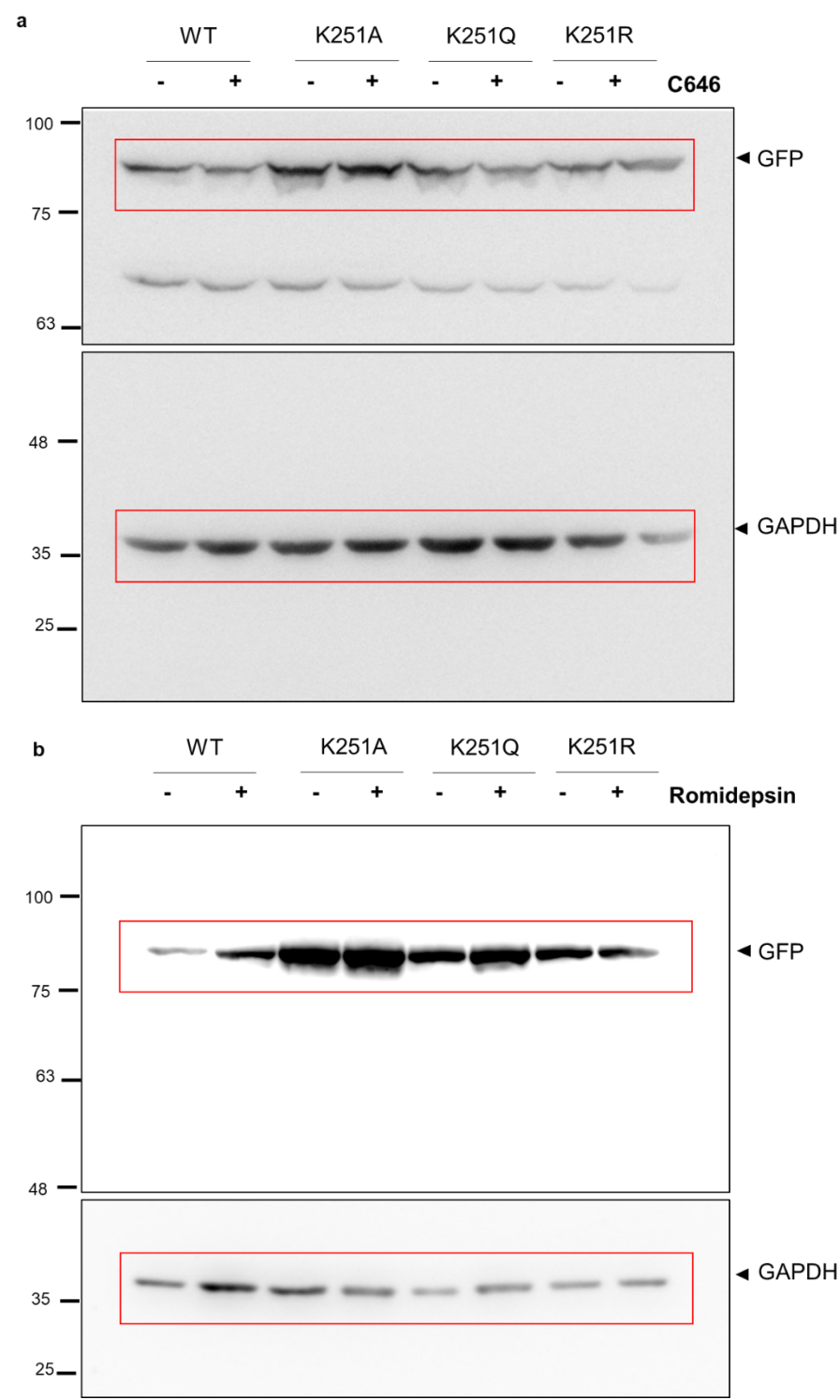
Supplementary Figure S8: Full blots for Figure 3f and 3g.



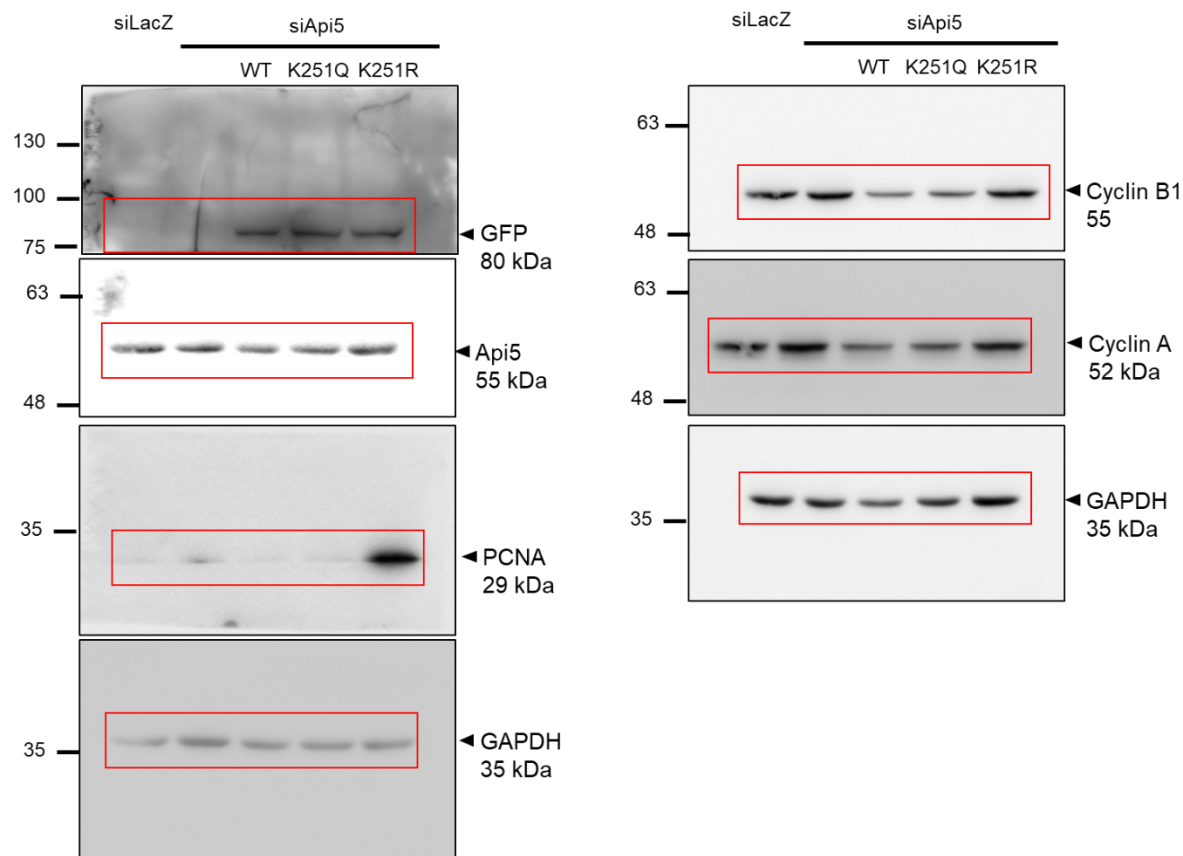
Supplementary Figure S9: Full blots for Figure 4a and b.



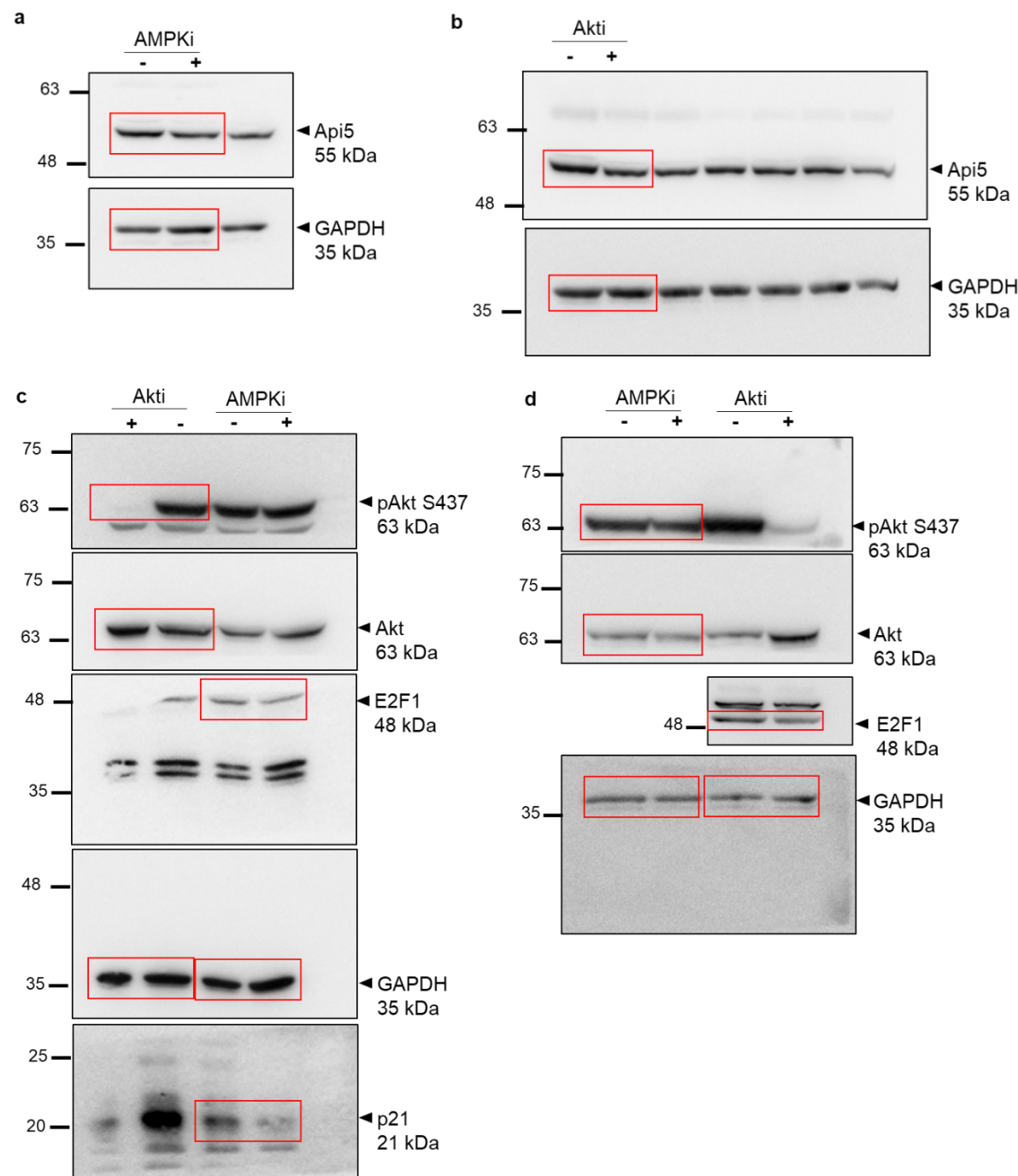
Supplementary Figure S10: Full blots for Figure 4c and e.



Supplementary Figure S11: Full blots for Figure 6b.



Supplementary Figure S12: Full blots for Figure 7.



Supplementary information

Primers used for cloning of Api5 into mVenus C1 and mCherry CSII-EF MCS vectors

mVenus Api5 For: GGAAGATCTATGCCGACAGTAGAGGAGCT

mVenus Api5 Rev: CCGGAATTCTCAGTAGAGTCTTCCCCGAC

mCherry Api5 For: AAGGAAAAAAGCGGCCGCATATGCCGACAGTAGAGGAGCT

mCherry Api5 Rev : GCTCTAGATCAGTAGAGTCTTCCCCGAC

Cloning of Api5 into mVenus C1 or mCherry MCS-CSII-EF

Full length Api5 was cloned into mVenus C1 vector after amplifying it using mVenus API5 forward and reverse primers. This wild type Api5 in mVenus C1 was further cloned into mCherry-CSII-EF-MCS vector for preparing lentiviral plasmids. Api5-mVenus C1 plasmid was PCR amplified (Supplementary Table S1) using mCherry Api5 forward and reverse primers with XbaI and NotI restriction site overhangs. PCR cycle was 95°C for 60 sec, 60°C for 1 min, 72°C for 3 mins sec and final extension for 5 min, repeated for 30 cycles. PCR products were purified using a PCR purification kit (Qiagen, USA) and digested using XbaI (New England Biolabs; NEB USA) and NotI HF (NEB) for 4 hours (Supplementary Table S2). mCherry-CSII-EF-MCS vector was also digested using same restriction enzymes at 37°C for 3 hours and calf intestinal phosphatase (CIP; NEB) treatment for 1 hr was performed. Digested plasmids were gel extracted after resolving on agarose gel using gel extraction kit (Sigma-Aldrich, USA). Api5 was ligated into mCherry-CSII-EF-MCS vector with an insert to vector ratio of 75 ng :25 ng using T4 DNA ligase (Takara Bio; USA) at 16°C overnight (Supplementary Table S3). Ligation mixture was used to transform DH5 α competent cells and incubated overnight at 37°C. Plasmid DNA was isolated from the obtained colonies and screened by PCR to check for the correct insert.

Supplementary Table S1: PCR amplification of wild type from API5-mVenus C1

Table contains the details of reagents used for PCR amplification of API5 from mVenus C1 for cloning into CSII-EF-MCS vector.

DNA Template	3 μ l
Forward Primer	3 μ l
Reverse Primer	3 μ l
10 mM dNTPs	12.5 μ l
10X Pfu buffer	10 μ l
DMSO	5 μ l
dH ₂ O	6.5 μ l
Pfu polymerase	2 μ l

Supplementary Table S2: Digestion of PCR product and CSII-EF-MCS-mCherry

Table contains the details of reagents used for digestion of PCR amplified API5 with XbaI and NotI overhangs and CSII-EF-MCS vector with mCherry tag.

DNA Template	5 μ g
10 X Cut smart buffer	5 μ l
Not 1 HF	5 μ l
Xba 1	5 μ l
dH ₂ O	To make up the reaction to 50 μ l

Supplementary Table S3: Ligation reaction of API5 into mCherry tagged CS-II-EF-MCS digested vector

Table contains the details of reagents used for ligating gel-extracted digested products of Api5 and CSII-EF-MCS-mCherry vector.

Insert	75 ng
Vector	25 ng
10X T4 Ligase buffer	1 μ l
T4 DNA Ligase	1 μ l
dH ₂ O	To make up the reaction to 10 μ l

Site-directed mutagenesis to generate siRNA resistant WT Api5 and K251 Api5 mutants.

The primers for site directed mutagenesis to generate siRNA resistant Api5 and Api5 K251 mutations were designed and ordered from Sigma-Aldrich, USA.

mut Api5 For: 5'-GCCGAACCTGGATCAGACCTTCA-3'

mut Api5 1-Rev: 5'-TGAAGGTCTGATCCACTTCGGC-3'

Api5K251Qfor: 5'-CAGTACCCCTCTTCTCTCAGAATGTCCATTCCACAAGGTT-3'

Api5K251Qrev: 5'-AACCTTGTGGAATGGACATTCTGAGAGAAGAGGGGGTACTG-3'

Api5K251Rfor: 5'-CAGTACCCCTCTTCTCTCGAAATGTCCATTCCACAAGGTT-3'

Api5K251Rrev: 5'-AACCTTGTGGAATGGACATTTTCGAGAGAAGAGGGGGTACTG-3'

Api5K251Afor: 5'-CAGTACCCCTCTTCTCTGCAAATGTCCATTCCACAAGGTT-3'

Api5K251Arev: 5'-AACCTTGTGGAATGGACATTTGCAGAGAAGAGGGGGTACTG-3'

PCR reaction was set up with WT Api5 mVenus C1 as template with mut Api5 forward and reverse primers to generate siRNA resistant Api5 (Supplementary Table S4). The PCR cycle was as follows: 95°C for 60 secs, 60°C for 1 min, 68°C for 20 mins and final extension for 5 min. The PCR product was DpnI digested at 37°C for 3 hrs and transformed into DH5 α cells. Colonies obtained were screened by sequencing. The siRNA resistant WT Api5 mVenus C1 plasmid was used as a template to generate K251 mutants of Api5 using specific primers and the same procedure.

Supplementary Table S4: PCR amplification cycle to generate mutants of Api5.

Table contains the details of reagents used for PCR amplification of API5-mVenus to generate siRNA resistant Api5 and K251 mutants of Api5.

DNA Template	500 ng
Forward Primer (25 μ M)	3 μ l
Reverse Primer (25 μ M)	3 μ l
10 mM dNTPs	12.5 μ l
10X Pfu turbo buffer	10 μ l
DMSO	5 μ l
dH ₂ O	6.5 μ l
Pfu turbo polymerase	2 μ l

ATR facilitates the degradation of Api5 through the ubiquitin-proteasome pathway via FBXW2 to regulate apoptosis upon DNA damage

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Key words: Api5, proteasomal pathway, FBXW2, DNA damage, ATR

Short title: FBXW2 ubiquitinates Api5 to regulate apoptosis upon DNA damage.

Summary

Apoptosis inhibitor 5 (Api5) is an inhibitor of apoptosis, which is found to be upregulated in several cancers and promotes invasion as well as metastasis. Over-expression of Api5 is positively co-related with poor survival of cancers and inhibition of DNA damage induced apoptosis in cancerous cells. Acetylation at lysine 251 (K251) on Api5 facilitates the stability of the protein and thus functionally provides resistance to cancer cells against chemotherapeutic or anti-cancerous agents. However, the regulation of Api5 upon DNA damage is not yet known. In this study, we demonstrate that Api5 undergoes degradation following DNA damage via the ubiquitin-proteasome system. Upon DNA damage, ATR was observed to phosphorylate Api5 at serine 138 which led to the cytoplasmic localisation of Api5. The E3-ubiquitin ligase, SCF-FBXW2 ubiquitinates Api5 leading to its proteasomal degradation.

Introduction

Apoptosis inhibitor 5 (Api5) is a ubiquitously present protein which was initially discovered as a negative regulator of apoptosis under stressed conditions (1). Api5 protein is comprised of α -helical repeats and has three main domains: LxxLL (leucine repeat domain) is responsible for maintaining the stability of the protein, a putative LZD (leucine zipper domain) that is generally known to bind to chromatin and NLS (Nuclear Localisation Sequence) that is responsible for the transport of the protein to the cytoplasm from the nucleus (2). Depending upon the cellular context, Api5 has been shown to engage in its anti-apoptotic function through interaction and regulation of multiple proteins. As mentioned earlier, Api5 was found to inhibit apoptosis upon serum starvation (1), however, later it was observed that Api5 could also play a role in inhibiting apoptosis during various stresses like viral infections, endotoxins and DNA damage. Api5 regulates Acinus-mediated DNA fragmentation due to acute DNA damage-induced apoptosis. Api5 binds with Acinus and prevents its activation (3). In human osteosarcoma cells, ectopic expression of Api5 was found to reduce E2F1-mediated apoptosis (4). Api5 has also been observed to aid in the degradation of Bim, a pro-apoptotic protein (5). It has also been reported that Api5 inhibits the transcription of APAF, one of the main components of the apoptosome complex. A recent study reported the interaction between Api5 and the zymogen, caspase 2. Upon binding with caspase 2, Api5 prevents its activation (6).

Acetylation at lysine 251 (K251) is the only reported post-translational modification of Api5 (2). This K251 acetylation provides stability to Api5. It has been observed that acetylation at lysine 251 on Api5 by p300 histone acetyl transferase stabilises while HDAC1-mediated de-acetylation leads to the degradation of the protein (bioRxiv, doi:<https://doi.org/10.1101/2020.11.22.393256>). In this study, we were interested to investigate whether Api5 undergoes any other post translational modification(s) during normal physiological conditions or upon DNA damage prior to its degradation. Ubiquitination is a well-known post-translational modification that leads to degradation of the target protein. It is a complex process and requires multiple enzymatic functions in order to achieve degradation of its target proteins (7). Ubiquitin ligases (E3) are the most heterogeneous type of enzymes that carry out degradation by attaching ubiquitin moieties to the proteins (8). The multi-protein E3 ubiquitin ligase Skp, Cullin, F-box containing complex (SCF complex) helps in

degradation of proteins that function during the cell cycle as well as in apoptosis (9). Most of the proteins that undergo ubiquitination also require phosphorylation as a secondary but necessary signal for their degradation. Since Api5 has earlier been reported to play a significant role in DNA damage induced apoptosis, it was important to investigate the mechanism of regulation of Api5 during DNA damage.

ATR is one of the sensor kinases that is involved in invoking a cell cycle arrest to allow for repair of damaged DNA (10). To perform its functions, ATR interacts with and phosphorylates BRCA1, CHK1, MCM2, RAD17, RPA2, SMC1 and p53/TP53 that collectively inhibit DNA replication as well as mitosis and promote DNA repair, recombination and apoptosis (11-17).

As earlier reports have shown Api5 to undergo post-translational degradation, it was pertinent to investigate the mechanism of its degradation during normal physiological conditions as well as during DNA damage. Our findings demonstrate Api5 to undergo poly-ubiquitination and thereby degrade via the proteasomal pathway during both normal physiological conditions as well as during DNA damage-induced apoptosis. We report FBXW2, a component of SCF (SKP1-Cullin1-F-box protein) E3 ubiquitin ligase to play a role during the proteasomal-mediated degradation of Api5. We also demonstrated that Api5 degradation upon DNA damage was regulated by ATR. Thus, we report ATR and FBXW2 as key regulators of Api5 function during physiological as well as during DNA damage conditions.

Results

Api5 undergoes ubiquitin proteasome-mediated degradation

To identify the potential pathway of Api5 degradation, cells were treated with different concentrations of chloroquine, a lysosomal pathway inhibitor and MG132, a proteasomal pathway inhibitor. Inhibition of the lysosomal pathway did not show any change in Api5 levels suggesting that Api5 degradation is not through the lysosomal pathway (Figure 1A and S1A). However, upon inhibition of the proteasomal pathway by varying the dose of MG132, showed increased levels of Api5 indicating that Api5 degradation is through the proteasomal pathway (Figure 1B and S1B). In order to obtain optimal accumulation of Api5, 10 μ M of MG132 was added to the cells and time-course experiments were performed. It was observed that inhibition of the proteasomal pathway for 4 hrs using 10 μ M of MG132 showed optimal accumulation of Api5 (Figure 1C-D). After standardising the optimal dose and time for MG132 treatment, all further experiments were performed with that particular dose and Api5 levels were analysed. To further validate that the increase in protein level upon inhibition of the proteasome pathway was not as a result of increased protein synthesis, cells were treated with 10 μ g/ml of cycloheximide, a protein synthesis inhibitor, for different time durations. Decrease in Api5 protein levels upon cycloheximide treatment confirmed the inhibition of Api5 protein translation (Figure 1E-F). To prove that the accumulation of Api5 is due to inhibition of proteasomal degradation and not due to increased protein synthesis, MCF7 cells were treated with a combination of cycloheximide and MG132. Accumulation of Api5 was observed upon treatment with MG132 even when protein synthesis was inhibited, thereby demonstrating that the degradation of Api5 is through the proteasomal pathway (Figure 1G-H). A plethora of studies have shown that most of the proteins that degrade via the proteasomal pathway undergo ubiquitination as a post-translational modification. Therefore, to verify whether Api5 undergoes ubiquitination, immuno-precipitation was performed using Api5 specific antibody. Immunoblotting of the Api5 immunoprecipitates revealed the presence of high mass ladders, which were further augmented following addition of MG132, indicating that Api5 undergoes poly-ubiquitination (Figure 1I).

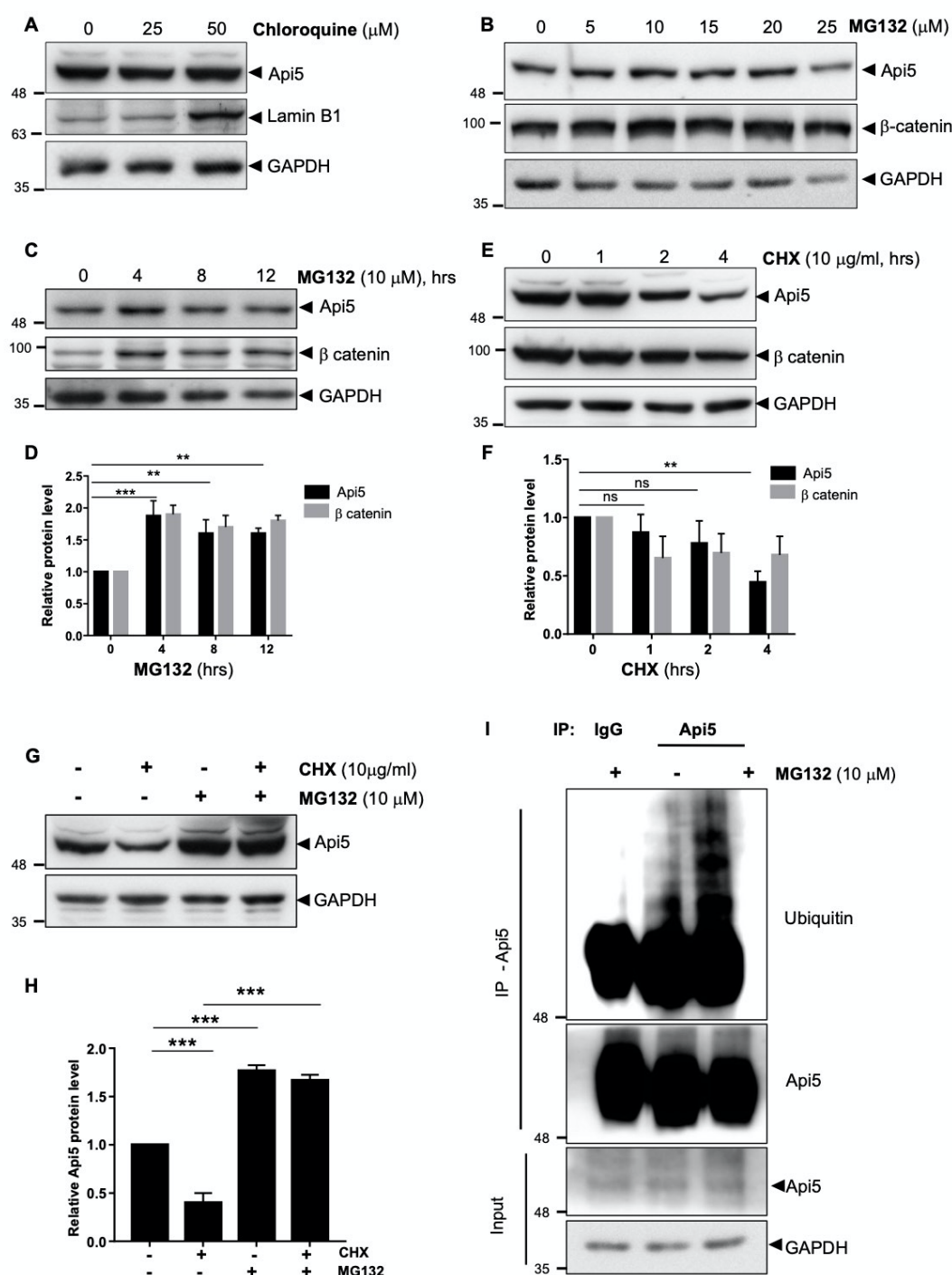


Figure 1. Api5 undergoes proteasomal degradation. (A) Western blot analysis showing protein levels of Api5 in MCF7 cells upon treatment with different doses of chloroquine. Lamin B1 was used as a positive control and GAPDH was used as a loading control. MCF7 cells treated with (B) varying doses of MG132 for 8hrs and (C) 10 μ M of MG132 for different time points. (D) Api5 levels were quantified and normalised to GAPDH. (E) Api5 levels were analysed in MCF7 cells treated with cyclohexamide for different time points and (F) quantification after normalising to

GAPDH. β catenin was used as the positive control for MG132 and cyclohexamide treatment. (G) MCF7 cells were treated with 10 μ g/ml of cycloheximide and 10 μ M MG-132 and Api5 levels were analysed using immunoblotting. (H) Api5 levels were quantified and normalised to GAPDH. (I) MCF7 cells treated with MG132 were lysed and immunoprecipitation was performed using Api5 specific antibody and immunoblotting using ubiquitin antibody.

F-box protein FBXW2 degrades Api5

F-box proteins are the substrate receptor component of SCF E3 ubiquitin ligases and they promote poly-ubiquitination-mediated degradation of their target proteins. To investigate the F-Box proteins involved in the degradation of Api5, cells were transfected with plasmids containing different F-box genes individually. Levels of Api5 were analysed following ectopic expression of myc- and flag-tagged FBOX proteins by immunoblotting. Of the 56 F-Box proteins screened, Api5 levels were significantly decreased following ectopic expression of FBXL6, FBXL8, FBXW2, FBXW4, FBXW9, FBXO3 and FBXO6 (Figure 2A). Quantification of Api5 levels revealed that the ectopic expression of FBXW2, FBXO3, and FBXO6 resulted in reduction of Api5 to its minimal level (Figure 2B).

To demonstrate a dose dependent degradation of Api5 by the selected F-Box proteins, cells were transfected with either vector control or plasmids containing FBXW2, FBXO3 and FBXO6 genes, and Api5 protein levels were analysed. It was observed that FBXW2, FBXO3 and FBXO6 degrade Api5 in a dose-dependent manner (Figure 2C-H). To prove that F-Box protein-mediated degradation of Api5 is through the proteasomal pathway, cells were treated with MG132 upon overexpression of FBXW2, FBXO3 and FBXO6. Api5 levels were restored upon MG132 treatment in the transfected cells suggesting that F-Box proteins degrade Api5 through the proteasomal pathway (Figure S2A-F).

In order for Api5 to undergo ubiquitin-mediated proteasomal degradation, the selected F-Box proteins must interact with Api5. To examine this, myc-tagged FBXW2, FBXO3 and FBXO6 were ectopically expressed and immuno-precipitation was carried out using anti-myc antibody. Presence of Api5 in the immune-precipitates of F-box proteins indicate that FBXO3, FBXO6 and FBXW2 interact with Api5 (Figure 2I).

Among the selected F-box proteins, FBXW2 has been reported to act as a tumour suppressor in lung carcinomas by degrading oncogenic SKP2 (18). Moreover there are multiple studies that suggest Api5 can act as a potential oncogene. We therefore speculated that FBXW2 may be potential candidate F-box protein to inhibit the anti-apoptotic function of Api5 and therefore we carried detailed studies to understand the mechanism of its regulation. First we examined the interaction of FBXW2 and Api5 at the endogenous level by co-immunoprecipitation assay. Immunoblotting results showed the presence of FBXW2 in the immuno-precipitates of Api5, indicating that Api5 and FBXW2 interact at the endogenous level (Figure 2J).

Next, we examined whether FBXW2 controlled basal levels of Api5. To address this, FBXW2 was depleted in cells using three independent shRNAs and a non-specific shRNA was used as control. Immunoblotting results revealed that expression levels of Api5 increased following depletion of FBXW2 (Figure 2K-L). Taken together, it may be concluded that FBXW2 plays a critical role in restricting the cellular levels of Api5 by directing the cells for proteasomal degradation.

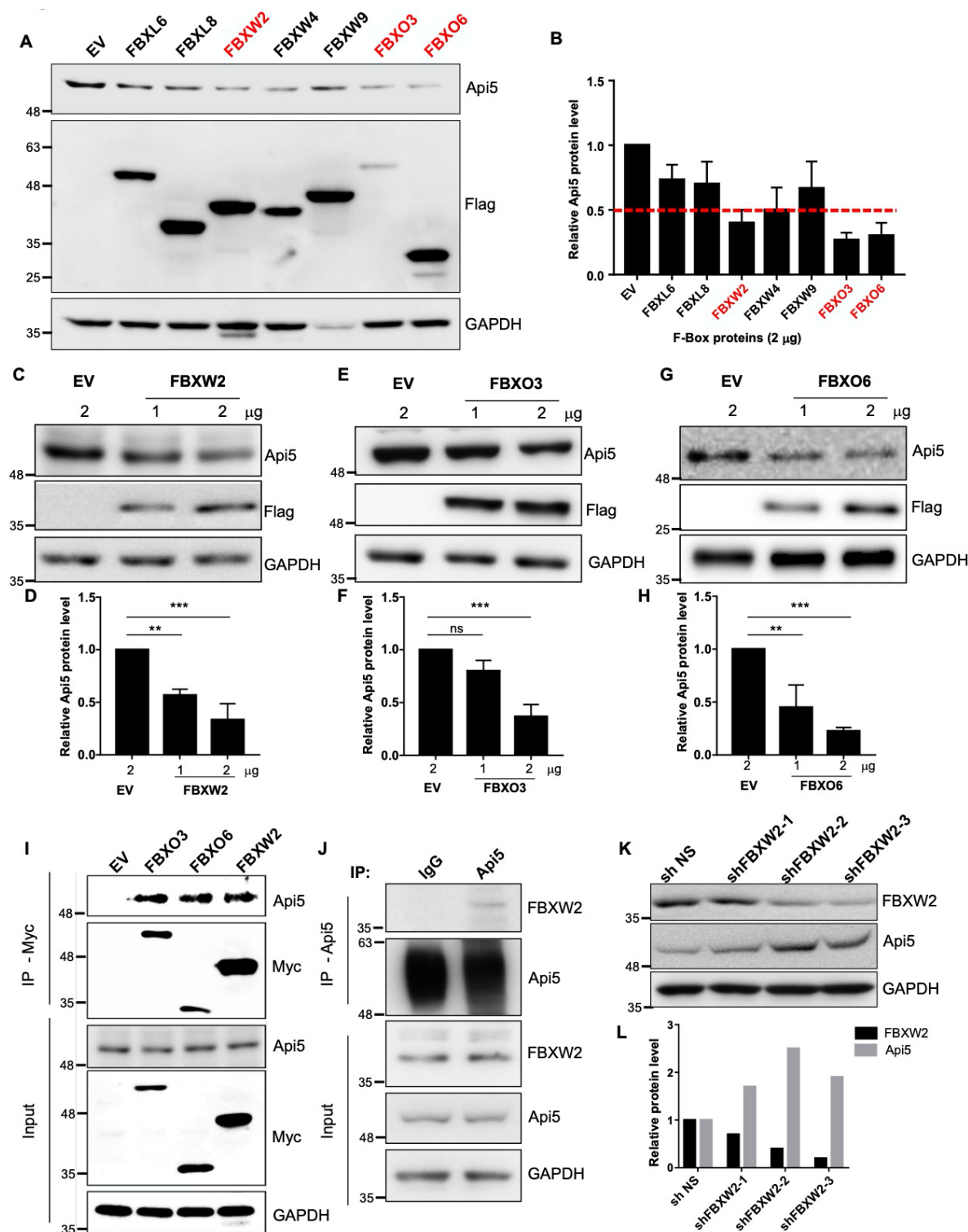


Figure 2. FBXW2 degrades Api5. Plasmids containing various flag-tagged F-box genes were ectopically expressed in MCF7 cells by transient transfection. These cells were lysed and Api5 levels were analysed using (A) immunoblotting and (B) quantified after normalising with GAPDH. Ectopic expression of F-box proteins was determined by immunoblotting with Flag. antibody MCF7 cells transfected with 1 and

2 µg of (C) FBXW2, (E) FBXO3 and (G) FBXO6 and Api5 levels were analysed using immunoblotting and (D), (F), (H) quantified after normalising with GAPDH. Empty vector (EV; 2µg) transfected cells lysates were used as control. (I) MCF7 cells transfected with myc-tagged FBXW2, FBXO3 and FBXO6 containing plasmids were lysed and immunoprecipitated using myc antibody. Api5 levels were analysed by immunoblotting with Api5 specific antibody. (J) Endogenous Api5 was pulled down in MCF7 cells and FBXW2 interaction was analysed using immunoblotting. (K) HeLa cells stably expressing various shRNA constructs of FBXW2 were lysed and Api5 and FBXW2 levels were analysed using immunoblotting. (L) FBXW2 and Api5 levels were quantified and normalised to GAPDH.

Api5 protein level reduces during DNA damage-induced apoptosis

To investigate the regulation of Api5 upon DNA damage, cells were treated with different concentrations of chemical and UV-radiation damage. Upon treatment with different doses of camptothecin (CPT) and etoposide (ETP), DNA damage was induced that was confirmed by observing the activation of Chk1 and Chk2 using immunoblotting as well as the presence of phosphorylated RPA foci by immunofluorescence (Figure S3A-D). Similarly, UV-irradiation led to the phosphorylation of Chk1 and the presence of pRPA foci, thus confirming DNA damage in the cells (Figure S3E-F). To demonstrate that DNA damage caused by chemicals as well as UV-irradiation can initiate apoptosis in the cells, apoptotic markers were analysed where increased levels of cleaved PARP and caspase 9 were observed (Figure S3 G-I). After standardising the dose and time for each DNA damaging agent, the levels of Api5 were analysed in the treated cells. It was observed that cells treated with camptothecin, etoposide and UV-irradiation showed reduced levels of Api5 (Figure 3A-F). This reduction in Api5 levels may be due to decrease in transcription of Api5. However, RT-PCR data demonstrated unaltered Api5 transcript levels upon DNA damage (Figure 3G-L), thereby indicating that Api5 may be regulated at the post-transcriptional level following DNA damage.

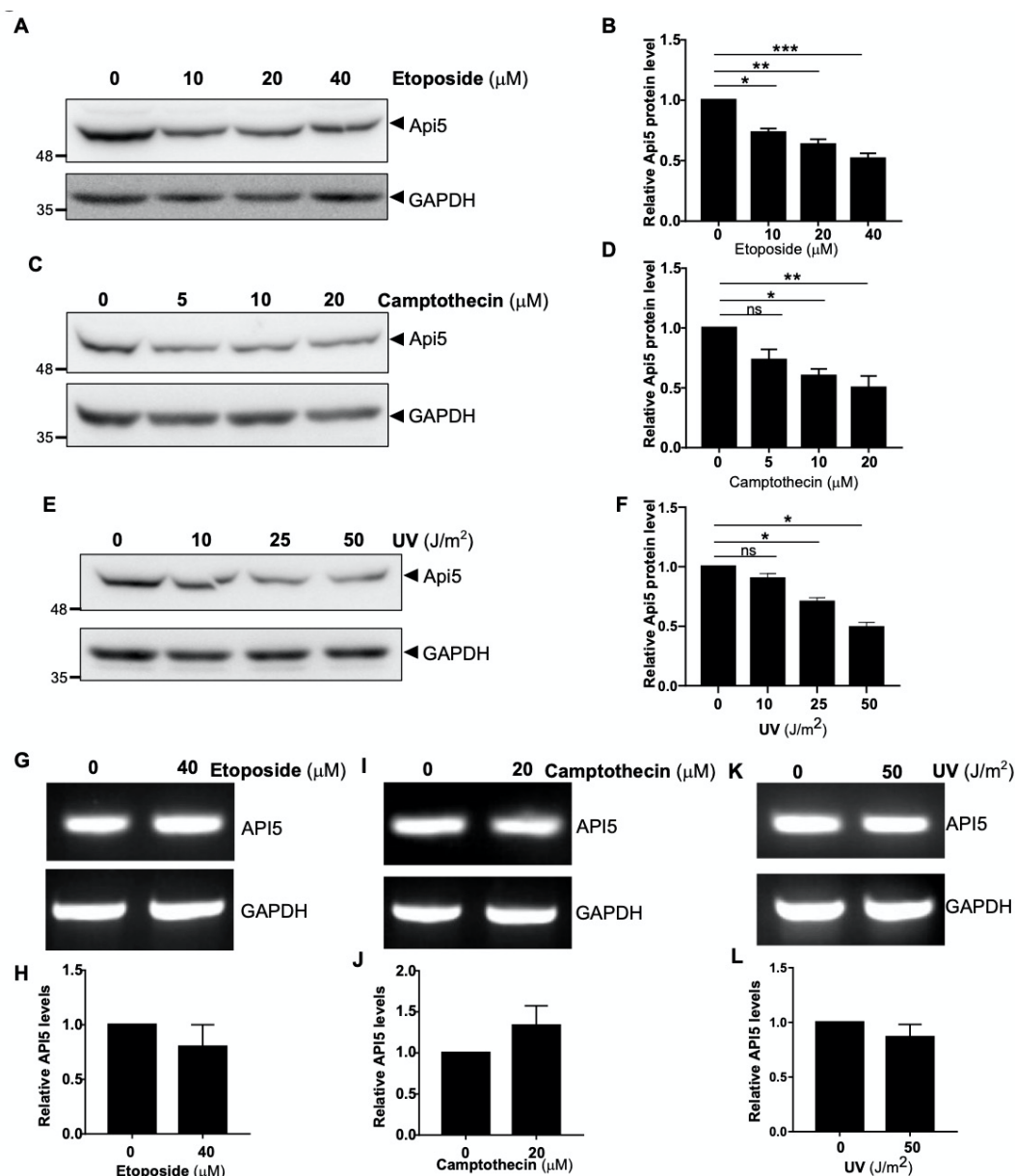


Figure 3. Api5 undergoes post-translational degradation upon DNA damage.

MCF7 cells treated with varying doses of (A) etoposide for 24 hrs, (C) camptothecin for 16hrs and (E) UV for 12 hrs were lysed and Api5 levels were analysed using immunoblotting and quantified after normalising with GAPDH in (B, D, and F). API5 transcript levels upon (G) etoposide, (I) camptothecin and (K) UV treatment were analysed using semi-quantitative PCR and (H, J, L) quantified after normalising to GAPDH.

Api5 undergoes DNA damage-mediated degradation through ubiquitin-proteasomal pathway by FBXW2

Previous experiments suggested that Api5 undergoes degradation via the proteasomal pathway during physiological conditions and Api5 levels reduce upon DNA damage. It was hypothesised that Api5 undergoes proteasomal mediated degradation upon DNA damage. As proteasome complex is localised in the cytoplasm while Api5 is a nuclear protein, we first investigated the localisation of Api5 following UV damage. In control cells, Api5 was observed to be present in the nucleus while upon UV treatment, most cells showed nuclear as well as cytoplasmic localisation of Api5 (Figure 4A-B). Interestingly, the increase in percentage of cells showing only cytoplasmic presence of Api5 upon UV-irradiation in the 8 and 12 hrs post treatment, suggest that Api5 compartmentalisation changes upon UV damage (Figure 4B).

To investigate whether Api5 undergoes proteasomal-mediated degradation upon UV damage, Api5 levels were analysed upon inhibition of the proteasomal pathway by MG132 treatment following UV damage. DNA damage was confirmed by the presence of activated pRPA32 by immunoblotting. Restored levels of Api5 were observed upon inhibition of the proteasomal pathway following UV damage suggesting that DNA damage-mediated degradation of Api5 was also through the proteasomal pathway (Figure 4C-F). To illustrate whether ubiquitination of Api5 precedes its degradation via the proteasomal pathway, mCherry-tagged Api5 overexpressing stable cells were immunoprecipitated using GFP-specific antibody. It was observed that Api5 undergoes poly-ubiquitination upon DNA damage (Figure 4G-H). These results overall suggested that Api5 undergoes proteasome pathway mediated degradation upon DNA damage.

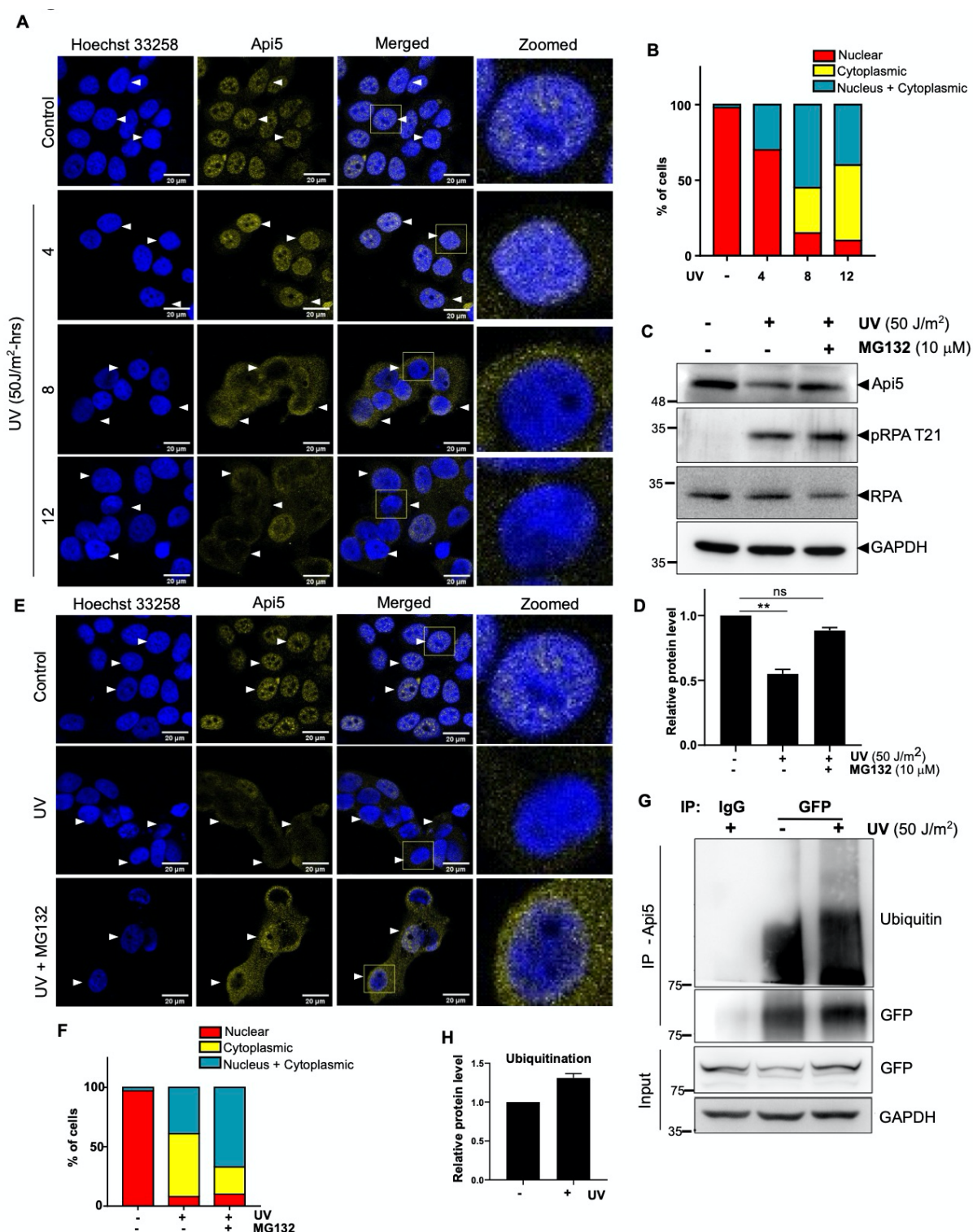


Figure 4. Api5 undergoes proteasomal pathway mediated degradation upon DNA damage. (A) MCF7 cells treated with 50 J/m² UV and fixed post 4, 8 and 12 hrs of irradiation. Immunofluorescence was performed using Api5 specific antibody and imaged in Leica SP8 microscope at 63X oil immersion objective (scale bar: 20 µm) and (B) cells with different Api5 staining pattern were manually counted and

analysed. (C) MCF7 cells treated with 50 J/m² UV and 10 μM MG132 for 4hrs were lysed after 12 hrs post UV treatment and Api5 levels were analysed using western blotting. (D) The fold change of Api5 was quantified after normalisation with GAPDH. (E) Immunofluorescence was performed on MCF7 cells irradiated with 50 J/m² UV and with and without MG132. (F) Api5 localisation changes were manually analysed. (G) mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV and 10 μM MG132 for 4 hrs were lysed 12 hrs post UV treatment and immunoprecipitation was performed using GFP specific antibody. Western blotting analysis was performed to analyse the ubiquitination status of Api5 using ubiquitin specific antibody. (H) Ubiquitin levels of GFP pull down lysates were quantified after normalising to GFP and represented as bar graph.

ATR regulates localisation and degradation of Api5 post DNA damage

Preceding results demonstrated that Api5 undergoes ubiquitin-proteasomal mediated degradation upon DNA damage. ATR kinase is one of central kinases responsible for relaying DNA damage response and repair. It phosphorylates numerous proteins associated with the DNA damage response and repair process. We investigated the role of ATR in proteasomal degradation of Api5 during DNA damage. Immunoblotting results showed that UV-induced reduction of Api5 was blocked following inhibition of ATR (Figure 5A-B). These results were further corroborated using immunofluorescence studies where the cytoplasmic localisation of Api5 upon UV damage decreased upon inhibition of ATR (Figure 5C-D). Interestingly Api5 was observed to form speckles inside the nucleus upon blocking the ATR kinase activity in UV-treated cells. These data prove that ATR regulates DNA damage mediated degradation of Api5. Immunoprecipitation studies were performed to examine the interaction between ATR and Api5 in cells stably expressing mCherry-tagged Api5 following UV damage. Results demonstrated the presence of ATR in the immunoprecipitates of Api5 (Figure 5E). This interaction was further confirmed by a reciprocal immunoprecipitation study (Figure 5F). Presence of Api5 in the ATR immunoprecipitates confirmed the interaction between Api5 and ATR.

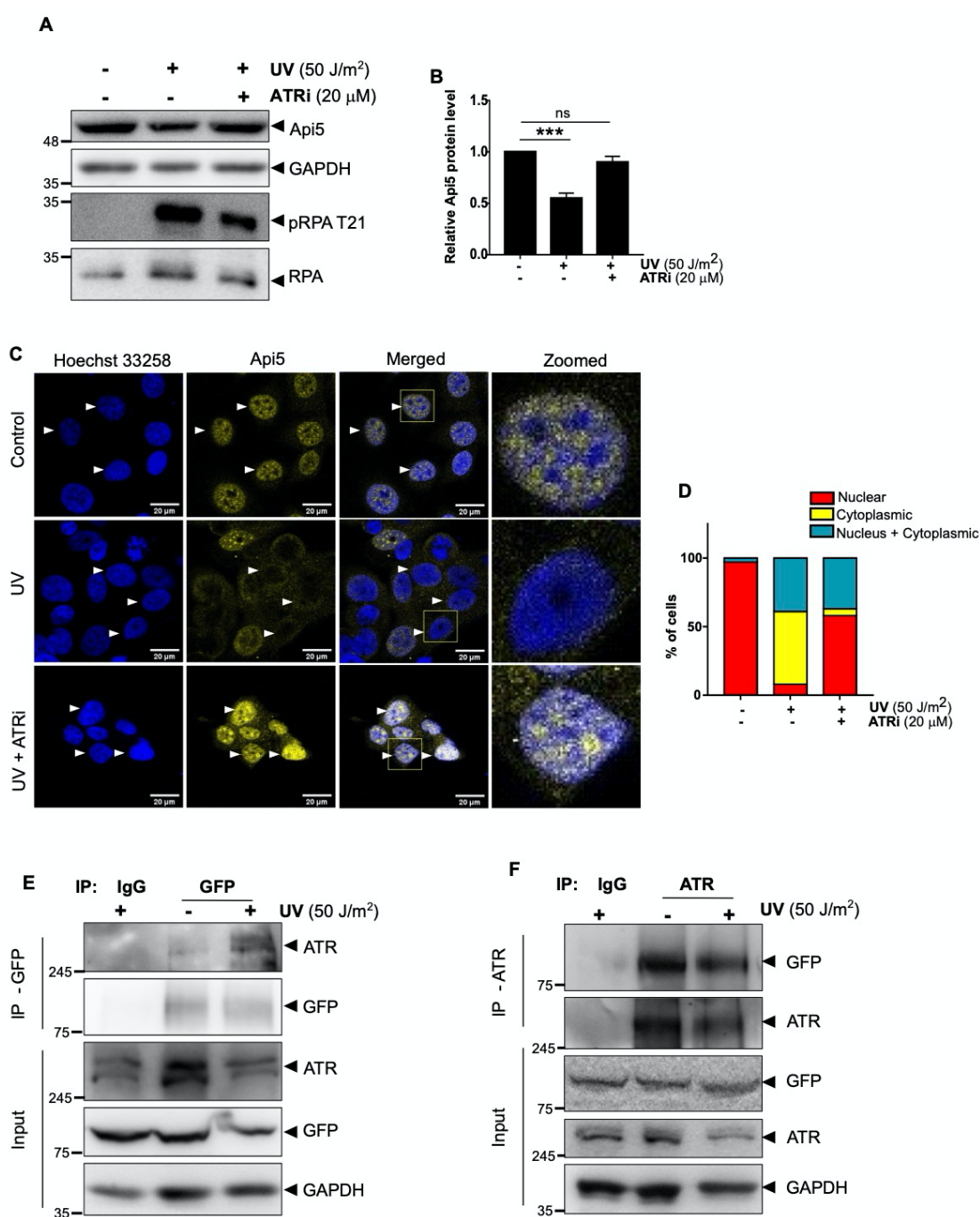


Figure 5. ATR interacts and regulates Api5 upon UV damage. (A) MCF7 cells were treated with 50 J/m² UV and ATRi (20µM VE821 for 12 hrs) were lysed 12 hrs post UV and levels of Api5, pRPA and RPA were analysed using western blotting. (B) The fold change of Api5 was quantified after normalisation with GAPDH. (C) Immunofluorescence assay was performed on MCF7 cells treated with 50 J/m² UV and ATRi (20µM VE821 for 12 hrs) using Api5 specific antibody and (D) Api5 localisation was manually analysed. mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV were lysed 12 hrs post treatment and

immunoprecipitation was performed using (E) GFP and (F) ATR-specific antibody. Western blotting analysis was performed to analyse the presence of ATR and GFP respectively.

ATR phosphorylates Api5 at serine 138 and regulates apoptosis upon DNA damage

Earlier studies have demonstrated that ATR/ATM phosphorylates their substrates at SQ/TQ sites. As ATR was found to be an interactor and regulator of Api5 upon DNA damage, we went ahead to investigate whether ATR can phosphorylate Api5 upon DNA damage. Analysis of Api5 amino acid sequence in Han et al., (2) showed the presence of only two (SQ/TQ) sites on Api5 where ATR could potentially phosphorylate Api5. One at serine 138 and the other at threonine 108 amino acid residues (Figure 6A). To assess whether ATR could phosphorylate Api5, mCherry-tagged Api5 stable cells were lysed and Api5 was immunoprecipitated with anti-GFP antibody and immunoblotting was performed using phospho-serine specific antibody. Presence of phospho-serine at the size of Api5 confirmed that Api5 undergoes phosphorylation upon DNA damage (Figure 6B-C). This phosphorylation could be a signal for Api5 to undergo ubiquitin-mediated proteasomal degradation. To understand the role of this phosphorylation on the stability of Api5, site-directed mutagenesis was performed to mutate serine 138 and threonine 108 to alanine. mVenus tagged wild-type, S138A and T108A mutants of Api5 were ectopically expressed and their stability were analysed upon DNA damage by immunoblotting. It was observed that upon DNA damage, protein levels of wild type and T108A Api5 reduced while Api5 S138A remained unaltered suggesting that phosphorylation at serine 138 of Api5 is responsible for its degradation (Figure 6D-E). In order to understand the function of Api5 S138A mutant upon UV damage, protein expression of various DNA damage repair and apoptotic proteins were analysed in cells expressing wild type and S138A Api5 mutant. Levels of pro-apoptotic proteins like cleaved PARP and caspase 9 were found to be elevated in wild-type cells upon UV damage while no change in protein levels of cleaved PARP or caspase 9 were observed in cells expressing Api5 S138A mutant, suggesting that ATR-mediated degradation of Api5 plays a significant role in the regulation of DNA damage induced apoptosis (Figure 6F-H). The reduction in the protein expression of apoptotic markers in Api5 S138A mutant cells may be due to reduced sensing of UV damage. It was therefore interesting to look for other DNA damage markers. As expected

there was a significant difference in levels of total p53 and activation of p53 at serines 15, 37 and 46 upon DNA damage. Cells expressing Api5 S138A mutant showed reduced activation of p-p53 S15, p-p53 S37, p-p53 S46 as well as total p53 when compared to cells expressing wild-type Api5 upon UV damage (Figure 6I-M), suggesting that phosphorylation of Api5 at serine 138 not only regulates the stability of the protein but also desensitises cells towards DNA damage as well as DNA damage induced apoptosis.

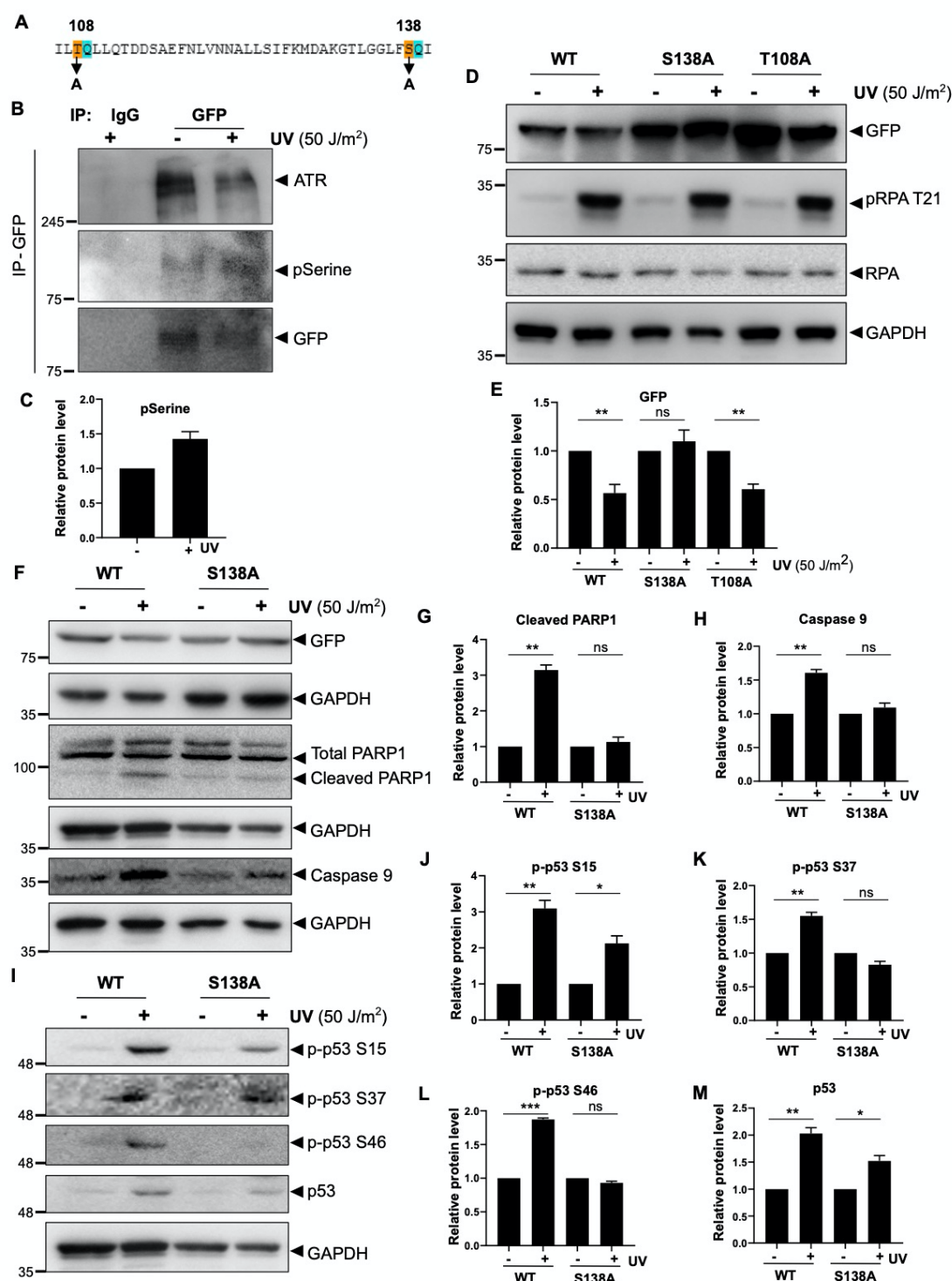


Figure 6. ATR phosphorylates Api5 at S138 to regulate DNA damage response.

(A) Sequence showing the potential ATR phosphorylation sites in Api5 protein highlighted at T108 and S138 residues. (B) mCherry-tagged Api5 over-expressing MCF7 cells treated with 50 J/m² UV were lysed 12 hrs post treatment and immunoprecipitation was performed using GFP-specific antibody. Western blotting

analysis was performed to analyse the presence of phospho-serine and ATR. (C) pSerine levels of GFP pulldown lysates were quantified after normalising to GFP and represented as bar graphs. Serine at 138 and threonine at 108 residues on Api5 were mutated to alanine by site-directed mutagenesis. MCF7 cells transfected with mVenus tagged-WT, S138A and T108A-Api5 mutants were UV irradiated (50 J/m²) post 24hrs of transfection and lysed post 12 hrs of UV treatment. Western blotting was performed to check the levels of (E) Api5, (F) PARP and caspase 9, (I) p53-S15, p53-S37, p53-S46 and total p53 and (E, G, H, J, K, L and M) quantified after normalising to the loading control GAPDH.

De-acetylated form of Api5 undergoes degradation during DNA damage

Studies from our laboratory have demonstrated that p300 and HDAC1 are the enzymes involved in the regulation of the stability of Api5. p300 acetylates Api5 at lysine 251 (K251) and maintain its stability while the HDAC1 deacetylates and leads to the degradation of Api5 (bioRxiv; doi: <https://doi.org/10.1101/2020.11.22.393256>). As our current data also reports Api5 to undergo ubiquitin-proteasome pathway mediated post translational degradation, it was interesting to investigate the role of p300-mediated acetylation and HDAC1 deacetylation on the regulation of stability and anti-apoptotic functions of Api5 following DNA damage. UV-irradiated cells were treated with romidepsin, a small chemical inhibitor of HDAC1 and levels of Api5 were analysed post UV damage. Api5 levels remained unaltered upon inhibition of HDAC1 in UV-irradiated cells suggesting that DNA damage induced degradation of Api5 requires de-acetylation by HDAC1 (Figure 7A-B). To check for other molecular players involved in the acetylation and stability of Api5 upon UV damage, cells were treated with UV-irradiation and protein levels of p300 and HDAC1 were analysed. p300 levels were found to be reduced upon UV damage while HDAC1 levels remained unaffected (Figure S4A-F). To demonstrate that acetylation of Api5 at K251 is required for maintaining stability of Api5 following DNA damage, mVenus-tagged K251 mutant of Api5, K251Q which is a constitutive acetylation mimic mutant was ectopically expressed along with wild-type Api5 followed by UV-irradiation and the levels of Api5 were analysed post DNA damage. Wild type Api5 levels were observed to reduce while the K251Q mutant Api5 protein levels did not alter upon UV-irradiation suggesting that acetylation at lysine 251 plays a crucial role in maintaining the stability of Api5 (Figure 7C-D). Stability of proteins is generally

considered to be a direct evidence for their functions, therefore the protein expression of various DNA damage and apoptotic markers were analysed in Api5 wild-type and K251Q mutant expressing cells upon UV damage. Wild-type Api5 showed increased levels of cleaved PARP while the other Api5 acetylation mutants failed to show any difference in the protein expression levels (Figure 7C-E). To understand the role of Api5 and K251 acetylation of Api5 on cell survival upon DNA damage, wild type and K251 mutants were stably expressed in Api5 knocked down stable cells. Cells with Api5 knock down showed reduced survival upon camptothecin and etoposide treatment compared to the control cells while upon rescue with wild type Api5, cell survival was restored (Figure 7 F-G). K251Q mutant of Api5 also facilitated cell survival similar to wild type Api5, however K251A mutant of Api5 was not able to rescue the cell survival upon DNA damage. These results suggest that Api5 acetylation at K251 plays a significant role during regulation of DNA damage-induced apoptosis.

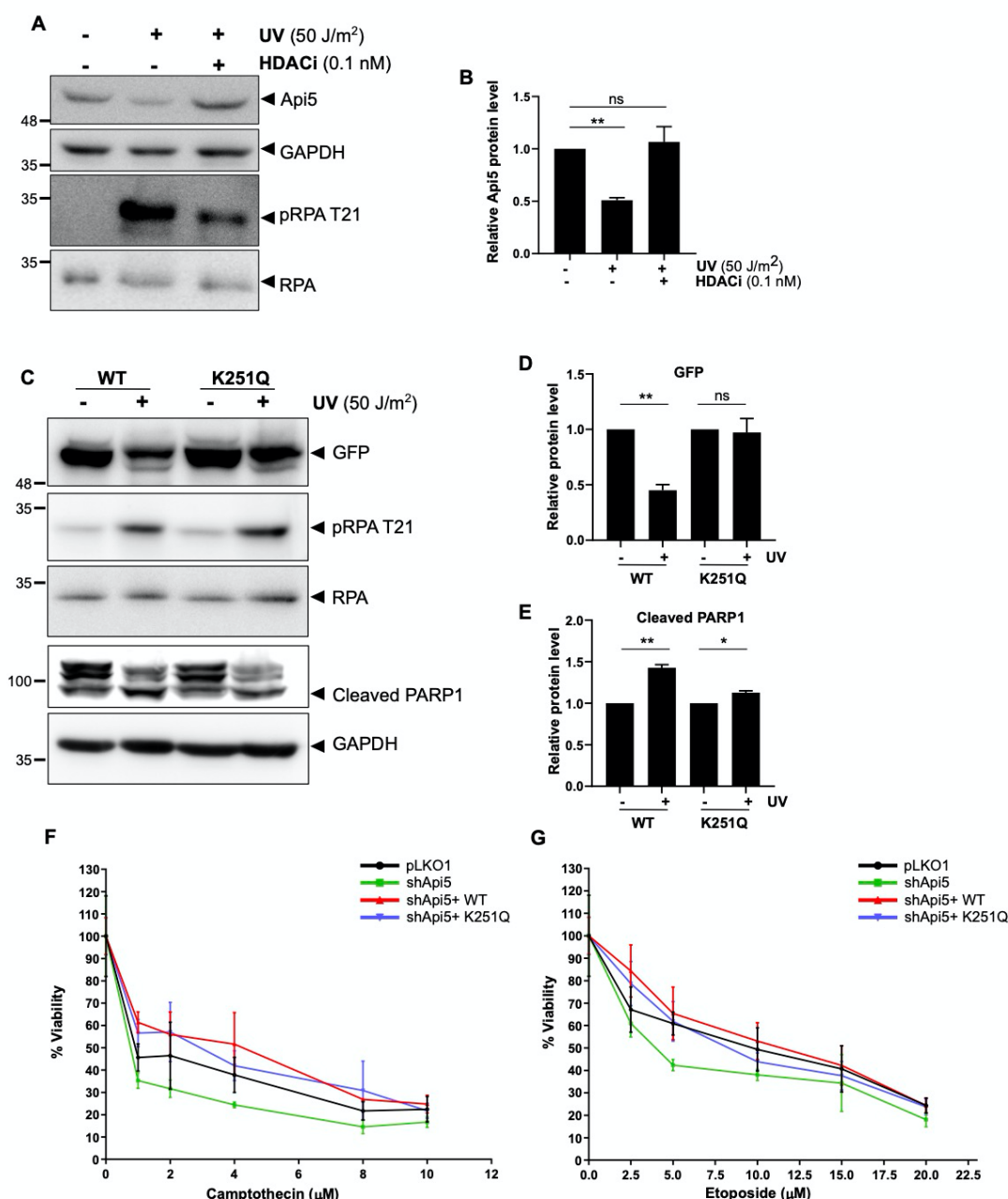


Figure 7- Acetylation of Api5 at K251 regulates DNA damage induced apoptosis. (A) MCF7 cells treated with HDACi (0.01nM romidepsin for 12hrs) and UV (50 J/m²) were lysed post 4 hrs of UV irradiation and Api5 levels were analysed using western blotting. (B) The fold change of Api5 was quantified after normalising to GAPDH. MCF7 cells were transfected with mVenus-tagged wild type and K251Q mutant constructs and treated with 50J/m² UV post 24 hrs of transfection and lysed 12 hrs post UV irradiation. Western blotting was performed to check for levels of (C) GFP, PARP. (D and E) quantification of the western blots after normalising to the loading control GAPDH. Viability of MCF7 cells stably expressing shApi5 and K251

mutants upon treatment with varying concentrations of (F) camptothecin and (G) etoposide for 24 hrs were measured using MTT assay.

Discussion

Api5 is an anti-apoptotic protein that helps in cell survival during serum starvation conditions (1). Later it was found that the anti-apoptotic function of Api5 was not only confined to serum starvation conditions but it also could potentially inhibit apoptosis during infectious and DNA damage conditions (19) (3) (6). However, the mechanism by which Api5 is able to regulate itself during stress conditions like serum starvation, infections and DNA damage is yet to be understood. In this study we report that Api5 undergoes degradation upon DNA damage induced apoptosis. UV-irradiation and chemical damage resulted in reduction in Api5 protein levels. Our results suggest that post translational degradation of Api5 upon DNA damage induced apoptosis is through the proteasomal pathway. Ubiquitination is the post translational modification that targets Api5 towards the proteasome machinery. However, we are not dismissing the possibility that Api5 may be regulated via other post-translational modifications and/or other pathways involved in the post-translational degradation of Api5.

FBXW2 is a SCF-type of E3 ubiquitin ligase that is involved in the ubiquitin-mediated degradation of a potential cell cycle regulator SKP2 (18). It has already been reported that in lung carcinomas, SKP2, a potential oncogenic protein gets degraded by FBXW2 which thus acts as a tumor suppressor. Api5 is also found to be upregulated in different types of cancers like cervical, oesophageal and lung cancers (18,20-22). Multiple studies by various groups suggest that Api5 can also be a potential oncogenic factor which upon its down regulation could make cells vulnerable towards DNA damage mediated cell death (3). Our studies confirmed that FBXW2 interacts with Api5 and promotes its degradation through ubiquitination during physiological conditions as well as during DNA damage induced apoptosis.

Phosphorylation is a secondary but an essential signal for degradation of multiple proteins via the proteasomal pathway. ATR is one of the main sensor kinase that upon sensing DNA damage, activates a cell cycle checkpoint response by phosphorylating specific proteins like Chk1 and p53 (10,12,13). Our results indicate that ATR binds to Api5 and phosphorylates it at the serine 138 residue.

Phosphorylation of Api5 at serine138 is required for its degradation during DNA damage induced apoptosis. Upon DNA damage Api5 first gets de-acetylated by

HDAC1. This de-acetylated Api5 undergoes post-translational degradation through the proteasomal pathway after getting phosphorylated by ATR. The phosphorylated form of Api5 localises to the cytoplasm and undergoes degradation post-DNA damage while the nuclear localised form of Api5 is acetylated and stabilised by p300 histone acetyltransferase. The acetylated form of Api5 that does not undergo degradation upon DNA damage may be playing a role in DNA repair and/or differential regulation of apoptosis upon DNA damage. In our studies we identified ATR as a major regulator of Api5 however we are also aware that other regulators like ATM and DNA-PK may also play a role that is yet to be investigated. Increased levels of Api5 are found to be associated with higher aggressiveness of cancers and poor cancer patient survival (23). Ectopic expression of Api5 can also promote properties like invasion, migration and metastasis in cancers (24). Depletion of Api5 induces apoptosis while over-expression of Api5 provides chemo-resistance to certain cancers (23). These studies demonstrate Api5 to be a potential oncogene and it is essential to understand the mechanism of regulation of stability as well as function of Api5 upon DNA damage. By understanding the molecular mechanism of regulation of Api5, multiple cancers can be targeted using techniques or molecules which specifically block the function of Api5. These molecules can be used as potential therapeutic agents to treat cancers where Api5 is over-expressed. Taken together we report FBXW2 and ATR as the novel regulators of Api5 upon DNA damage. Upon DNA damage HDAC1 de-acetylates Api5 and this de-acetylated Api5 undergoes post-translational degradation. Upon UV irradiation, ATR phosphorylates Api5 at S138 residue. This signals FBXW2 E3 ubiquitin ligase to target the protein for ubiquitination and degradation as summarised in Figure 8.

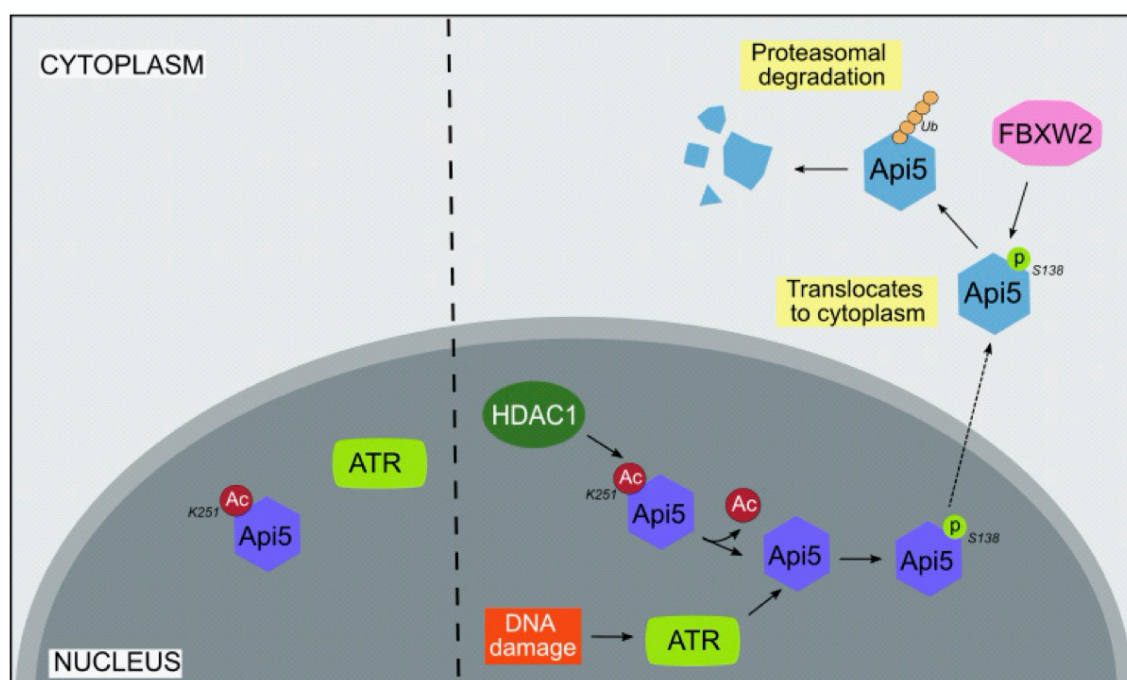


Figure 8: Schematic representation summarising the regulation of Api5 upon DNA damage. A proposed model summarising the pathway of regulation of Api5 by its key regulators HDAC1, ATR and FBXW2 upon DNA damage.

Materials and methods

Chemicals and antibodies

Camptothecin (C9911), Etoposide (E1383), Protease inhibitor cocktail (P2714), dimethyl sulfoxide (DMSO), propidium iodide (P4170) Chloroquine (C6628), MG132 (M7449), Cyclohexamide (C1988), polybrene (Hexadimethrine bromide; H9268), thiazolyl blue tetrazolium (MTT; M5655) and dimethyl sulfoxide (DMSO; D8418) were purchased from Sigma-Aldrich; romidepsin (FK228, Depsipeptide) was from Selleck Chemicals, USA and the selective ATR kinase ATR inhibitor (VE 821) was purchased from Axon Medchem, USA. Lipofectamine 2000 (H668), Alexa flour 488 goat anti-rabbit (A11034) and Phalloidin 568 (A12380) were purchased from Thermo Fisher Scientific, USA. FBXW2 (ab 83467), GFP (ab290), p300 (ab10485), ATR (ab2905), RPA T21 (ab61065), Caspase 9 (ab2324) antibodies were obtained from Abcam, USA. HDAC1 (sc-8410), Chk1 (G4, sc-8408), Ubiquitin (sc-8017) were purchased from SantaCruz while Api5 (PAB7951) was from Abnova, Taiwan. Chk1 S345 (2348), Chk2 T68 (2661), Chk2 (3440), p53 S15 (9284), p53 S37 (9289), p53 S46 (2521), RPA 32 (2208) and c-Myc (9402) were from Cell Signaling Technology, USA while p53 (247A) and GAPDH (G9545) were from Bethyl Laboratories, USA and Sigma-Aldrich respectively. Hoechst 33258 (H3569) was purchased from Molecular Probes, Thermo Fisher Scientific, USA.

Plasmids

CSII-EF-MCS plasmid was a gift from Dr. Sourav Banerjee (NBRC, Manesar, India). pCAG-HIVgp and pCMV-VSV-G-RSV-Rev plasmids were purchased from RIKEN BioResource Centre, Japan. pLKO.1 eGFP was a kind gift from Dr. Sorab Dalal, ACTREC, India. shApi5 sequence was cloned into the same using Addgene pLKO.1 protocol. Lentiviral pGIPZ shFBXW2 plasmids were a kind gift from Prof. Michael R. Green, University of Massachusetts Medical School, USA. psPAX2 and pMD2.G were obtained from Dr. Didier Trono (Addgene, Watertown, MA, USA). Human cDNA encoding FBOX genes were procured from Origene (Rockville, MA, USA). mVenusC1 was gifted by Dr. Jennifer Lippincott-Schwartz, NIH, USA in which Api5 was cloned. Api5 was made siRNA-resistant and K251 and S138A mutants were generated using site directed mutagenesis.

Cell culture and drug treatments

MCF7 cells were obtained from the European Collection of Cell Cultures (ECACC). HEK293T and HeLa cells were a gift from Dr. Jomon Joseph (NCCS, Pune) and Dr. Sorab Dalal respectively. The cells were maintained in 100 mm dishes (Thermo Fisher Scientific, USA, 172931) and were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Lonza Group AG, Switzerland, 12-604F) containing 10% heat inactivated FBS (Thermo Fisher Scientific, USA, 10270106) and 100 units/mL penicillin-streptomycin (Lonza Group AG, Switzerland, 17-602E) and incubated at 37°C in humidified 5% CO₂ incubators (Eppendorf, GmbH or Thermo Scientific, USA).

To induce DNA damage, 7 x 10⁵ cell were seeded on 35mm dishes 16 hrs prior to treatment. MG132 and cycloheximide treatments were for 4hrs, Chloroquine for 8 hrs, HDACi, ATRi and UV treatments were for 12 hrs, camptothecin for 16 hrs and etoposide for 24 hrs with concentrations mentioned except for time dependent experiments.

Plasmid Transfection

RING-finger E3 ubiquitin ligase screen was performed in MCF7 cells as described previously (25). For transfections, 6 x 10⁵ cells were seeded in 35mm dishes and incubated at 37°C overnight. 2 µg of Api5 K251 mutants or Api5 S138A plasmids which were generated by site directed mutagenesis were transfected in MCF7 cells using Lipofectamine 2000 (Thermo Fisher Scientific, USA) using manufactures protocol.

Generation of stable cell lines

MCF7 cells stably expressing mCherry-tagged Api5 were generated using lentiviral mediated transduction as mentioned previously (bioRxiv; doi: <https://doi.org/10.1101/2020.11.22.393256>). MCF7 cells stably expressing pLKO1.eGFP and shApi5.eGFP was generated using lentiviral transduction. shApi5 was cloned into pLKO1.eGFP vector as per Addgene protocol using the following primers: Forward Primer: 5'-CCGGAAGACCTAGAACAGACCTTCACTCGAGTGAAGGTCTGTTCTAGGTCTTTT TTTG-3', Reverse Primer: 5'-

AATTCAAAAAAGACCTAGAACAGACCTTCACTCGAGTGAAGGTCTGTTCTAGG TCTT - 3'. Briefly, 7.5×10^5 HEK293T cells were seeded on 35mm dish and transfected with 1 μ g of pLKO1.eGFP or shApi5-eGFP plasmid along with 1 μ g of psPAX2 and 0.5 μ g pMD2 packaging plasmids using Lipofectamine 2000. 1 ml DMEM containing 30% FBS was added to the cells 24 hours post transfection. 5×10^5 MCF-7 cells were seeded on 35 mm dish for transduction. Viral supernatant was collected 48 hours post transfection and filtered through a 0.45 μ m filter to get rid of the cell debris. Filtered viral supernatant containing media along with 1 ml fresh media was added to the MCF-7 cells. 4 μ g polybrene was added to the cells to increase the transduction efficiency. Cells were replenished with fresh medium 48 hours post transduction. Transduced MCF7 cells were passaged and sorted using BD Aria Fusion sorter (BD Biosciences, USA). Lentiviral particles containing mCherry-tagged siRNA resistant Api5 wild type and K251 mutants were generated as described previously (bioRxiv; doi: <https://doi.org/10.1101/2020.11.22.393256>). shApi5 cells after sorting was transduced with lentiviral particles containing mCherry-tagged siRNA resistant Api5 wild type and K251 mutants and sorted for mCherry fluorescence. HeLa cells expressing shFBXW2 were generated as described previously (26).

MTT assay

For the MTT assay, 1×10^4 cells were seeded in each well of a 96 well plate. 16 hours post seeding, cells were treated with different concentrations of camptothecin and etoposide and incubated for 24 hours at 37°C. After removing the drug containing medium, 0.5 mg/ml thiazolyl blue tetrazolium (MTT) containing DMEM was added to the cells and incubated at 37°C after covering with aluminum foil. 4 hours post MTT addition, MTT-DMEM medium mixture was aspirated from the wells and 100 μ l of DMSO was added to dissolve the purple MTT-formazan crystals. The absorbance was recorded at 570nm using the multimode Varioskan Flash plate reader (Thermo Scientific, USA).

Immunoblotting and Immunoprecipitation

mCherry-tagged Api5 over-expressing stable cells with or without treatments were lysed in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors.

Immunoprecipitations were performed as described earlier (27) using Api5, Myc, GFP and ATR specific antibodies. Western blotting was performed with Api5, GFP, ATR, Ubiquitin, pSerine and GAPDH antibodies as previously mentioned (28). Immunoblots include all the experimental lanes of a said experiment. There are no composite images where two or more blots were merged together. The blots were processed as horizontal cut strips in the respective antibody dilutions and imaged as individual strips in ImageQuant LAS4000 (GE Healthcare, now Cytiva, USA).

Semi-quantitative PCR

RNA extraction and cDNA synthesis of camptothecin, etoposide and UV treated cells were performed as described previously (29). Semi-quantitative PCR was performed using API5 specific primers were described previously (bioRxiv; doi: <https://doi.org/10.1101/2020.11.22.393256>). GAPDH was used as endogenous control.

Immunofluorescence

For immunofluorescence, 6×10^5 cells were seeded on coverslips placed in 35mm dishes. After treatment, cells were fixed in 4% paraformaldehyde and permeabilised using 0.5% Triton X after PBS washes. After PBS-Glycine wash, cells were blocked using 10% FBS for 1 hr and incubated overnight with Api5 or pRPA antibody at 4°C. Cells were incubated with secondary antibodies conjugated with Alexa Fluor for 1 hr at room temperature along with phalloidin. Nuclei was stained using Hoechst 33258 for 5 mins and washed twice using PBS. The cells were mounted using mounting media and imaged under 63X oil immersion objective of the SP8 confocal microscope (Leica, Germany).

Statistical analysis

Densitometry analysis of western blots were performed using ImageJ software. Results were analysed from a minimum of three independent experiments and plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Student's t-test and Mann Whitney U test was used to test the significance in difference of Api5 levels between two samples and One-way Anova, Dunnett's multiple comparisons or Tukey's multiple comparisons test for more than two samples. p value > 0.05 was

considered non-significant. *, ** and *** correspond to $p < 0.05$, $p < 0.001$, and $p < 0.001$ respectively.

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Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

V.K.S, M.K.S and M.L conceived and conceptualised the project. V.K.S performed and analysed the experiments with contribution from S.I, J.B, S.M and D.P. M. K.S gave valuable comments on the first draft of the manuscript. V.K.S and M.L wrote the paper.

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Small molecule-mediated induction of
endoplasmic reticulum stress in cancer cells†Shalini Pandey,^{ab} Virender Kumar Sharma,^c Ankur Biswas,^a
Mayurika Lahiri ^c and Sudipta Basu ^{*b}

The endoplasmic reticulum (ER) is one of the crucial sub-cellular organelles controlling myriads of functions including protein biosynthesis, folding, misfolding and unfolding. As a result, dysregulation of these pathways in the ER is implicated in cancer development and progression. Subsequently, targeting the ER in cancer cells emerged as an interesting unorthodox strategy in next-generation anticancer therapy. However, development of small molecules to selectively target the ER for cancer therapy remained elusive and unexplored. To address this, herein, we have developed a novel small molecule library of sulfonylhydrazide-hydrazones through a short and concise chemical synthetic strategy. We identified a fluorescent small molecule that localized into the endoplasmic reticulum (ER) of HeLa cells, induced ER stress followed by triggering autophagy which was subsequently inhibited by chloroquine (autophagy inhibitor) to initiate apoptosis. This small molecule showed remarkable cancer cell killing efficacy in different cancer cells as mono and combination therapy with chloroquine, thus opening a new direction to illuminate ER-biology towards the development of novel anticancer therapeutics.

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Introduction

The endoplasmic reticulum (ER) is a vital organelle that is known to govern the synthesis, folding and processing of over a third of all the cellular proteins.^{1–4} Several ER associated chaperones assist in ensuring proper folding and modification of these proteins before they traffic out of the ER.^{5–7} Despite the robustness of this protein folding machinery, the success rate for optimum protein folding is low owing to the wide range of cellular disturbances that disrupt its efficiency.⁸ As a result, several unfolded and misfolded proteins start accumulating inside the ER lumen leading to a state known as ER stress.^{9,10} In response to this burden of unfolded proteins, a cytoprotective program known as the unfolded protein response (UPR) is launched by the cell.^{11–13} Together, the three mechanistically different branches of the UPR regulate the expression of numerous genes that resolve this ER stress and maintain homeostasis or induce apoptotic signals in case

the stress remains unmitigated.¹⁴ Very recently, this deregulated ER homeostasis has been implicated in various pathological states and particularly with cancer.^{15,16} The baseline activity level of the ER stress response system is elevated in cancer cells as compared to normal cells. Thus, the ER stress signalling is involved in tumorigenesis and development leading it to be the new Achilles heel in the development of cancer therapeutics.^{17–19}

Over the last couple of decades, small molecules have emerged as important tools to illuminate myriads of biological phenomena in cancer cells leading to the development of novel anti-cancer therapies.^{20,21} In this context, few small molecules have been explored that target various aspects of the unfolded protein response and ER physiology.^{22–30} Molecules like salubrinal, GSK2606414, and sunitinib are well reported to manipulate one or more arms of ER stress pathways.³¹ However, due to the scarcity of the targeting moieties, the development of ER targeted therapeutics is still in its infancy and therefore the presence of such ER stress inducers and UPR inhibitors is limited. Hence, there is a serious need to develop novel small molecules that can serve as ER stress modulators. Very recently, a few molecules were demonstrated to accumulate inside the ER in cancer cells to induce ER stress or as a tool to understand ER physiology.^{32–35} For instance, Rocchi *et al.* reported the development of HA15 that serves as an ER stress inducer and overcomes BRAF-inhibitor resistance in melanoma cells.³⁶ On the other hand, Xiao *et al.* reported an

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† Electronic supplementary information (ESI) available: ¹H NMR, ¹³C NMR, and HR-MS spectra, cell viability, fluorescence spectra, western blot quantification, and cell viability assay. See DOI: 10.1039/d1md00095k

ER-H₂O₂ probe to understand the generation of hydrogen peroxide at the ER during apoptosis.³⁷ A remarkable highlight in these small molecules is the presence of sulfonamide moieties. Furthermore, it was reported that the sulphonamide moiety facilitates the internalization of small molecules in the intracellular ER owing to the presence of sulphonamide receptors on the ER surface.^{38,39} Moreover, sulphonamide as well as hydrazide-hydrazone containing small molecules demonstrated diverse biological activities making them privileged structures for natural and non-natural products (Scheme 1a).^{40–47}

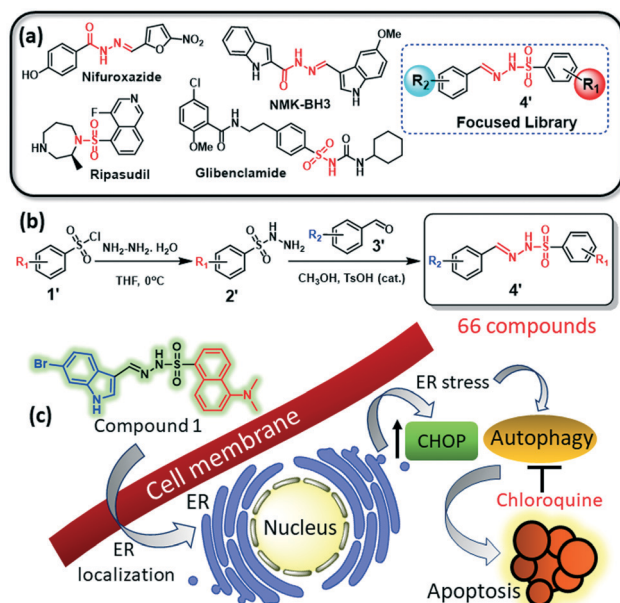
Inspired by the scarcity of novel ER stress modulators, we develop a focussed library of sulfonylhydrazide-hydrazone based small molecules (4', Scheme 1a) to identify novel ER stress inducers in cancer cells. To address this, herein, we report a short and concise synthesis of a library of sulfonylhydrazide-hydrazone based small molecules comprising 66 compounds (Scheme 1b). Upon screening these compounds in the HeLa cell line, we identified four molecules as potential ER stress inducers. One of the four lead molecules was found to be fluorescent in nature and localized inside the subcellular ER to induce ER stress through CHOP and IRE-1 α pathways (Scheme 1c). Interestingly, the ER stress induced by the lead molecule triggered autophagy followed by apoptosis in HeLa cells. Further inhibition of autophagy by chloroquine (CQ), a classic inhibitor that decreases the autophagic flux by inhibiting the autophagosome-lysosome fusion,⁴⁸ resulted in improved cell killing in lung and breast cancer cells including drug resistant-breast cancer cells.

Results and discussion

Synthesis of the small molecule library

The synthesis of sulfonylhydrazide-hydrazone molecules is shown in Scheme 1b. Briefly, commercially available substituted aromatic sulfonyl chlorides (1') were reacted with hydrazine monohydrate at 0 °C to obtain substituted aromatic sulfonyl hydrazides (2') in 60–70% yield (Fig. S1, ESI†). Further, these aromatic sulfonyl hydrazides were reacted with different aromatic aldehydes (3') in the presence of *p*-toluenesulfonic acid as a catalyst to afford 66 different sulfonylhydrazide-hydrazone (4') in 50–80% yield (Fig. S2, ESI†). The final products were characterized by ¹H NMR, ¹³C NMR and HR-MS (Fig. S3–S200, ESI†).

To evaluate their cancer cell killing activity, we first incubated these library members in HeLa cervical cancer cells at a concentration of 30 μ M for 24 hours. The cell viability was quantified by the MTT assay. Interestingly, only four compounds (compounds 1, 10, 17 and 55) were found to induce more than 60% HeLa cell killing even at 30 μ M concentration (Fig. S201, ESI†). After finding out the potential lead compounds, we evaluated their cancer cell killing ability in a dose dependent manner. HeLa cells were incubated with compounds 1, 10, 17 and 55 for 24 h in different concentrations and the cell viability was evaluated by the MTT assay. It was observed that at 24 h post incubation, compounds 1, 10, 17 and 55 induced HeLa cell killing with IC₅₀ values of 9 μ M, 16 μ M, 18 μ M and 19 μ M respectively (Fig. 1). From this cell viability assay, it was evident that compound 1 showed the best cell killing efficacy with the lowest IC₅₀ value. Interestingly, we observed that the electron donating groups (Cl, Br, dansyl) on the benzene ring attached to the sulfonyl moiety increased the cytotoxicity effect, whereas, the electron withdrawing group (NO₂) reduced the cytotoxicity. Moreover, we also observed that the 6-bromoindole moiety also increased the cytotoxicity of the compounds.⁴⁷ However, a detailed structure–activity relationship (SAR) analysis needs to be performed to understand the effect of the functional groups in the sulfonylhydrazide-hydrazone library. Moreover, compound 1



Scheme 1 (a) Structures of the biologically active sulphonamide natural products and sulfonylhydrazide-hydrazone focused library. (b) Synthetic scheme of the sulfonylhydrazide-hydrazone library. (c) Schematic representation of the mechanism of action of compound 1 in cancer cells to induce ER stress.

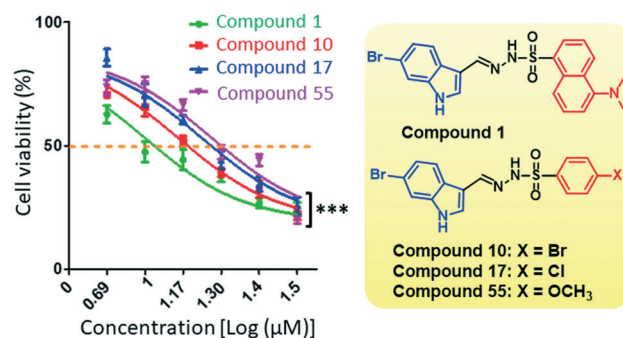


Fig. 1 Cell viability assay of compounds 1, 10, 17 and 55 in HeLa cells at 24 h post-incubation in a dose dependent manner. ****p* < 0.0001, the *p* value is calculated using two-way ANOVA.

was found to be fluorescent with λ_{max} (emission) at 538 nm (Fig. S202, ESI†). This inherent fluorescence property of compound **1** will aid in its cellular tracking into the endoplasmic reticulum (ER), and hence we chose to carry out further studies using compound **1**.

ER homing

We hypothesized that the presence of the sulphonamide moiety in compound **1** will enable its localization specifically inside the subcellular ER.^{49,50} To validate this hypothesis, we incubated the HeLa cells with compound **1** (concentration = 5 μM) (green fluorescent) for two different time points of 3 h and 6 h followed by staining the ER with ER Tracker Red. We then visualised the cells using confocal laser scanning microscopy (CLSM). It was observed that compound **1** started accumulating in the ER within 3 h and the accumulation increased at 6 h (Fig. 2 and S203, ESI†) which was observed from the yellow fluorescence resulting from merging of red and green fluorescence. The ER localization of compound **1** was further quantified from the CLSM images through Pearson's coefficients of 0.84 and 0.86 and Mander's coefficients of 0.98 and 0.97 at 3 h and 6 h, respectively (Table S1, ESI†). These confocal images and quantification showed that indeed compound **1** homed into the subcellular ER in HeLa cancer cells over 6 h.

ER stress

Once internalized inside the ER, compound **1** is expected to induce ER stress in the HeLa cervical cancer cells. Elevated ER

stress levels lead to an increment in the expression levels of protooncogene CHOP which is a marker for ER stress induced apoptosis.^{51,52} To demonstrate the onset of ER stress we evaluated the levels of CHOP in HeLa cells by an immunofluorescence assay. We treated HeLa cells with compound **1** for 24 h. We then incubated the cells with a primary antibody specific to CHOP for 4 h followed by incubation with an Alexa-Fluor 594 (red fluorescence) tagged secondary antibody. The nuclei of the cells were stained with DAPI (blue fluorescent). We then visualized the cells with confocal microscopy. The fluorescence microscopy images showed that the control cells expressed negligible CHOP which was evident from no red fluorescence signals (Fig. 3a). However, the cells treated with compound **1** showed remarkably increased red fluorescence signals indicating that compound **1** increased (1.7 fold) the expression of CHOP protein in HeLa cells. Furthermore, the overlap of red and blue fluorescence to give purple fluorescence indicated the accumulation of CHOP in the nucleus which is a marker of ER stress induction.⁵² Interestingly, the immunofluorescence staining of CHOP showed a characteristic punctate structure after treatment with compound **1** which is the hallmark of ER stress (Fig. S204, ESI†). We also evaluated the expression of CHOP by gel electrophoresis. We treated HeLa cells with compound **1** for 24

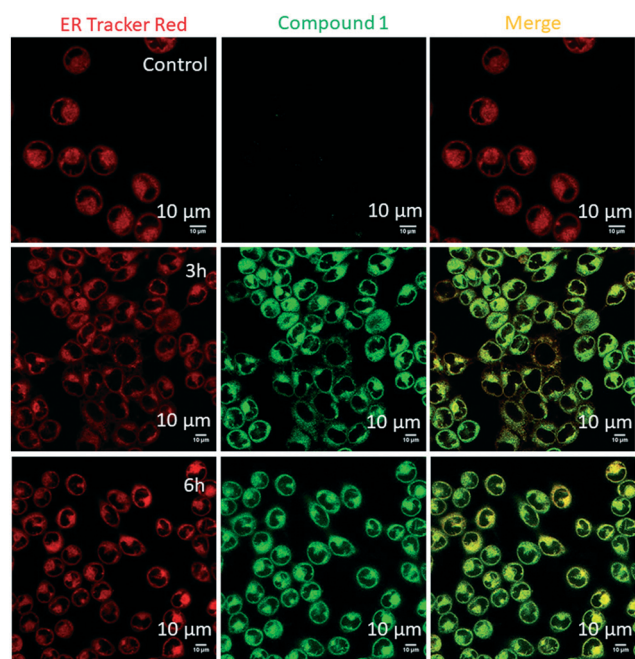


Fig. 2 Confocal microscopy images of HeLa cells at 3 h and 6 h post-incubation with compound **1** (green fluorescence). The cells were counter stained with ER-Tracker Red dye. Scale bar = 10 μm .

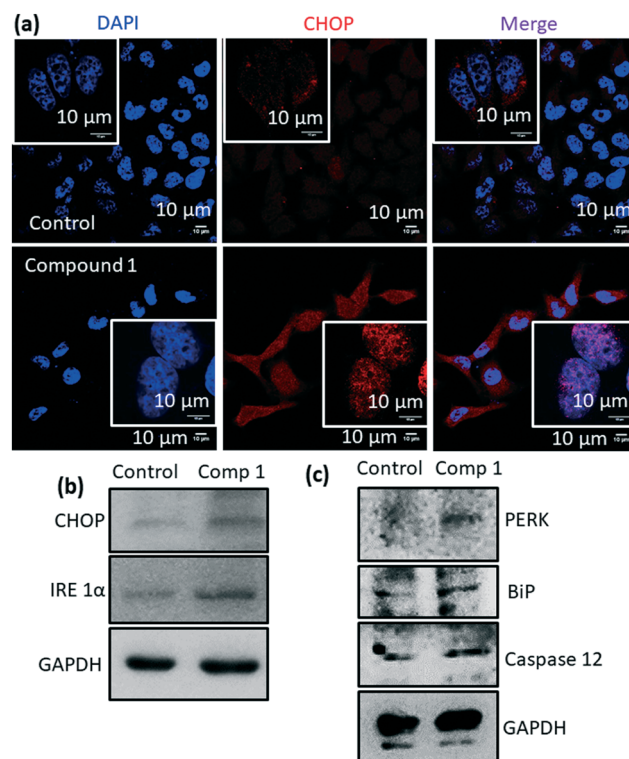


Fig. 3 (a) Confocal images of HeLa cells after treatment with compound **1** for 24 h followed by treatment with a CHOP primary antibody and an Alexa Fluor 594-tagged secondary antibody (red). Nuclei were stained with DAPI (blue). Scale bar = 10 μm . (b and c) The western blot images of CHOP, IRE1 α , PERK, BiP and caspase-12 proteins as the markers of ER stress in HeLa cells after treatment with compound **1** for 24 h.

h, followed by lysis of the cells. We separated the sub-cellular proteins to perform western blot analysis. In accordance with the confocal microscopy study, the cells after treatment with compound **1** showed a significant increase (1.6 fold) in the expression of CHOP compared to the non-treated control cells (Fig. 3b and S205a, ESI†). To further estimate the ER stress, we evaluated the expression levels of another ER stress marker IRE1- α .⁵³ We found that the cells treated with compound **1** exhibited an enhanced expression level of IRE1- α as compared to the control cells (1.8 fold) (Fig. 3b and S205b, ESI†). We also evaluated the expression of PERK and BiP as ER stress markers and activation of caspase-12 as an ER stress-mediated apoptosis marker by gel electrophoresis.^{10,36,54} We found that compound **1** induced enhanced expression of PERK (1.6 fold), BiP (1.6 fold) and caspase-12 (1.4 fold) compared to the non-treated control cells (Fig. 3c and S205c–e, ESI†). These immunofluorescence assays and gel electrophoresis revealed that compound **1** indeed induced ER stress in HeLa cells.

The ER is the major site of lipid metabolism as several enzymes involved in it are compartmentalized in the ER.⁵⁵ As a result, excessive ER stress is known to increase lipid accumulation inside the cell.⁵⁶ We anticipated that HeLa cells treated with compound **1** will also show an enhanced lipid content in them. To validate this hypothesis, we performed an Oil Red O staining experiment.^{57,58} We treated HeLa cells with compound **1** for 24 h followed by staining the cells with Oil-Red-O dye. Oil Red O is a lipid soluble lysochrome dye which stains the lipid droplets in cells or frozen tissues.⁵⁹ We also stained the nucleus of the cells with blue fluorescent DAPI. We visualised the Oil-Red-O stained cells by confocal microscopy. The confocal microscopy images showed that the control cells contained traces of lipid molecules as expected (Fig. 4). However, compound **1** treatment remarkably increased the level of the lipids inside the cells, which was clear from the increased red fluorescence signals from the Oil-Red-O dye in Fig. 4.

To confirm that the fluorescence signal from compound **1** does not interfere with the Oil-Red-O, we further treated the HeLa cells with non-fluorescent compound **10** for 24 h and performed the Oil-Red-O staining. From the confocal imaging

it was evident that compound **10** also enhanced the red fluorescence signal significantly compared to the non-treated control cells (Fig. S206, ESI†). This confocal microscopy exhibited that indeed the lipidic content was increased inside the cells after treatment with compound **1** and compound **10** because of elevated ER stress.

Autophagy induction

ER stress induction acts as a potent trigger for autophagy which is primarily a cytoprotective secondary response to the excessive protein build up in the ER.^{60–62} To investigate if autophagy was launched as a survival mechanism by the HeLa cells in response to treatment with compound **1**, we evaluated the cellular expression level of LC3B, a key autophagy marker, by an immunofluorescence assay.⁶³ We treated HeLa cells with compound **1** for 24 h followed by fixing the cells with paraformaldehyde. These fixed cells were then permeabilized with a buffer containing 0.1% Tween-20 followed by incubation with a primary antibody specific to LC3B for 4 h. The cells were then incubated with a secondary antibody tagged with Alexa-Fluor-488 (green fluorescent). The nuclei of the cells were stained with blue fluorescent DAPI. The cells were visualised by confocal microscopy. The green fluorescence signals were hardly seen in the control cells (Fig. 5a). However, the cells treated with compound **1** exhibited highly enhanced green fluorescence (5 fold) compared to the control cells. More interestingly, we observed the formation of autophagosomes (green puncta in the images) which is regarded as the hallmark of autophagy.⁶⁴ To confirm that the fluorescence signal of compound **1** does not interfere with the immunofluorescence assay, we treated the HeLa cells with non-fluorescent compound **10** for 24 h. The cells were then fixed, permeabilized and stained with a primary antibody specific to LC3B and a secondary antibody tagged with Alexa-Fluor-488. The control cells were treated in a similar manner without compound **10**. The confocal imaging clearly showed that compound **10** also increased the expression of LC3B remarkably in the HeLa cells compared to the control cells (Fig. S207, ESI†).

We also evaluated the expression level of Beclin, another autophagy marker, by immunofluorescence.⁶⁵ HeLa cells were treated with compound **1** for 24 h followed by incubation with a Beclin specific primary antibody. The cells were then incubated with a secondary antibody tagged with Alexa-Fluor-594 (red fluorescent). The nuclei of the cells were stained with DAPI. We visualised the cells by confocal microscopy and found that an increased red fluorescence signal was observed in the compound **1** treated cells compared to that in the non-treated control cells (8 fold) (Fig. 5b). The enhanced expression levels of LC3B and Beclin in the treated cells as evaluated by the immunofluorescence assay evidently demonstrated that autophagy was induced upon elevation of ER stress in the HeLa cells.

We further validated the expression levels of LC3B and Beclin by western blot analysis. We treated HeLa cells with compound **1** for 24 h followed by cell lysis. The cellular proteins were then subjected to gel electrophoresis. The western blot

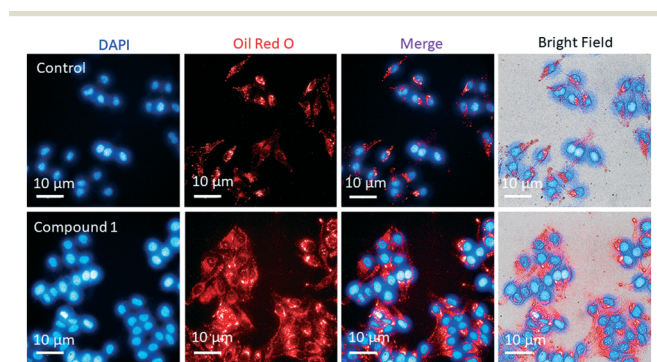


Fig. 4 The confocal microscopy images of HeLa cells after incubation with compound **1** for 24 h followed by staining with Oil Red O dye (red) and DAPI. Scale bar = 10 μ m.

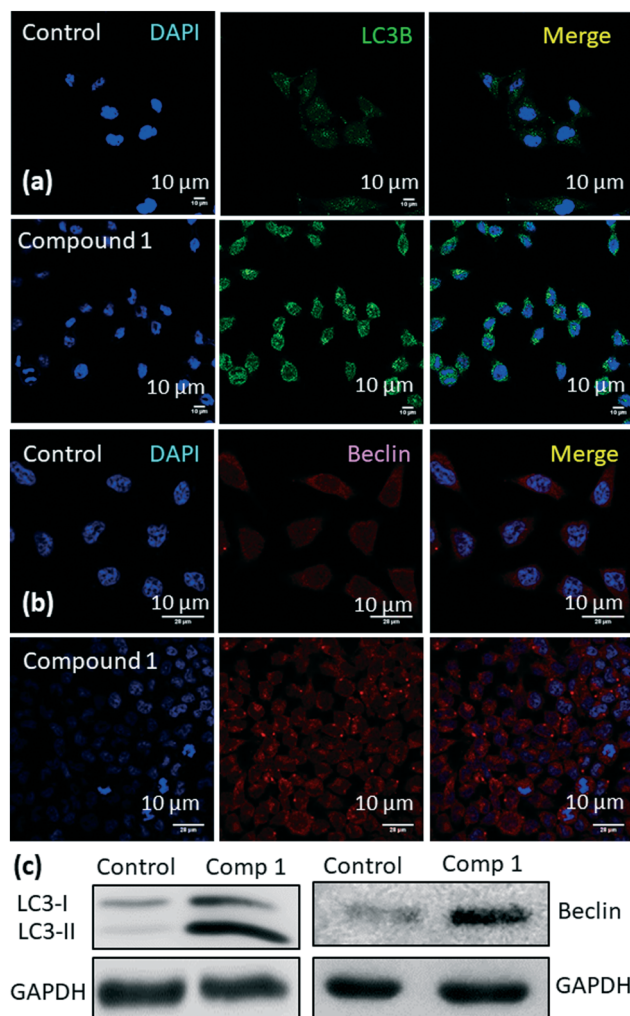


Fig. 5 (a and b) Confocal images of HeLa cells after treatment with compound **1** for 24 h followed by LC3B and Beclin specific primary antibodies followed by an Alexa Fluor 488 tagged secondary antibody (green) and an Alexa Fluor 594 tagged secondary antibody (red), respectively. Scale bar = 10 μm. (c) Western blot images of the LC3-I, LC3-II and Beclin protein expressions in HeLa cells after treatment with compound **1** for 24 h.

analysis revealed that treatment with compound **1** resulted in increased levels of LC3-I (2.6 fold), LC3-II (10 fold) and Beclin (2 fold) indicating the onset of autophagy (Fig. 5c and S208a–c, ESI†). Moreover, the high LC3-I/LC3-II ratio (3.8 fold) clearly confirmed the induction of autophagy by compound **1**. The immunofluorescence assay and western blot analysis thus confirmed that autophagy was triggered upon ER stress induction on treatment with compound **1** which is in agreement with our previous reports as well as other reports.^{36,49,50}

Induction of apoptosis

Induction of ER stress is expected to induce apoptosis in HeLa cells. To estimate the apoptosis induced by compound **1** in HeLa cells, we performed a flow cytometry assay. We

treated HeLa cells with compound **1** for 24 h followed by staining the apoptotic and necrotic cells with Annexin V-FITC and propidium iodide (PI), respectively. The cells were then analysed by flow cytometry. We found that treatment with compound **1** resulted in 64.6% cells in the early apoptotic stage and 17.8% cells in the late apoptotic stage compared to 1.26% and 0.08% in the control cells, respectively (Fig. 6a). We anticipate that compound **1** was not as effective to induce apoptosis due to induction of autophagy as a survival mechanism. We thus hypothesized that the concomitant inhibition of autophagy will improve the apoptotic outcome in HeLa cells. To validate this hypothesis, we cotreated HeLa cells with compound **1** and 50 μM chloroquine, an autophagy inhibitor, for 24 h and performed a flow cytometry assay.⁶⁶ We observed that autophagy inhibition indeed improved the apoptotic outcome with 28.6% and 62% cells in the early and late apoptotic stages, respectively (Fig. 6a). The flow cytometry data thus confirmed that compound **1** induced apoptosis in HeLa cells which further improved upon inhibition of autophagy with chloroquine.

To further investigate the effect of autophagy inhibition on the cell killing efficacy of compound **1**, we performed a cell viability assay. We cotreated HeLa cells with compound **1** in a dose dependent manner along with 50 μM chloroquine for 24 h. The cell viability was measured by the MTT assay. We found that inhibiting autophagy improved the IC₅₀ value of compound **1** from 9 μM to 4 μM in HeLa cells (Fig. 6b). We also evaluated the cell killing efficacy of compound **1** on three different cancer cell lines by the MTT assay. We treated MCF-7 (human breast cancer), A549 (human lung carcinoma) and MDA-MB-231 (drug resistant triple negative breast cancer) with compound **1** in a dose dependent manner for 24 h. We found that compound **1** exhibited IC₅₀ values of 15 μM, 10 μM and 17 μM in MCF-7, A549 and MDA-MB-231, respectively (Fig. 6b). Furthermore, inhibition of autophagy by treatment with chloroquine resulted in enhanced cell killing and improved IC₅₀ values of 10 μM, 5 μM and 13 μM in MCF-7, A549 and MDA-MB-231, respectively (Fig. 6b). Compound **1** thus efficiently induced cell killing in

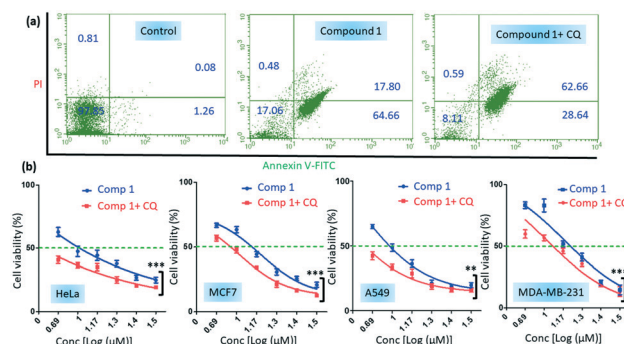


Fig. 6 (a) Flow cytometry analysis of compound **1** and CQ in HeLa cells to evaluate apoptosis. (b) Dose dependent cell viability assay of compound **1** and CQ in HeLa, MCF7, A549 and MDA-MB-231 cells at 24 h post incubation. ****p* < 0.001 and ***p* < 0.01, the *p* value is calculated using two-way ANOVA.

different cancer cell lines which further improved upon autophagy inhibition.

We also evaluated the inhibition of autophagy along with induction of ER stress with the other lead molecules (compounds **10**, **17** and **55**) on HeLa cells. We treated HeLa cells with CQ in combination with compounds **10**, **17** and **55** in a dose dependent manner for 24 h and the cell viability was measured by the MTT assay. We observed that the combination treatment of CQ with compounds **10**, **17** and **55** induced much improved HeLa cell killing with IC_{50} = 4.4 μ M, 5.5 μ M and 16.1 μ M respectively (Fig. S209, ESI†).

To be successful in future cancer therapy, compound **1** should selectively induce cancer cell death without collateral damage to the non-cancerous healthy cells. To evaluate the effect of compound **1** on healthy cells, we incubated HEK293 human embryonic kidney cells with compound **1** in a dose dependent manner for 24 h and quantified the cell viability by the MTT assay. To our surprise, we found that compound **1** showed only 52.8% cell viability even in 30 μ M concentration with IC_{50} = 31.7 μ M, which was quite high compared to the IC_{50} value in different cancer cells (Fig. S210, ESI†). We further calculated the selectivity index (SI) of compound **1** in HeLa, MCF7, A549 and MDA-MB-231 cells to understand its effect on cancer cells compared to the healthy cells.⁶⁷ It was found that the SI of compound **1** was 3.52, 2.11, 3.17 and 1.86 in HeLa, MCF7, A549 and MDA-MB-231 cells, respectively, which indicated that compound **1** is more selective for HeLa and A549 cells, compared to the other tested cancer cells.

Finally, as a proof of concept, to confirm that ER stress induction resulted in apoptosis, we treated HeLa cells with compound **1** and 4-phenyl butyric acid (4-PBA), a chemical chaperone that alleviates both toxicity and proteomic alterations induced by an ER stress inducer, by aiding in protein folding in the ER.^{68–70} Although 4-PBA is also known as a potent HDAC inhibitor, it was shown to be non-toxic even at a higher concentration in HeLa cells.⁷¹ We co-treated HeLa cells with compound **1** along with 15 μ M 4-PBA for 24 h and then performed the cell viability assay. We found that cotreatment with 4-PBA resulted in reduced cell killing (IC_{50} = 28 μ M) in HeLa cells as compared to the treatment with compound **1** only (Fig. S211, ESI†). From these cell viability assays, it was evident that compound **1** triggered apoptosis after induction of autophagy. Furthermore, co-treatment of compound **1** and an autophagy inhibitor would induce improved cancer cell killing without inducing significant damage to the non-cancerous cells.

Conclusions

In conclusion, we synthesised a library comprising 66 small molecules based on sulfonohydrazide-hydrazones through short and concise synthetic steps. Upon screening, we identified four molecules as potential ER stress inducers. Further studies with the dansyl based sulfonohydrazide-hydrazone (compound **1**) revealed its accumulation in the ER

within 3 h in HeLa cervical cancer cells. The identified small molecule efficiently induced ER stress mediated apoptosis and autophagy in HeLa cells. It also induced ER stress associated apoptosis in lung, breast and drug resistant breast cancer cell lines. Finally, the treatment in combination with an autophagy inhibitor, chloroquine, remarkably improved the cell killing efficacy of the small molecule in the cancer cell lines. We envision that these small molecules can be employed in understanding the ER biology and functions in cancer and pave the way for the development of novel ER stress inducers thereby improving the cancer therapeutics.

Experimental section

Materials

All the chemicals were purchased from commercial suppliers unless otherwise noted. MCF-7 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, UK). HeLa, HEK-293, MDA-MB-231 and A549 cells were obtained from the National Centre for Cell Science (NCCS) (Pune, India).

Synthesis of sulfonohydrazides

The sulfonohydrazides were prepared according to a previously reported procedure.⁷² Briefly, to a solution of sulfonyl chloride (1 equiv.) in THF at -30 °C, hydrazine monohydrate (5 equiv.) was added dropwise. The solution was then allowed to stir for 30 minutes. The progress of the reaction was monitored through TLC and after the reaction was over, ethyl acetate was added into the cold reaction mixture. The reaction mixture was washed multiple times with ice cold 10% brine solution. The organic layer was collected and dried over anhydrous Na_2SO_4 and the solvent was then evaporated under reduced pressure. The solid obtained was washed with pentane three times and the product was stored at 4 °C for further use.

Synthesis of sulfonohydrazide-hydrazones

To a solution of sulfonohydrazide (1 eq.) in ethanol, aldehyde was added (1 eq.) along with a catalytic amount of *para*-toluene sulfonic acid and the reaction was allowed to stir at room temperature overnight. The extent of reaction was monitored by TLC. On completion, the solvent ethanol was evaporated, and the residue obtained was dissolved in organic solvents (DCM/ethylacetate). The organic layer was washed with water, collected and evaporated. The residue obtained was then purified using column chromatography. The procedure was utilized to generate a library of 66 small molecules.

Author contributions

SP, VKS and AB performed all the experiments and collected the data. SP, ML and SB wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Esterase Activatable Synthetic M^+/Cl^- Channel Induces Apoptosis and Disrupts Autophagy in Cancer Cells

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Abstract: The supramolecular esterase activatable synthetic M^+/Cl^- ion channel is reported which can form self-assembled rosette ion channel in the bilayer membrane by the action of esterase enzymes. The channel formation switches the cancer cells to apoptosis via an intrinsic pathway. The more interesting feature is that along with apoptosis this system disrupts autophagy in cancer cells via alkalization of lysosomes, which was confirmed through cell imaging and western blot analysis.

Cell death plays a pivotal role in the normal pathophysiological processes in the biological systems. Dysregulation of cell death results in pathogenesis of a wide range of diseases like neurodegenerative diseases and cancer.^[1] Three classical forms of cell death include necrosis, apoptosis, and autophagy. The necrosis is often considered as accidental cell death while as apoptosis is a well-known programmed pathway to regulate normal functioning by wiping out the damaged cells.^[2] In contrary autophagic cell death (ACD) refers to cell death that is inhibited *via* specific blockage of the autophagic pathway. The cancer cells have decreased apoptosis rate and high autophagy rate than normal cells, helping cancer cells to proliferate at high rate. The anticancer treatments engage autophagy as a cytoprotective answer in response to toxic dose to mitigate cellular stress, whereas in some cases the results are opposite. So, the apoptosis-inducing agents such as e.g. obatoclax, cannabinoids, etc which target autophagy have great applications to evade cancer-related problems.^{[3],[4]} Obatoclax is an analog of prodigiosin, and its mechanism of action is linked to the alkalization of lysosomes, thereby, disturbing the autophagy process.^[5] The synthetic ion transporters have apoptosis inducing activity in cancer cells.^[2b, 6] However, synthetic transporters that play a dual role in inducing apoptosis and disrupting autophagy are very rare.^[2b, 7]

Very recently, considerable efforts have been invested in the design of stimuli-responsive ion transporters aiming to target cancer cells selectively. In this regard pH,^[8] light,^[9] enzymes,^[10] glutathione,^[11] etc. have drawn significant attention. Rotello and coworkers reported that the site of activation of prodrugs is very

important in the development of biomedicine.^[12] According to them, the extracellular activation is important when cell impermeable substrates are used as prodrugs and are activated by extracellular machinery. In contrast, intracellular activation is required when intracellular machinery is targeted for activation. Jeong and coworkers reported a series of glycoside tethered urea molecules as enzyme-responsive procarriers.^[10] However, the crucial limitation of these systems is associated with their choice of enzymes for activation. It is well-known that the levels of intracellular glycosidases are higher compared to their extracellular levels.^[13] As a result, the release of active transporters was done by adding the respective enzymes in the extracellular media. Moreover, if applied *in vivo* these water soluble protransporters are expected to be excreted easily by the kidney.

Therefore, we considered a strategy for activating amphiphilic protransporters by intracellular enzyme, and selected esterase enzyme for activating the synthetic channel-mediated ion transport. This enzyme is overexpressed in cancer cells,^[14] and therefore, applied significantly in the activation of prodrugs and drug delivery.^[15] The inactive form **1** of the channel forming molecule was designed by connecting methyl pivalate at the C-2 position of N^1,N^3 -dihexyl-2-hydroxyisophthalamide **2** so that the system fails to proper self-assembly (Figure 1). We have recently demonstrated the ion channel formation by **2** activated by glutathione.^[11a] The ion channel activation by glutathione was confirmed using MCF-7 and *INS-1E* cells. The detailed mechanistic study corroborated to the apoptosis of MCF-7 cells due to the disruption of ion homeostasis. Therefore, we realized that upon reaction with intracellular esterases, methyl pivalate moiety of compound **1** would be cleaved^[16] releasing **2** that would form self-assembled M^+/Cl^- ion channels in the membrane resulting in cancer cell death via the perturbation of ion homeostasis. Therefore, the strategy would

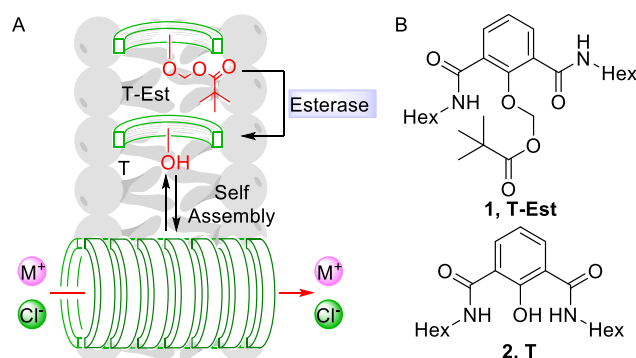


Figure 1. Representation of esterase activatable M^+/Cl^- channel (A). Structure of inactive ester form **1** (T-Est) and channel forming form **2** (T) (B).

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provide a new being higher in lysosome compared to the cytoplasm,^[17] an activation of such ion transport across lysosomal membranes would also perturb the functions of these organelles. So the channel can act as a double-edged sword for inducing apoptosis and disrupting autophagy in cancer cells.

The synthesis of **1** was carried out by reaction of compound **2** with iodimethyl pivalate presence of triethylamine^[18] (Scheme S1, ESI). The compound was characterized by ¹H NMR, ¹³C NMR, and high-resolution mass spectrometry.

After the successful synthesis of compound **1**, the release of compound **2** from **1** upon enzymatic cleavage was studied using ¹H NMR studies. The compound **1** (1 mM) in phosphate buffer saline (PBS) was treated with 4 U/mL of porcine liver esterase enzyme (Sigma Aldrich) prepared in PBS at pH 7.2 for different time intervals. The disappearance of the peak for H_{b'} at δ = 5.6 ppm, the shift of peak for H_a at δ = 8.0 ppm to 7.98 ppm as well as the reappearance of the peak for H_c around 14.4 ppm was monitored and it was found that almost 95% of the cleavage takes place within three hours of reaction time (Figure 2). These results suggest the release of **2** from compound **1** is a very feasible process when incubated in cells due to higher expression of esterases.

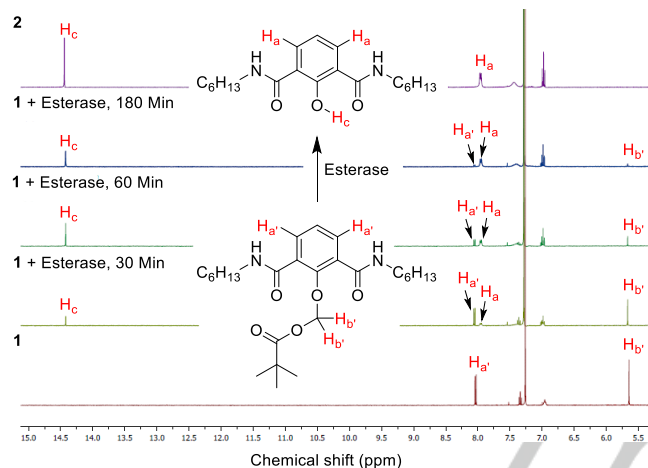


Figure 2. Esterase mediated release of **2** from **1** monitored by ¹H NMR experiments.

The ion transport properties of **1** and **2** were evaluated using lucigenin based fluorescence assay using large unilamellar vesicles (LUVs) composed of egg-yolk phosphatidylcholine (EYPC).^[19] The compound **1** was found to be almost inactive than compound **2** for ion transport at identical concentrations (Figure 3). However, upon treatment with esterase enzyme, the transport activity was regained showing the efficient release of the active channel forming compound **2** from **1**. These studies encouraged us to further carry out the cellular experimentations. The compound **1** was incubated in the human epithelial breast cancer cells MCF-7, for 30 min and then the cells were observed under a confocal microscope (Leica SP8). The results suggested that there is an enhancement of blue fluorescence in the cells (in the cytoplasm and near the membrane) incubated with **1** compared to control (Figure 4) suggesting that compound **2** gets released from **1** by intracellular esterases.

The results of the cell-imaging assay encouraged us to evaluate the cell viability of cancer cells in the presence of compound **1**. MCF-7 cells and HeLa cells were incubated with compound **1** in a dose-dependent manner for 24 hours and then the cell viability was evaluated by MTT assay.^[6b] The results suggested that compound **1** displayed enhanced cytotoxicity with increase in concentration of the compound giving IC₅₀ values of 15 μ M and 20 μ M for MCF-7 and HeLa cells respectively. The higher IC₅₀ value of the esterase activatable

compound (*IC*₅₀ value of 15 μ M for MCF-7 cells) compared to our previously reported glutathione activatable compound (*IC*₅₀ value of 1 μ M for MCF-7 cells)^[11a] may be corroborated to (i) better permeability of the 2,4-dinitrobenzenesulfonyl (DNS) linked protransporter and (ii) better cleavability of DNS group (*i.e.*, faster activation. The effect of *in situ* generated by-products (formaldehyde and pivalic acid as 4-methoxyphenol is proved to be non-toxic to cells)^[20] on cell viability was evaluated independently in MCF-7 cells and the compound **6** showed negligible cytotoxicity compared to **1** (Figure S2).

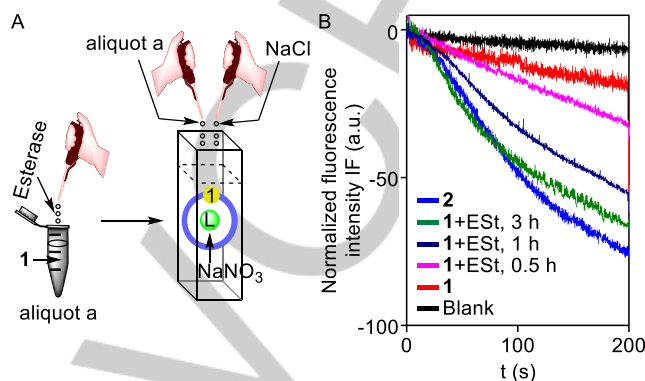


Figure 3. Schematic representation of assay (A) and comparison of Cl⁻ ion influx into EYPC-LUVs containing lucigenin by compounds **2** and **1** treated with esterase enzyme for specific time intervals (B).

The above results encouraged us to further evaluate the mechanism of cell death *i.e.*, whether cell death is being mediated by necrosis or apoptosis. We evaluated the apoptotic pathway starting with monitoring the mitochondrial membrane potential (MMP) change on treated cells using JC-1 dye.^[21] The enhanced green fluorescence in treated cells compared to control cells indicated that the MMP is damaged (Figure 5A, B, Figure S3). This change in MMP leads to disturbances in the electron transport chain in the mitochondrial respiratory cycles, which ultimately results in the generation of reactive oxygen species (ROS).^[22] Therefore, we monitored the ROS produced in the mitochondria using mitoxon^[23] (Figure S4) and overall cellular ROS using H₂DCFDA probes^[24] (Figure S5, S6). These results indicated the production of ROS in the cells treated with compound **1**.

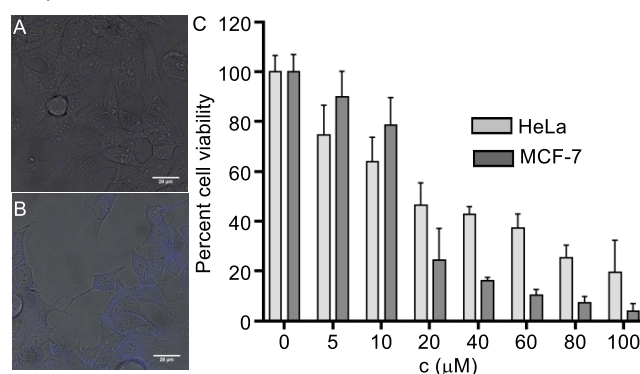


Figure 4. Live cell imaging of MCF-7 cells upon incubation with 0 μ M (A) and 15 μ M (B) of **1**. Dose-dependent viability of MCF-7 and HeLa cells incubated with **1** evaluated by MTT assay (C).

It is reported that the intrinsic apoptotic pathway initiates from the mitochondria and releases cytochrome c which binds to the Apaf-1 to form an apoptosome.^[6b] The apoptosome activates the caspase 9 pathway, which switches the cells to apoptosis. Therefore, to confirm the apoptotic pathway is functional we

probed the activation of the caspase 9 pathway using immunoblot analysis of MCF-7 cells upon treatment with compound **1** (15 μ M). GAPDH was used as the loading control. The results showed an increase in expression of cleaved caspase 9 (Figure 5E), which confirms the activation of the intrinsic pathway of apoptosis. To further support, the expression of cleaved poly(ADP-ribose) polymerase (PARP) was also monitored, which showed a significant amount of degradation of full-length PARP-1 (116 kDa) with a concomitant increase of cleaved PARP-1 (86 kDa)^[25] (Figure 5F) supporting activation of the caspase 9 pathway of apoptosis.

The chloride concentration in lysosomes (80 mM) maintains the lysosomal pH (4.5–5.0),^[17] and any disturbance in this chloride concentration leads to a change in the lysosomal pH, which causes the disturbance in the activity of the lysosomal enzymes, leading to autophagy disruption.^[26] Therefore, to evaluate the effect of compound **1** on lysosomes, we first monitored the pH of lysosomes of treated cells using acridine orange (AO) dye.^[27] AO is a cell-permeable dye which accumulates in acidic compartments, such as lysosomes, and shows a characteristic orange fluorescence emission while as emits green fluorescence at higher pH, such as in the cytosol. Upon treatment with **1** followed by AO staining the MCF-7 cells showed complete loss of granular orange fluorescence (Figure 5G) compared to control (Figure 5F), indicating the alkalization of lysosomes.^[28]

The alkalization is known to be associated with a decrease in the activity of lysosomal enzymes, hence, autophagy.^[7] Therefore, we probed the effect of compound **1** on autophagy using immunoblot analysis. One of the markers of autophagy is Lamin B1, which decreases during autophagy.^[29] The treatment of MCF-7 cells with **1** (15 μ M) for 24 hours showed a marked decrease in the expression of Lamin B1 (Figure 5H) compared to control, indicating that autophagy is being induced. To further corroborate this data, the expression of microtubule-associated protein 1 light chain 3 (LC3) was monitored. Typically increased expression of LC3 indicate the induction of autophagy in cells.^[30] The treatment of MCF-7 cells with **1** showed increased expression of LC3 (Figure 5I) compared to control. Together, these results suggest the activation of the autophagy process upon treatment with compound **1**.

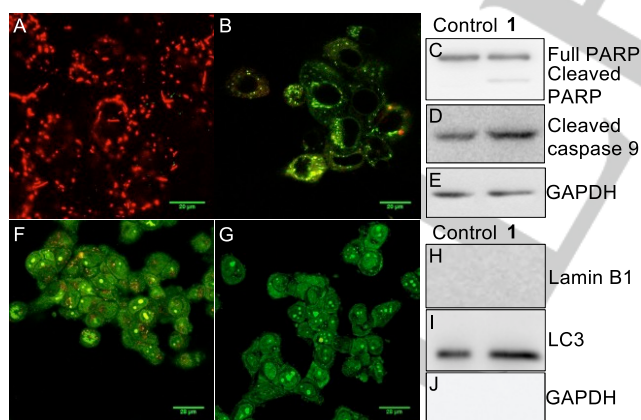


Figure 5. Live cell imaging of MCF7 cells upon treatment with 0 μ M (A) and 15 μ M (B) of **1** for 24 h followed by staining with JC-1 dye. Red and green channel images were merged to generate the displayed image. Immunoblot assay for PARP cleavage (C) in MCF7 cells, and active caspase 9 (D) after 24 h incubation with 0 μ M and 15 μ M of **1** with GAPDH as a loading control (E). Live cell imaging of MCF 7 cells incubated with **1** at 0 μ M (F), and 15 μ M (G) concentrations followed by staining with acridine orange. Immunoblot assay for a decrease in Lamin B1 expression (H) and an increase in LC3 expression (I) in MCF 7 cells, with GAPDH as a loading control (J).

In summary, we have reported the novel concept of enzyme activatable synthetic ion channel which can be activated by

intracellular esterase enzymes. The successful activation of ion channel inside cells leads to ion transport across the plasma membrane, which switches the cells to apoptosis through the mitochondrial pathway. Furthermore, this transport can dissipate the pH gradient of the lysosomes, which leads to lysosomal dysfunctions, and ultimately leads to autophagy disruption in MCF 7 cells.

Acknowledgements

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Keywords: Supramolecular chemistry • Ion Channel • Enzyme responsive • Apoptosis • Autophagy

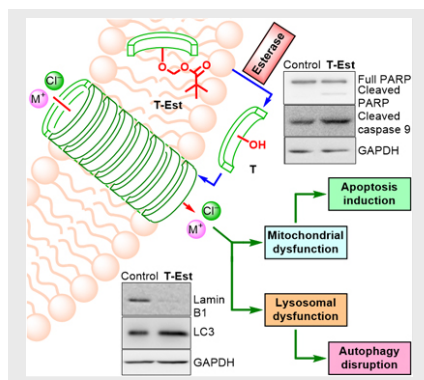
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COMMUNICATION

Esterase cleavage of a protransporter leads to ion channel formation in membrane. The activation causes apoptosis and disrupts autophagy in cancer cells.



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Title: Esterase Activatable Synthetic M^+/Cl^- Channel Induces Apoptosis and Disrupts Autophagy in Cancer Cells

Supporting Information

Esterase Activatable Synthetic M^+/Cl^- Channel Induces Apoptosis and Disrupts Autophagy in Cancer Cells

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I. General Methods:

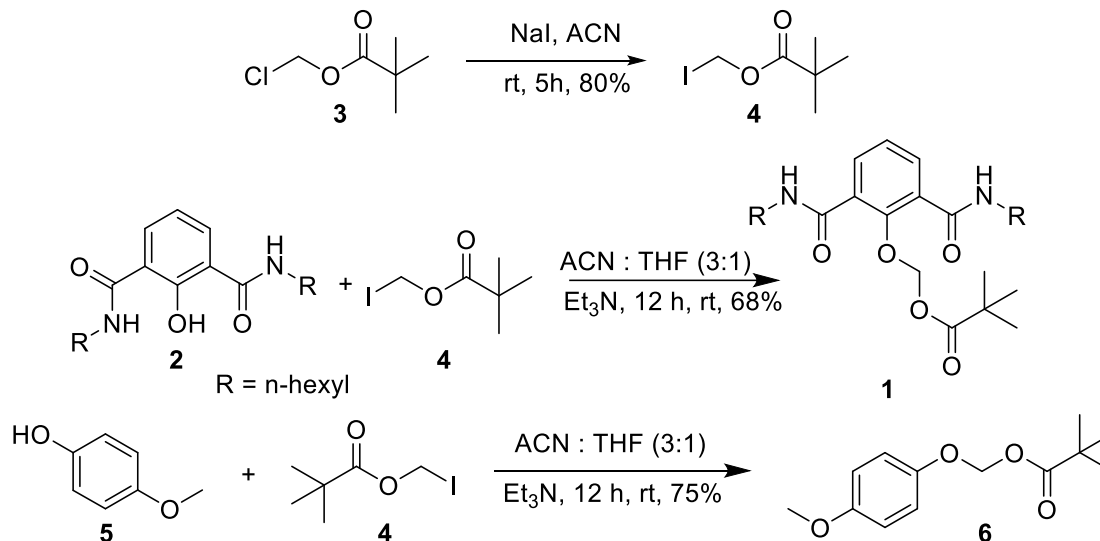
All reactions were carried out under the nitrogen atmosphere. All the chemicals were purchased from commercial sources and were used as received unless stated otherwise. Solvents were dried by standard methods prior to use or purchased as dry. Thin layer chromatography (TLC) was carried out with E. Merck silica gel 60-F₂₅₄ plates and column chromatography was performed over silica gel (100-200 mesh) obtained from commercial suppliers. Egg yolk phosphatidylcholine (EYPC) lipid was purchased from Avanti Polar Lipids as a solution dissolved in chloroform (25 mg/mL). Lucigenin dye, Triton X-100, NaOH and all inorganic salts of molecular biology grade were purchased from Sigma. Size exclusion chromatography was performed on a column of Sephadex G-50. Large unilamellar vesicles (LUV) were prepared from EYPC lipid by using a mini extruder, equipped with a polycarbonate membrane either of 100 nm or 200 nm pore size, obtained from Avanti Polar Lipids. The JC1 dye was the generous gift from Dr. Shilpy Sharma at Savitribai Phule Pune University. The H₂DCFDA was purchased from Sigma Aldrich.

II. Physical Measurements:

The ¹H and ¹³C NMR spectra were recorded on 400 MHz Jeol ECS-400 (or 100 MHz for ¹³C) spectrometers using either residual solvent signals as an internal reference or from internal tetramethylsilane on the δ scale relative to chloroform (δ 7.26), dimethylsulphoxide (δ 2.50 ppm), acetone (δ 2.05) for ¹H NMR and chloroform (δ 77.20 ppm), dimethylsulphoxide (δ 39.50 ppm), acetone (δ 29.84 and 206.26) for ¹³C NMR. The chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The following abbreviations are used: s (singlet), d (doublet) m (multiplet), and td (triplet of doublet) while describing ¹H NMR signals. High-resolution mass spectra (HRMS) were obtained from a MicroMass ESI-TOF MS spectrometer. Fluorescence spectra were recorded by using Fluoromax-4 from Jobin Yvon Edison equipped with an injector port and a magnetic stirrer. All melting points were measured in open glass capillary and values are uncorrected. All fluorescence data were processed either by Origin 8.5 or KaleidaGraph and finally, all data were processed through ChemDraw Professional 15. MTT assay was recorded in a microplate reader (Varioskan Flash). Western blot was visualized in ImageQuant LAS 4000 (GE Healthcare). Cell images were taken using Leica sp8 confocal microscope and Nikon

Eclipse TS 100 fluorescence microscope. The cell images were processed by image j software. The Grapgpad Prism 7 was used for plotting the data from biological assays.

III. Synthesis:



Scheme S1. Synthesis of compounds **1** and **6**.

Synthesis of compound 4: Synthesis of compound **4** was done according to the known protocol.^[S1]

Synthesis of compound 1: In a clean dry round bottom flask was added compound **2** (50 mg, 0.14 mmol) in dry acetonitrile (ACN, 3 mL) and tetrahydrofuran (THF, 1 mL) mixture (3:1) at room temperature. To this solution, was added iodomethyl pivalate **4** (70 mg, 0.28 mmol) and K_2CO_3 (60 mg, 0.42 mmol) at room temperature and the reaction mixture was stirred at room temperature overnight. After the TLC showed complete consumption of starting material, the compound was washed with brine and extracted with dichloromethane (DCM). The compound was purified by silica gel column chromatography to yield compound **1** as yellowish viscous semi-solid (45 mg, 68 %). **^1H NMR (400 MHz, CDCl_3)** δ 7.99 (d, J = 7.7 Hz, 2H), 7.30 (t, J = 7.7 Hz, 1H), 7.00 (t, J = 5.4 Hz, 2H), 5.63 (s, 2H), 3.41 (td, J = 7.3, 5.8 Hz, 4H), 1.59 (dd, J = 14.9, 7.6 Hz, 4H), 1.43 – 1.25 (m, 12H), 1.11 (s, 9H), 0.88 (t, J = 7.0 Hz, 6H). **^{13}C NMR (101 MHz, CDCl_3)** δ 177.79, 164.95, 151.26, 133.93, 128.90, 125.76, 89.71, 40.28, 38.94, 31.55, 29.48, 26.90, 26.80, 22.62, 14.09. **HRMS (ESI):** Calc. for $\text{C}_{26}\text{H}_{42}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$: 463.3178; Found: 463.3173.

Synthesis of compound 6: In a clean dry round bottom flask was added compound 5 (100 mg, 0.80 mmol) in dry acetonitrile (ACN, 6 mL) and tetrahydrofuran (THF, 2 mL)) mixture (3:1) at room temperature. To this solution, was added iodomethyl pivalate 4 (390 mg, 1.6 mmol) and K₂CO₃ (330 mg, 2.4 mmol) at room temperature and the reaction mixture was stirred at room temperature overnight. After the TLC showed complete consumption of starting material, the compound was washed with brine and extracted with dichloromethane (DCM). The compound was purified by silica gel column chromatography to yield compound 6 as yellowish viscous oil (133 mg, 75 %). **¹H NMR (400 MHz, CDCl₃)** δ 6.96 (d, J = 9.0 Hz, 2H), 6.82 (d, J = 9.0 Hz, 2H), 5.68 (s, 2H), 3.76 (s, 3H), 1.19 (s, 9H). **¹³C NMR (101 MHz, CDCl₃)** δ 177.53, 155.29, 151.04, 117.70, 114.60, 86.98, 55.63, 38.89, 26.96. **HRMS (ESI):** Calc. for C₁₃H₁₈O₃ [M+Na]⁺: 245.1132; Found: 245.1263.

IV. Esterase mediated cleavage of 1 to release compound 2 using ¹H NMR spectroscopy.

The compound 1 in DMSO (1 mM) was dissolved in phosphate buffer solution (10 mM, pH = 7.2). To this solution was added porcine liver esterase enzyme (Sigma Aldrich, 4U/mL) at 37 °C and this enzymatic reaction was continued for different time intervals. After each interval, the compound was extracted using ethyl acetate, concentrated *in vacuo* and submitted for ¹H NMR spectroscopy.

V. Ion Transport Studies:

Buffer and stock solution preparation: Phosphate buffer (10 mM) was prepared using autoclaved water followed by adjustment of pH = 7.0 by adding NaOH solution. The stock solution of the compound was prepared in Acetonitrile.

Determination of chloride ion influx by lucigenin assay.^[S2, S3]

Preparation of EYPC-LUVs and Lucigenin for concentration-dependent assay and symport assay: A solution (1 mL) of EYPC lipid (25 mg/mL) lipid dissolved in CHCl₃ was taken in a clean and dry small round bottom flask. The solvents were evaporated slowly by a stream of nitrogen, followed by drying under vacuum for at least 5 h. After that 1 mL of 1 mM *N,N'*-dimethyl-9,9'-biacridinium dinitrate (lucigenin) in 200 mM NaNO₃ (dissolved in 10 mM phosphate buffer with pH=7.0) was added, and the suspension was hydrated for 1 h with

occasional vortexing of 4-5 times and then subjected to freeze-thaw cycle (≥ 15 times). The vesicle solution was extruded through a polycarbonate membrane with 200 nm pores for minimum 19 times (must be an odd number), to give vesicles with a mean diameter of ~ 200 nm. The extracellular lucigenin was removed from the vesicles by size exclusion column chromatography (Sephadex G-50) using 200 mM NaNO₃ as eluent. The obtained vesicles were diluted to 4 mL with 200 mM NaNO₃.

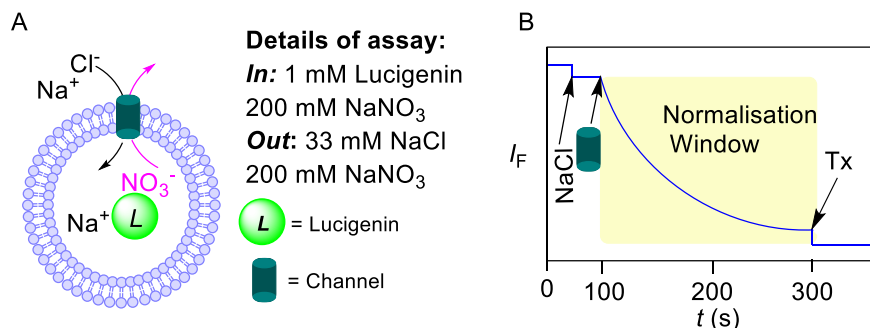


Figure S1. Schematic illustration of chloride influx assay using EYPC-LUVs⊃Lucigenin (A) and representative fluorescence kinetics experiment of the corresponding assay (B).

The time axis was normalized according to Equation S1:

$$t = t - 100 \quad \text{(Equation S1)}$$

The time of compound addition can be normalized to $t = 0$ s and time of Triton-X 100 addition was normalized to $t = 200$ s.

Chloride influx into EYPC-LUVs⊃Lucigenin: In a clean and dry fluorescence cuvette, 50 μ L of above lipid solution and 1950 μ L of 200 mM NaNO₃ solution was taken and kept in slowly stirring condition by a magnetic stirrer equipped with the fluorescence instrument (at $t = 0$ s). In this assay, the time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 455$ nm). 25 μ L of 2 N NaCl was added to the cuvette at $t = 50$ s to make the salt gradient between the intravesicular and extravesicular systems. Compound **1** (extracted after enzymatic reaction) was added at $t = 100$ s and finally at $t = 300$ s, 25 μ L of 10% Triton X-100 was added to lyse all vesicles for 100% chloride influx. Fluorescence intensities (F_t) were normalized to fractional emission intensity I_F using Equation S2.

$$\text{Normalized Fl Intensity } (I_F) = [(F_t - F_0) / (F_\infty - F_0)] \times (-100) \quad \text{(Equation S2)}$$

VI. Biological Studies:

General: All tissue culture treated plastic wares used in this study were purchased from Eppendorf and Thermo scientific, unless mentioned otherwise. DMEM, PBS and Penicillin-Streptomycin were purchased from Lonza. Fetal bovine serum and Trypsin were purchased from Invitrogen. MTT dye was purchased from Sigma Aldrich.

A. Cell culture protocol:

The cells were grown in High Glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen or Lonza) containing 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Invitrogen) and 100 units/mL penicillin-streptomycin (Invitrogen). Cells were maintained in 100 mm tissue culture treated dishes (Corning) at 37 °C in humidified 5% CO₂ incubator (Thermo Scientific).

B. Live cell imaging:

The MCF 7 cells were seeded in glass bottom 35 mm dishes at the concentration of 5×10^5 cells per plate. After attaining the confluency, the cells were focused on a Lieca sp8 confocal microscope and then the DMEM media containing 15 μ M of **1** was added. The blue fluorescent compound was seen in DAPI region using blue LASER. The time course to enter the cell was monitored using real-time analysis.

C. MTT-based cytotoxicity assay: ^[S4]

Cells were dispersed in a 96-well flat bottom tissue culture treated plates (Corning) at a density of 10^4 cells/well (per 100 μ L) and incubated at 37 °C in a 5% CO₂ incubator for 16 h. Compounds were added to each well in different concentrations by maintaining the maximum amount of DMSO at 2 μ L and incubated for 24 h. DMEM solution containing compounds in each well was replaced with 110 μ L of MTT-DMEM mixture (0.5 mg MTT/mL of DMEM) and incubated for 4 h in identical conditions. After 4 h, the MTT solution was removed and 100 μ L of DMSO was added in each well to dissolve the formazan crystals. The absorbance was recorded in a microplate reader (Varioskan Flash) at the wavelength of 570 nm. All experiments were performed in triplicates, and the relative cell viability (%) was expressed as a percentage of untreated cells.

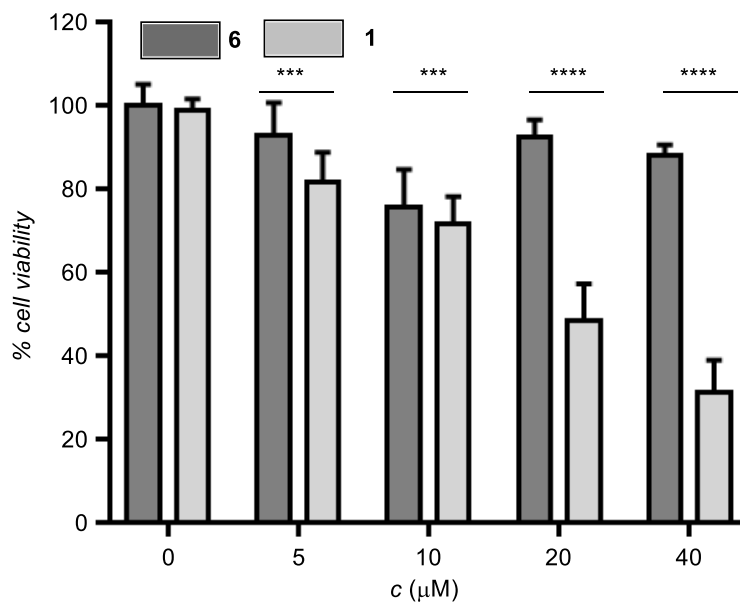


Figure S2. Cell viability of MCF7 cells treated with **6** and **1** in a dose dependent manner. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

D. Mitochondrial membrane depolarization:

The MCF 7 cells were seeded in glass bottom 35 mm dishes at the concentration of 5×10^5 cells per plate. Cells were incubated with 15 μM of **1** for 24 h. After that cells were washed thoroughly and incubated with JC-1 at the final concentration of 50 nM for 30 min. Fluorescence images were acquired after washing with PBS in both red and green channels using a Lieca sp8 confocal microscope.

For quantification, MCF 7 cells were resuspended in a 96-well flat bottom tissue culture plates (Corning) at a density of 10^4 cells/well (per 100 μL) and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Post-incubation, the media was replaced with fresh media. Then, the compound **1** was added after four hours to each well in increasing concentration by maintaining the maximum amount of DMSO at 1.5% (1.5 μL per 100 μL) and was incubated further for 24 h. The cells were again washed twice with PBS and the JC1 dye (50 nM) was added in DMEM and incubated for 30 minutes. Finally the cells were washed with PBS and 100 μL of PBS was added and the plates were read with a multi-mode plate reader (Perkin Elmer) at 529 nm (green) and 590 nm (red). All experiments were performed in triplicates, and the ratio of red/green was expressed as a percentage of untreated cells. Statistical analysis was done using one-way analysis of variance (ANOVA).

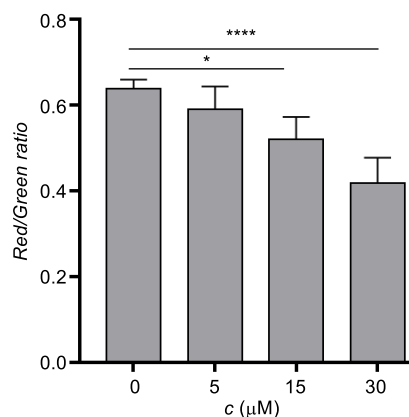


Figure S3. Quantification of Red/Green ratio of treated cells treated with **1** in a dose dependent manner. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

E. Mitosox assay: ^[S5] The MCF-7 cells were dispersed in a 96-well flat bottom tissue culture treated plates (Corning) at a density of 10^4 cells/well (per 100 μ L) and incubated at 37 °C in a 5% CO₂ incubator for 24 h. Fresh media was added to the cells. Then, the compound **1** was added to each well in different concentrations by maintaining the maximum amount of DMSO at 1.5 % (1.5 μ L) and incubated for 24 h. The cells were washed with PBS and mitosox (5 μ M) in PBS was added to each well and incubated for 20 minutes. The cells were again washed twice with PBS and then 100 μ L of PBS was added and the cells were read with a plate reader at 580 nm ($\lambda_{ex} = 510$ nm). All experiments were performed in triplicates, and the relative ROS (%) was expressed as a percentage of untreated cells. Statistical analysis was done using one-way analysis of variance (ANOVA).

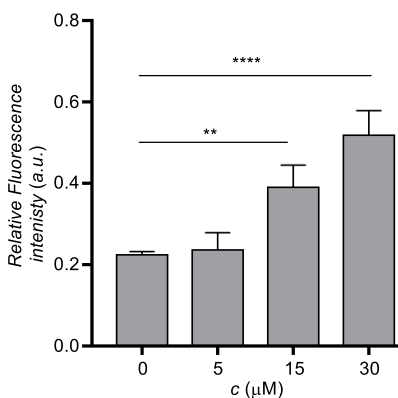


Figure S4. Quantification of mitochondrial ROS generated in cells treated with compound **1** in a dose dependent manner for 24 h evaluated by mitosox dye. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

F. ROS generation: ^[S6]

The MCF 7 cells were seeded in glass bottom 35 mm dishes at the concentration of 5×10^5 cells per plate. Cells were incubated with 15 μM of **1** for 24 h. After that cells were washed thoroughly and incubated with DCFDA at the final concentration of 0.5 μM for 20 min. The cell images were acquired after washing with PBS in time dependent manner using a green channel with Nikon Eclipse TS 100 fluorescence microscope.

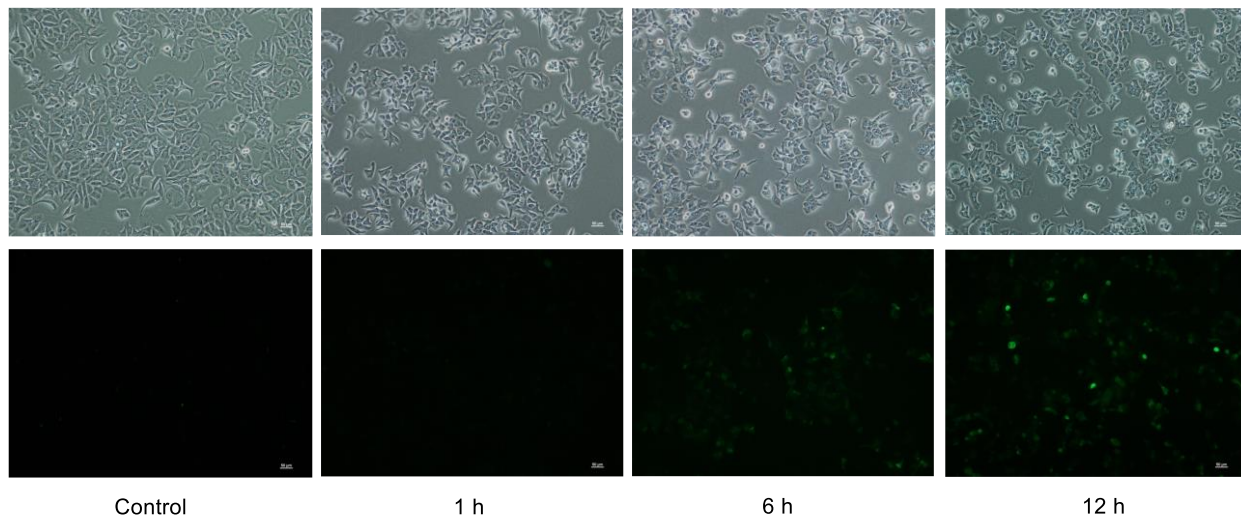


Figure S5. Live cell images of MCF-7 cells incubated with compound **1**, and H_2DCFDA as ROS probe (scale bar 50 μm) in a time dependent manner.

For quantification, the MCF-7 cells were resuspended in a 96-well flat bottom tissue culture treated plates (Corning) at a density of 10^4 cells/well (per 100 μ L) and incubated at 37 °C in a 5% CO₂ incubator for 24 h. The cells were washed with PBS and fresh media was added to the cells. Then, the compound **1** was added after four hours to each well in different concentrations by maintaining the maximum amount of DMSO at 1.5 % (1.5 μ L) and incubated for 24 h. The cells were again washed twice with PBS and the DCFDA in PBS was added and incubated for 20 minutes. Finally the cells were washed with PBS and 100 μ L of PBS was added and the cells were read with a plate reader at 535 nm (λ_{ex} = 488 nm). All experiments were performed in triplicates, and the relative ratio (%) was expressed as a percentage of untreated cells. Statistical analysis was done using one-way analysis of variance (ANOVA).

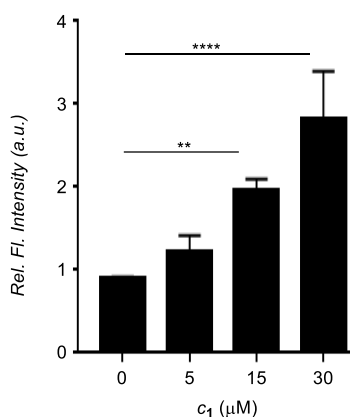


Figure S6. Quantification of ROS generated in cells treated with compound **1** in a dose-dependent manner for 24 h evaluated by H₂DCFDA dye (C). * represents $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

G. Immunoblot analysis:

MCF 7 cells were seeded at a density of 6×10^5 cells per well in 6-well tissue culture treated plates (Corning) and maintained at 37 °C for 24 h. Cells were then treated with 15 μ M of **1** by direct addition of the compound to the culture medium for 24 h. Control cells were treated with an equivalent volume of DMSO. After 24 h treatment, medium containing **1** was aspirated and cells were washed once with 1X phosphate buffered saline (PBS; PAN-Biotech GmbH). Cells were lysed in sample buffer containing 60 mM Tris (pH 6.8), 6% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT) and 0.006% bromophenol blue and lysates were stored at - 40 °C.

Cell lysates were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Blocking was performed in 5% (w/v) skimmed milk (SACO Foods, USA) prepared in 1X Tris buffered saline containing 0.1% Tween 20 (1X TBS-T) for 1 h at room temperature. Blots were incubated for 16 h at 4 °C temperature in the primary antibody solution. Following washes, blots were incubated with peroxidase-conjugated secondary antibody solution prepared in 5% (w/v) skimmed milk in 1X TBS-T for 1 h at room temperature following which blots were developed using Immobilon Western Detection Reagent kit (Millipore) and visualized using ImageQuant LAS 4000 (GE Healthcare).

H. Acridine Orange Staining: ^[S7]

MCF-7 cells (10^6 cells/mL) were seeded in 35 mm glass plates for 24 h and then after compound **1** (15 μ M) was added. DMSO was added to control cells. After 1 hour of incubation, cells were washed twice with PBS and incubated in 5 μ g/mL acridine orange solution (Sigma-Aldrich) for 30 min at room temperature. Finally, cells were washed with PBS-10% FBS three times and fluorescence was examined with a confocal microscope (Leica sp8) under green and red channels.

I. Expression of Lamin B1 and LC3 by Immunoblot Analysis: ^[S8, S9]

MCF-7 cells were seeded at a density of 6×10^5 cells per well in 6-well tissue culture treated plates (Corning) and maintained at 37 °C for 24 h. Cells were then treated with 15 μ M of **1** by direct addition of the compound to the culture medium for 24 h. Control cells were treated with an equivalent volume of DMSO. After 24 h treatment, medium containing **1** was aspirated and cells were washed once with 1X phosphate buffered saline (PBS; PAN-Biotech GmbH). Cells were lysed in sample buffer containing 60 mM Tris (pH 6.8), 6% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT) and 0.006% bromophenol blue and lysates were stored at - 40 °C. Cell lysates were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). Blocking was performed in 5% (w/v) skimmed milk (SACO Foods, USA) prepared in 1X Tris buffered saline containing 0.1% Tween 20 (1X TBS-T) for 1 hour at

room temperature. Blots were incubated for 16 h at 4 °C temperature in the primary antibody solution. Following washes, blots were incubated with peroxidase-conjugated secondary antibody solution prepared in 5% (w/v) skimmed milk in 1X TBS-T for 1 h at room temperature following which blots were developed using Immobilon Western Detection Reagent kit (Millipore) and visualized using ImageQuant LAS 4000 (GE Healthcare).

VII. NMR Spectra.

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RBB-01-090

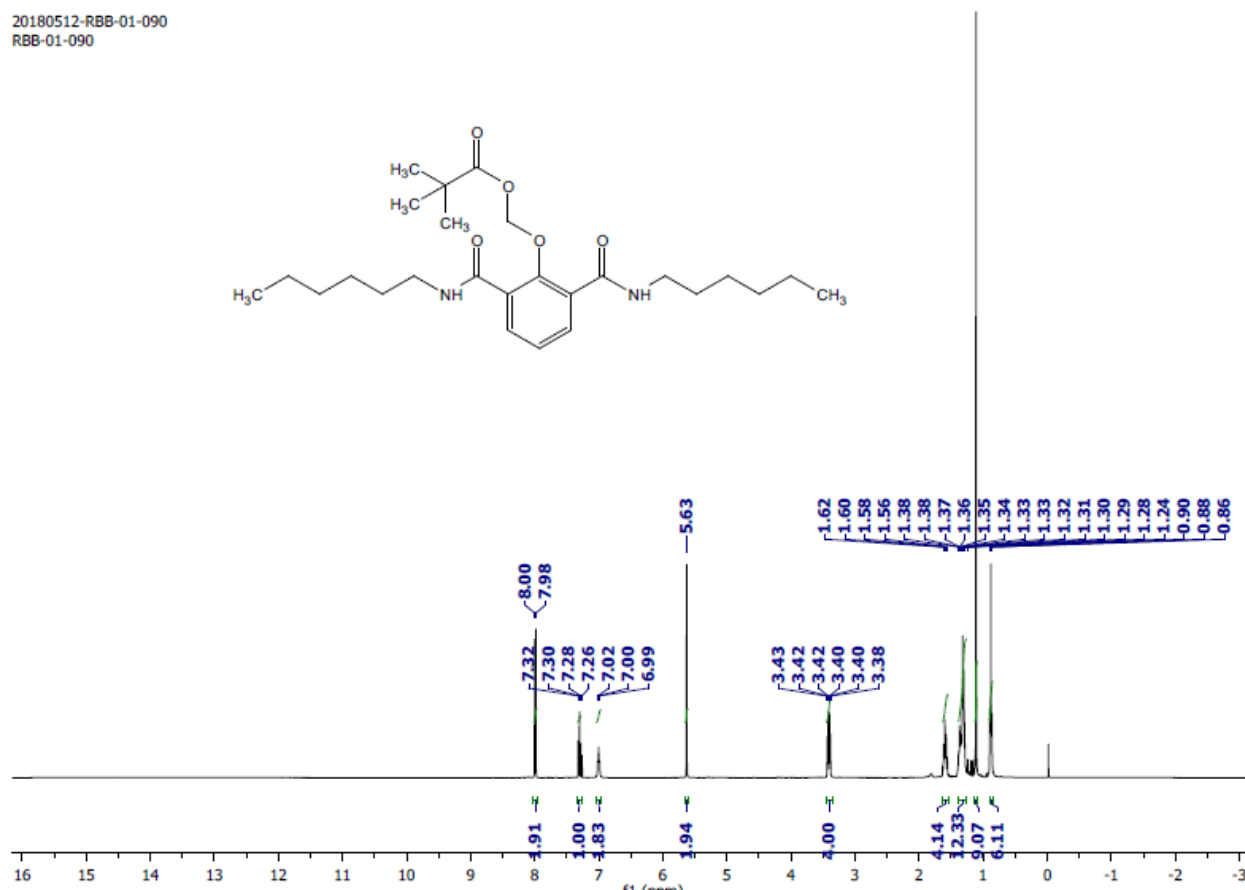


Figure S7. ¹H NMR spectrum of **1** in CDCl₃.

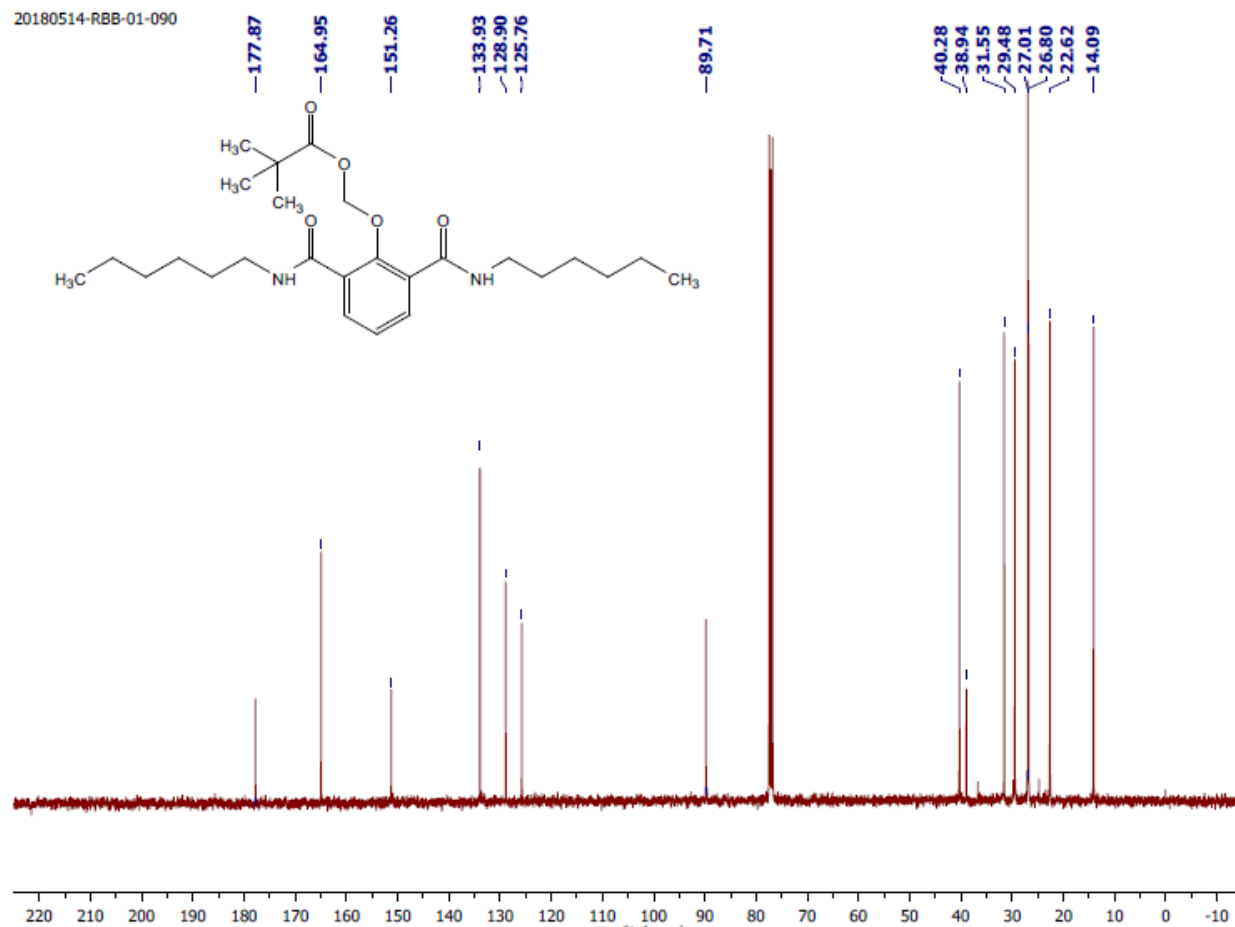


Figure S8. ^{13}C NMR spectrum of **1** in CDCl_3 .

20200206-JVD-04-104

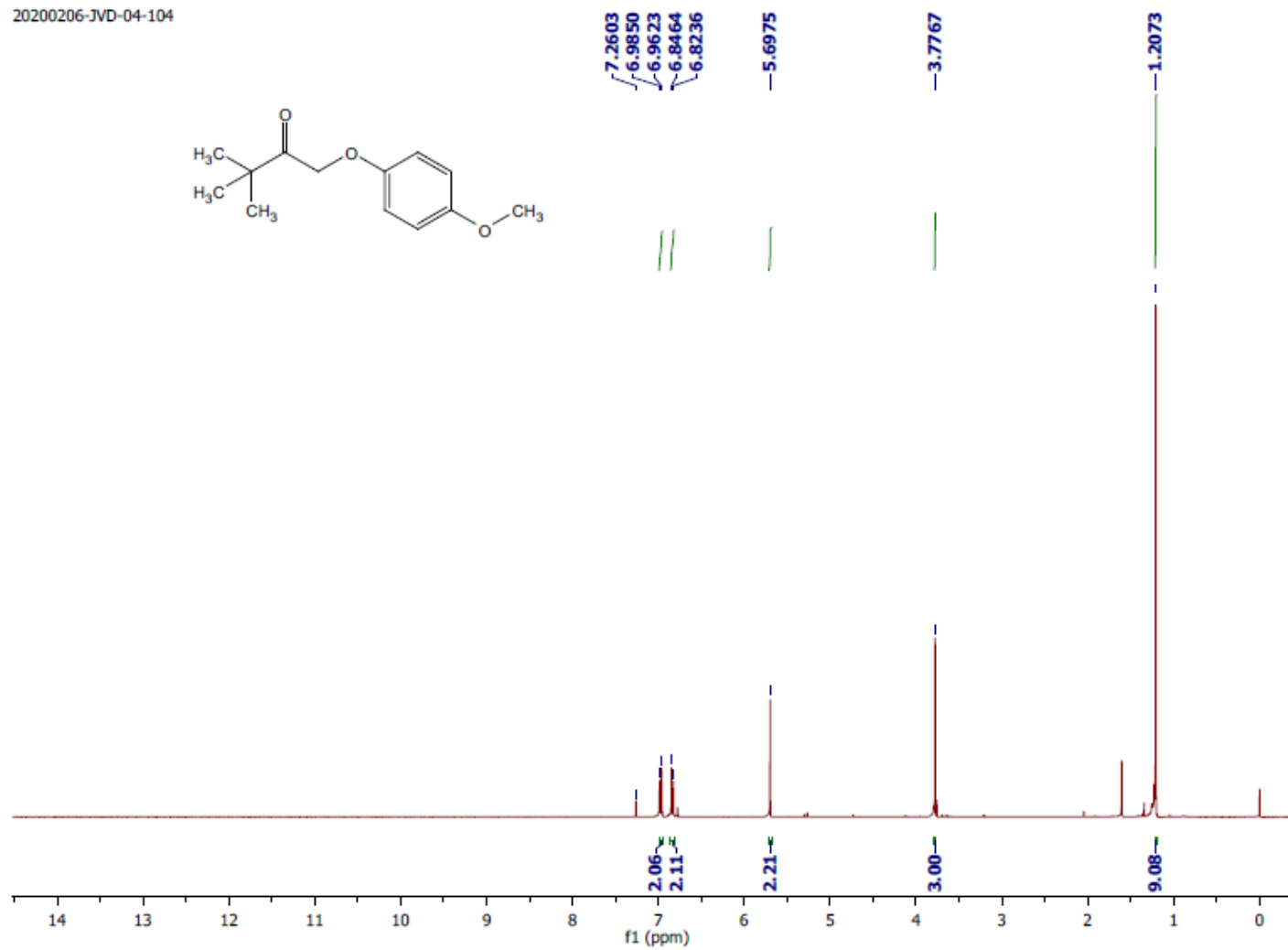


Figure S9. ¹H NMR spectrum of **6** in CDCl₃.

20200209-JVD-04-104

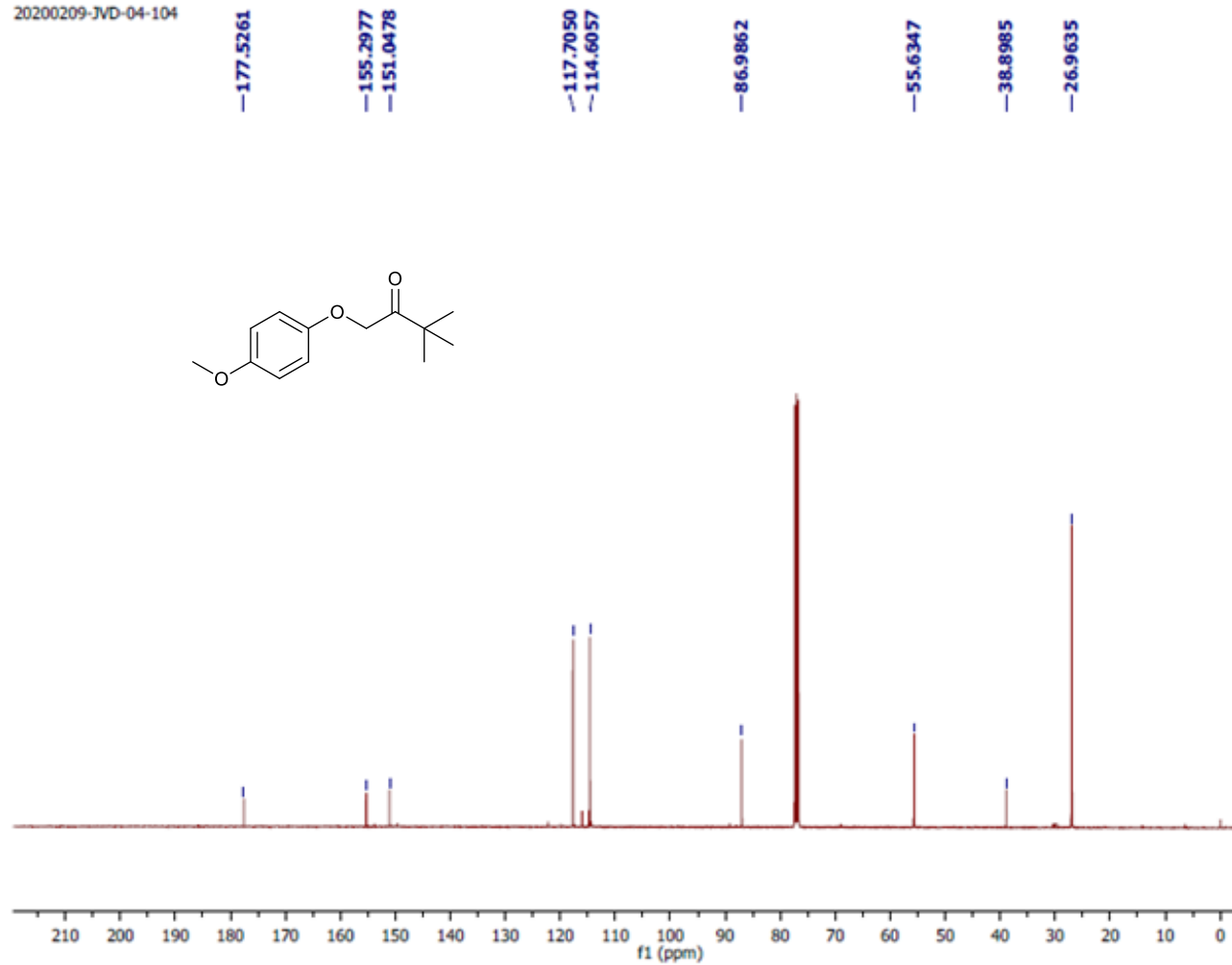


Figure S10. ¹³C NMR spectrum of **6** in CDCl₃.

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