Universidade de Lisboa

Faculdade de Farmácia

Subcellular localisation of APOBEC3A, APOBEC3B and APOBEC3G in human cervical cancer cell line

Maria Vieira da Silva Rafael

Trabalho de Campo orientado pela Professora Doutora Nika Lovsin, Professora Assistente no Departamento de Bioquímica Clínica na Faculdade de Farmácia da Universidade de Ljubljana, e coorientado pela Professora Doutora Graça Soveral, Professora Catedrática da Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

As proteínas APOBEC pertencem a uma família de deaminases de citidina e são membros cruciais na resposta imunitária inata, estando envolvidas, simultaneamente, na origem e progressão de muitas patologias. O mecanismo pelo qual estas atuam é através da sua capacidade de se ligarem e editarem cadeias simples de ADN e ARN. Esta família de deaminases inclui uma deaminase induzida por ativação (AID) e quatro subfamílias- APOBEC1, APOBEC2, APOBEC3 e APOBEC4. O foco deste estudo serão as proteínas da subfamília APOBEC3.

As proteínas APOBEC3 constituem uma parte integrante do nosso sistema imunitário, atuando ao nível de uma variedade de mecanismos subjacentes às respostas imunitárias. No entanto, quando a sua expressão se encontra desregulada pode provocar o aparecimento de mutações indesejáveis, originando instabilidade genómica e, consequentemente, levar ao desenvolvimento da carcinogénese de células humanas e ao aumento da replicação de certos vírus. Por consequência, estas enzimas tornaram-se alvos promissores de estudo para terapêuticas anticancerígenas e antivirais. Neste contexto, é necessária uma maior compreensão destas enzimas de modo a aperfeiçoar a formulação de potenciais estratégias terapêuticas e de forma a obter uma informação mais aprofundada relativa ao mecanismo por detrás da carcinogénese e replicação viral associado a estas enzimas.

A localização destas proteínas a nível celular é um dos primeiros passos a seguir para uma melhor compreensão dos potenciais mecanismos reguladores das diferentes APOBEC3. Assim, o principal objetivo deste estudo é estimar a localização celular de algumas proteínas relevantes neste campo - APOBEC3A, APOBEC3B e APOBEC3G. A literatura atual referente ao papel que a subfamília APOBEC3 desempenha, bem como sua localização a nível celular é escassa. Desta forma, focámo-nos em conceber um protocolo para a sua visualização e co-localização com outras estruturas celulares numa linha de células HeLa. Resultados preliminares indicam que APOBEC3A se localiza tanto no núcleo como no citoplasma, APOBEC3B localiza-se predominantemente no núcleo, sendo que a localização da APOBEC3G não foi estabelecida. Neste trabalho, investigamos e reportamos dados sobre a localização celular das enzimas APOBEC3A, APOBEC3B e APOBEC3G com o intuito de estabelecer uma compreensão mais abrangente da sua biologia bem como dos mecanismos subjacentes às mesmas.

Palavras-chave: APOBEC3A; APOBEC3B; APOBEC3G; Localização celular; Imunofluorescência.

Abstract

Apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) proteins belong to a family of cytidine deaminases that are crucial members of the innate immune response but, simultaneously, can be the cause of various diseases. The particularity of these proteins and the underlying mechanism by which they rule is their common ability to bind and edit single-stranded DNA and RNA. This family of cytidine deaminases comprises an activation-induced deaminase (AID) and four subfamilies - APOBEC1, APOBEC2, APOBEC3, and APOBEC4. The focus of this study is on the proteins of the APOBEC3 subfamily.

APOBEC3 proteins are a part of our innate immune system and underpin a variety of immune defences. Nonetheless, when dysregulated, they can cause undesired RNA modifications and begin to erroneously target the human genome, leading to viral replication and carcinogenesis. Thus, APOBEC3 enzymes are promising antiviral and anticancer targets. A more holistic understanding of these enzymes is needed and will help improve the design of novel therapeutic strategies and lead to a deeper knowledge of the mechanisms underlying carcinogenesis and viral replication in some viruses.

Localising the protein within the cell is one of the first steps in understanding the potential regulatory mechanisms of APOBEC3 functions. Therefore, the main goal of this study is to estimate the cellular localisation of some relevant proteins in this field - APOBEC3A, APOBEC3B, and APOBEC3G.

Works studying the properties of this protein, such as cellular localisation, are scarce. Hence, we focused on establishing a protocol for the visualisation of APOBEC3 enzymes and their co-localisation with other cellular structures in a human cervical cancer cell line (HeLa Cells). The visualisation was successfully accomplished through an immunofluorescence (IF) protocol and subsequent imaging with fluorescence microscopy. In order to capture immunofluorescence images, an immunostaining protocol was carried out and optimised by diluting the concentration of specific antibodies to obtain a proper signal.

Here, we investigate and report data on the subcellular localisation of A3A, A3B, and A3G enzymes, hoping to move towards a more comprehensive understanding of APOBEC3 biology as to the detailed mechanisms of their regulation and their exact functions.

Keywords: APOBEC3A; APOBEC3B; APOBEC3G; Subcellular localisation; Immunofluorescence.

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Last but not least, I would like to thank my family and friends who have always been there for me, and their encouragement has been essential through these years.

List of Abbreviations

AF	Alexa fluor
AID	Activation Induced Deaminase
APOBEC	Apolipoprotein B mRNA Editing Catalytic Polypeptide-like
A3A	APOBEC3A
A3B	APOBEC3B
A3C	APOBEC3C
A3D	APOBEC3D
A3F	APOBEC3F
A3G	APOBEC3G
A3H	APOBEC3H
BSA	Bovine serum albumin
CD	Cytidine deaminase
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr Virus
FBS	Foetal bovine serum
GFP	Green fluorescent protein
HA	Hemagglutinin
HBV	Hepatitis B virus
HIV-1	Human Immunodeficiency virus-1
HPV	Human Papillomavirus
ICC	Immunocytochemistry
IF	Immunofluorescence

KO	Knockout
NLS	Nuclear localisation signal
pAb	Primary antibody
pDNA	Plasmid deoxyribonucleic acid
pA3A	Plasmid APOBEC3A
pA3B	Plasmid APOBEC3B
pA3G	Plasmid APOBEC3G
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RFP	Red fluorescent protein
sAb	Secondary antibody
SBS	Single base substitution
SBS2	Single base substitution 2
SBS13	Single base substitution 13
ssDNA	Single-stranded DNA
SGs	Stress Granules
ssRNA	Single-stranded RNA
Vif	Viral infectivity factor
WT	Wildtype
ZDD	Zinc dependent deaminase

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 APOBEC: Family of Enzymes

APOBEC (Apolipoprotein B mRNA Editing Catalytic Polypeptide-like) is a family of cytosine deaminases that are part of the human immune system and play a variety of vital functions in human health and disease. The specificity in APOBEC proteins is their intrinsic ability to bind and edit, through several mechanisms, both foreign and self RNA and single-stranded (ss)DNA (1,2). This grants them antiviral functions in mammalian cells through lethal editing in the genomes of endogenous and exogenous retroviruses like Hepatitis B virus (HBV), small DNA viruses such as Human Papillomavirus (HPV), large DNA viruses such as the γ-herpesviruses Epstein-Barr Virus (EBV), and potentially those of RNA viruses such as coronaviruses (3–9).

Besides having an active role in inhibiting viral replication, APOBEC family has also recently proved to be a major endogenous source of mutation in various cancers, namely bladder, breast, cervix, head/neck, esophagus, and lung cancers (2,10–12).

The clinical impact of APOBEC-associated mutations appears to be significant, which correlates to the overarching focus and study that is being made in this field. Understanding and undercover the regulatory process of APOBEC expression and characterisation of their functions and interactions with other substrates and macromolecules could be the key to developing and discovering novel therapeutical interventions, mainly in the oncology and antiviral field (13,14).

1.2 APOBEC Structure and Mechanism

The APOBEC family in humans consists of eleven genes: APOBEC1 (A1), APOBEC2 (A2), seven APOBEC3s (A3A, A3B, A3C, A3D, A3F, A3G, A3H), APOBEC4 (A4), and activation-induced deaminase (AID) (2).

All APOBECs share a one or two zinc-dependent deaminase sequence motif (ZDD) within an invariant five-stranded β-sheet (β1–5) and six α-helices (h1–6) that form the core catalytic site of a cytidine deaminase (CD) domain. Each CD core structure contains a highly conserved

spatial arrangement of the catalytic or pseudo-catalytic centre, which consists of an H -E-X$_{23-28}$-P-C-X$_{2-4}$-C motif (being X an amino acid), where the H and two C residues coordinate a Zn atom, and the E residue is essential for polarising a water molecule near the Zn-atom for catalysis (15).

DNA cytosine deamination is the fundamental biochemical activity of the APOBEC family by which they execute diverse biological processes, including adaptive and innate immunity, genetic mutations, and various associated diseases. Nine out of the eleven APOBEC subfamilies can catalytically deaminate cytosine to uracil, to cause C-to-U change on DNA or/and RNA. For this process, it is required a minimum of five contiguous deoxy-nucleotides - 3 nucleotides on the 5' end of the target cytosine and one on its 3'end (16,17). Cytosine to uracil (C-to-U) deamination occurs through a zinc-mediated hydrolytic mechanism, in which a conserved glutamic acid deprotonates water, and the resulting zinc-stabilized hydroxide ion attacks the 4-position of the cytosine nucleobase, yielding an unstable tetrahedral intermediate. A DNA uracil nucleobase is then achieved through the formation of the C4-O olefin and the release of NH3 (1,18).

Despite having a common CD core and a conserved Zn-centre, each APOBEC member multimerise with different styles and mechanisms and consequently presents different functions.
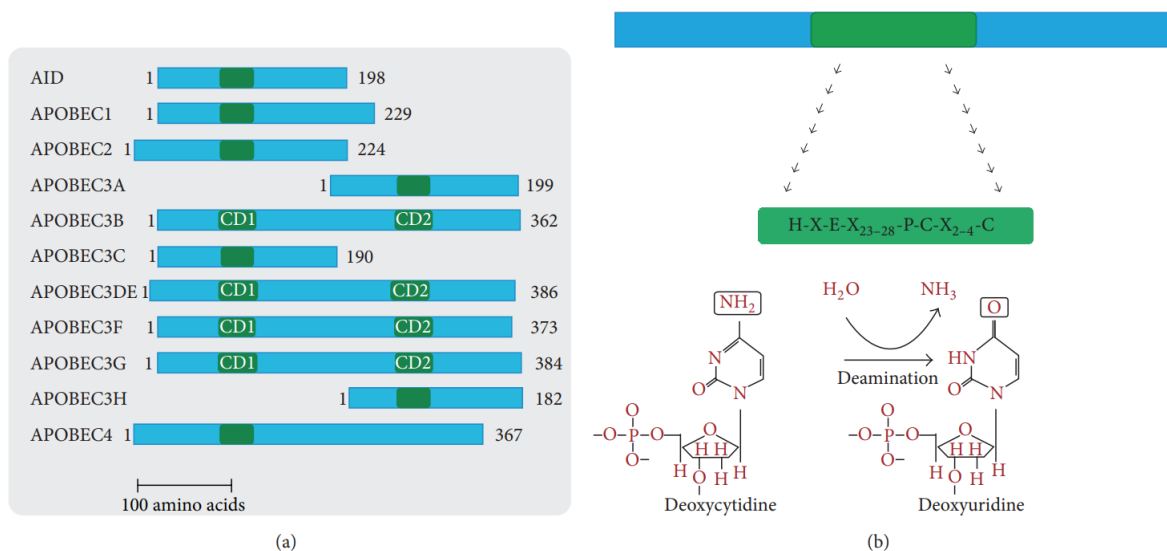
**Figure 1. Structure of human APOBEC proteins.**
(a) Schematic representation of the structure of APOBEC family of proteins. The zinc-binding motifs, represented by the catalytic domain (CD) that can be found in single or double copies, are depicted in green. In the proteins that harbour two CD copies, the N- and C-terminal domains are named CD1 and CD2, respectively. APOBEC proteins are drawn to scale, and each

2

version's total number of amino acids is shown to the right. The length of the scale bar represents 100 amino acids. (b) The active site of the catalytic domain core is enlarged, and its formula is depicted. The hydrolytic deamination reaction mediated by these enzymes is illustrated at the bottom of the figure. Adapted from (19).

1.3 APOBEC3 Subfamily
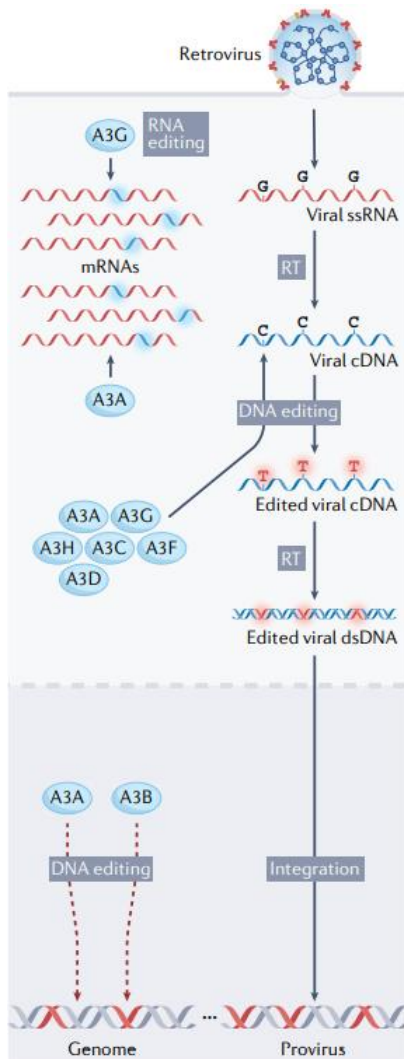
APOBEC3 is located on chromosome 22 in locus 22q13.1 and encodes seven different proteins designated APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3D (A3D), APOBEC3F (A3F), APOBEC3G (A3G), and APOBEC3H (A3H). These proteins are specific to mammals and play an active role in restricting viruses and genomic mobile elements (2,20–22).

The retroviral restriction activity is primarily due to the ability to inflict C-to-U deamination in viral genomes and through deaminase-independent mechanisms. Upon the infection of the cell by a retrovirus, its viral genome gets released as single-stranded RNA (ssRNA); it then undergoes reverse transcription converting it to cDNA. The single-stranded viral cDNA becomes a target for cytosine deamination by APOBEC3 proteins leading to C-to-U base modification. This edited viral genome is then degraded (if heavily edited) or integrated into the genome as a provirus (20,21,23).

Figure 2. APOBEC3 activity in leukocytes.
Once a retrovirus infects a cell, it releases its viral genome as single-stranded RNA (ssRNA), which is retro-transcribed to cDNA. APOBEC3 proteins proceed to deaminate the viral single-stranded DNA (ssDNA) trough cytosine deamination. The edited viral genome can then be integrated into the genome as a provirus. Adapted from (21).

Accumulating evidence also suggests that certain members of APOBEC3 subfamily can also target the host genome creating genomic instability patterns consistent with the development and progression of certain types of cancer. Consequently, APOBEC3 enzymes are tantalising

targets for the development of chemical probes and therapeutic molecules to harness mutational processes in human disease (13,22,24).

Although APOBEC3 proteins appear to have a highly homologous structure and a common evolutionary origin, they are very diverse in their impact on human health and disease (2).

1.3.1   APOBEC3A

APOBEC3A is a myeloid lineage-specific DNA cytosine deaminase localised either in the nucleus or in the cytoplasm of cells (25).

A3A is the most potent human DNA cytosine deaminase playing a massive role in restricting the replication of several transposons and viruses. For instance, A3A has been shown to limit the retrotransposition of L1 and Alu elements (26,27), and it has been reported to inhibit the replication of certain DNA viruses, including HBV (8,28), HPV (9,29), parvoviruses (30,31), as well as herpesviruses (32,33).

Beyond these restriction activities, A3A can catalyse mutation and actively drive carcinogenesis in vivo. The primary mechanism for which A3A rules is the hypermutation of nuclear DNA, creating double-stranded DNA breaks (34,35). Analyses of cancer mutation data sets have proven A3A to be a potent inducer of carcinogenesis, and its genotoxicity is widely reported in ovarian and breast cancer (36,37).

1.3.2   APOBEC3B

APOBEC3B (A3B) is the only human DNA deaminase family member with an apparent steady-state nuclear location (38).

A3A and A3B are 91% identical at the protein level, but their molecular mutagenic mechanisms are different, and therefore their mutational signatures are statistically distinguishable (39–41).

APOBEC3B is up-regulated in over half of all cancers, making it a leading candidate for APOBEC mutagenesis in cancer due to several independent lines of evidence, including overexpression in tumours and cancer cell lines, nuclear localisation, modulation by tumour-promoting viruses, and associations with poor clinical outcomes. In addition, recent data showed present A3B mutational signatures in breast, head/neck, lung, bladder, cervical, and ovarian cancers, and it is also likely to be a more modest but still significant contributor to many other cancers (42–47).

4

In terms of viral infections, its role has been mostly studied in the context of HBV(6,7), and EBV (48,49), and little is known about A3B signature mutations in other viral infections.

1.3.3 APOBEC3G

The first APOBEC3 protein to be discovered was APOBEC3G, and it is the one that shows the highest expression levels out of all A3 family members (2).

Regarding its role in viral infections, A3G is the protein that contributes the most to the inhibition of Human immunodeficiency virus-1 (HIV-1) replication in the absence of the viral infectivity factor (Vif). Thanks to its cytidine deaminase activity, A3G makes it possible to convert cytidine to uridine in ssDNA generated during reverse transcription, resulting in G-to-A substitutions in the (+) strand DNA that eventually leads to blocking viral replication due to the incorporation of missense or stop-codons. HIV-1 overcomes A3G restriction by expressing Vif, which targets A3G for polyubiquitination and proteasomal degradation (23,50–52).

In addition, APOBEC3G also inhibits other viruses, such as HBV (6,7,53,54), human T-lymphotropic virus type 1 (HTLV-1) (55), and Alu retrotransposons (56).

1.4 The importance of Subcellular Localisation

As previously stated, A3 proteins are found in different subcellular locations. Single-domain APOBEC3s such as A3A, A3C, and A3H are small enough (~25kDa) to passively enter and exit the nucleus; consequently, they are found either in the cytoplasm or in the nucleus compartment. In contrast, larger (>50kDa) double-domain APOBEC3s like A3F, A3D, and A3G cannot passively enter the nucleus, so they are restricted to the cytoplasm. Lastly, and because of its N-terminal nuclear localisation signal (NLS), A3B is the only deaminase with a predominant location in the nucleus (19,21,38,57).

Their distinctive locations allow them to protect both compartments through restriction of nuclear (such as the herpes simplex virus and non-LTR retrotransposons) or cytoplasmic (like HBV, retroviruses, and LTR retrotransposons) replicating elements.

Regardless of an AID/APOBEC protein's inherent ability to bind and deaminate ssDNA, ssRNA, or both substrates, its ability to do so in cells will depend on its subcellular localisation and access to the specific substrate. For instance, mRNA, viral RNA, and viral DNA can all be deaminated in either the nucleus or the cytoplasm, whereas nuclear-localized family members can only deaminate the host genome. As a result, analysing APOBEC subcellular localisation

is an essential step toward understanding additional levels of APOBEC regulation and how these enzymes will contribute to the innate immune response, carcinogenesis, and viral infection (21,58).

1.5 APOBEC mutations in Cancer

APOBEC-associated mutational signatures are among the most widespread in cancer, making this family of enzymes a prominent putative source of mutations in cancer. This correlative fact is corroborated by extensive data showing APOBEC-associated mutational signatures expressed in more than 70% of cancer types and around 50% of all cancer genomes (59,60).

Due to its significant clinical impact, the study of APOBEC mutagenesis in cancer aroused the scientific field with the hope of developing strategies to leverage this knowledge for prognostic and diagnostic tests and developing novel therapeutics in the oncology field.

1.5.1 Signature 2 and Signature 13

Mutagenic processes in cancer leave DNA footprints called mutation signatures. Mutation signatures have been crucially important in revealing a variety of endogenous and exogenous mutational processes in cancer and enabling elucidation of the biological origins and natural history of numerous tumour types.

Two distinct signatures of genome-dispersed mutations associated with APOBEC deamination were discovered first in breast cancer in 2012 (61,62) and later across multiple cancer types (63). These mutational signatures are designated Signature 2 and Signature 13 and are highly relevant in cancer mutagenesis, given that they show to be present in over half of all human tumours.

Signature 2 is characterised by C>T transitions, whilst signature 13 is characterised by C>G and C>A transversions, both at TpCpN sites (thymine preceding the mutated cytosine). These SBS (single base pair substitution) signatures derive from the activity of the A3A and A3B deaminases and are almost always present together in individual cancers, albeit in varying proportions (59,60,64,65).
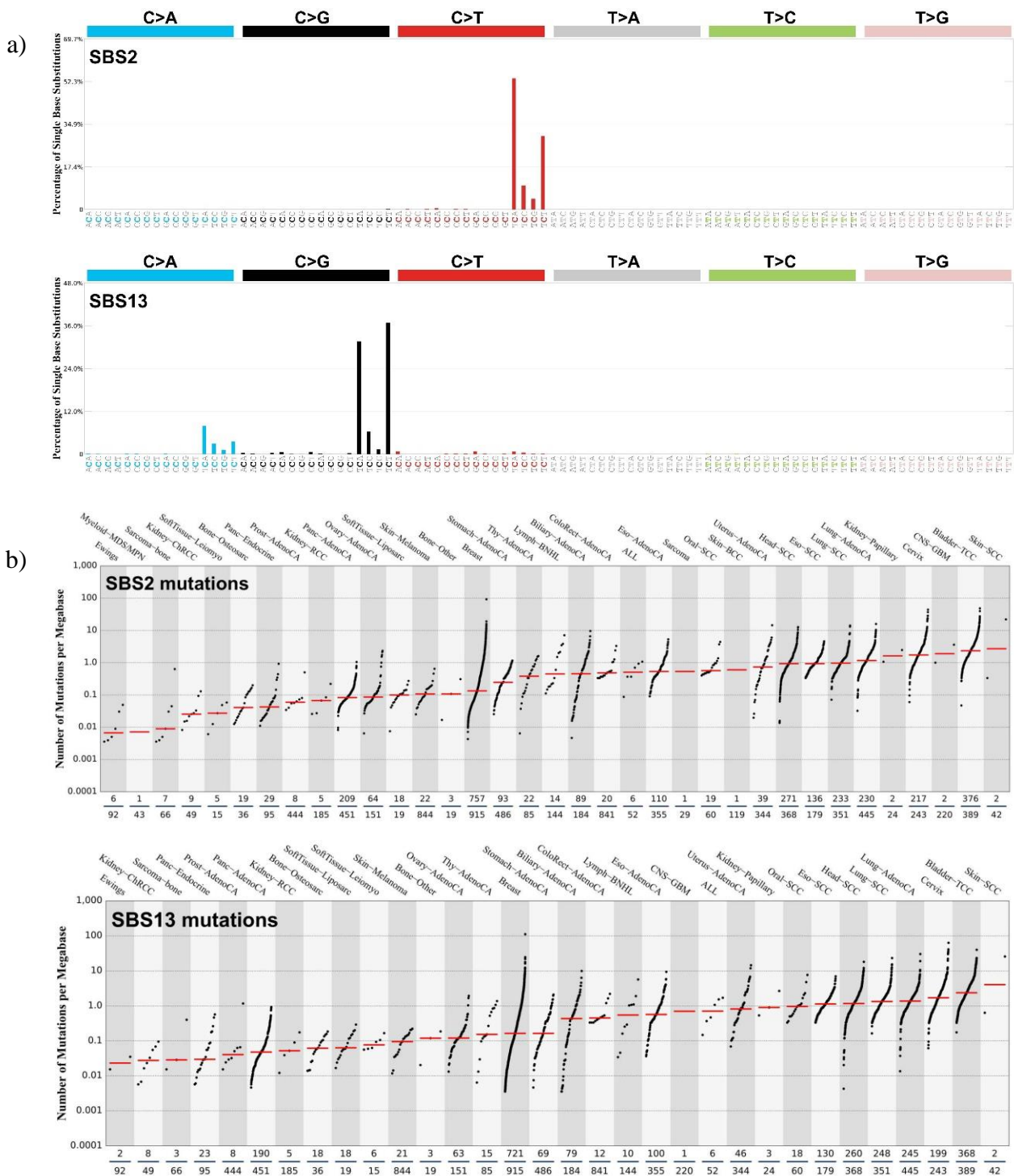
Figure 3. APOBEC-associated genome wide signatures SBS2 and SBS13.
a) Each signature is displayed on horizontal axes according to the alphabetical 96-substitution classification, defined by the six colour-coded substitution types (C>A, C>G, C>T, T>A, T>C, and T>G) and sequence context immediately 5' and 3' to each mutated base. Vertical axes indicate the percentage of mutations attributed to specific mutation types and are adjusted to the actual frequencies of each trinucleotide in the reference human genome version GRCh37. b) Numbers of mutations per megabase attributed to the mutational signature across the cancer types in which the signature was found. Each dot represents a single sample, and only samples where the signature is found are depicted. Images acquired from (66).

Given the high prevalence of SBS2 and SBS13 in human cancer, the nature of the biological factors that instigate APOBEC mutagenesis is an important issue that warrants further investigation to better understand cancer aetiology and potential implications for prevention and treatment.

1.6 Future Perspectives

Our knowledge of the APOBEC family of proteins has been growing at a fast rate over the last decade, and today the role and importance of these proteins in cancer mutagenesis and viral infections are all but certain. We are just now beginning to appreciate the extent to which APOBEC family has in the mutational processes and clinical and public health applications.

Since the APOBEC3 subfamily was identified as a potential antiviral and anticancer target (18), the field has been primed for the discovery of novel inhibitors, and numerous assays are being conducted to that end.

Nevertheless, the study of APOBEC signatures brings some challenges. Cellular inhibition of A3 enzymatic activity has no obvious or immediate phenotype, so A3 enzymes are not traditional anticancer drug targets. This makes assessing the biological role of A3 enzymes and the effect of A3 inhibitors in vivo difficult. These issues are less pronounced when assessing A3 inhibition in viruses because viral genomes acquire A3-catalyzed mutations at a faster rate, allowing for real-time in vivo assessments of co-treatment.

Despite the challenges that come within and although research in this field is still in early stages, APOBEC enzymes have the potential to become a revolutionary new strategy to maximise clinical success in the management of both retroviral infections and cancer.

2 Aim of the work

The present study's ultimate goal is to detect and analyse the subcellular localisation of members of the APOBEC3 family of proteins (namely APOBEC3A, APOBEC3B, and APOBEC3G) in order to better understand their unique functional characteristics as well as their impact on the biology of cells.

We aimed to establish and optimise a protocol for overexpression of APOBEC3 proteins and further transfection into HeLa Cells, along with its subcellular localisation using immunofluorescence methods.

In this essay, we will test the following hypothesis:

1. APOBEC3A is located in the nucleus and cytoplasm, as it is probably the main place of action of the protein.

2. APOBEC3B is primarily located in the nucleus as it is probably the main place of action of the protein.

3.APOBEC3G is primarily located in the cytoplasm as it is probably the main place of action of the protein.

3 Materials and Methods

3.1 Chemicals and Reagents

Chemicals:

- Dulbeccos'modified medium (DMEM) (L0064-500, Biowest, Nuaillé - France)
- 10% Foetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, Massachusetts, USA)
- 4% Paraformaldehyde (PFA) (Sigma Aldrich, MO, USA)
- 0,1% Triton X-100 (9036-19-5, Sigma Aldrich, MO, USA)
- Trypsin (L0931-100, Biowest, Nuaillé - France)
- Invitrogen ProLong Gold Antifade Mountant with DAPI (P36941, ThermoFisher Scientific)
- PolyJet^{TM} In Vitro DNA Transfection (SignaGen® Laboratories)
- 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)
- 3% Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA)
- 0,2% Tween (Sigma-Aldrich, Overijse, Belgium)
- Antibiotic/antimycotic (L0010-020, Biowest, Nuaillé - France)
- Phosphate-buffered saline (PBS) (Sigma Aldrich, MO, USA)
- DAPI (ThermoFischer Scientific, MA, USA)

Plasmids:

- pGFP
- pA3A
- pA3G
- pA3B

Antibodies:

- Anti-APOBEC3B antibody produced in rabbit (HPA 066719, Sigma-Aldrich)
- Anti-APOBEC3A antibody produced in rabbit (HPA 043237, Sigma-Aldrich)
- Anti-HA antibody, mouse monoclonal (H3663, Sigma-Aldrich)
- Anti-rabbit IgG Alexa Fluor 647 (A27040 ThermoFisher Scientific)
- Anti-mouse IgG Alexa Fluor 555 (A31570, Invitrogen Molecular Probes)
- Anti-cMyc produced in goat (NB600-335, Novus Biologicals)
- Anti-rabbit IgG Alexa® Fluor 488 (A11008, Invitrogen Molecular Probes)

Cell Lines:

- HeLa Cells

3.2 HeLa Cells

For this experiment, HeLa Cells were used. HeLa Cells are cervical cancer cells isolated from the American cancer patient Henrieta Lacks, from whom they got their name. HeLa Cells were the first immortal human cell line to be isolated and are a remarkably durable and prolific line of cells that have supported most medical research fields throughout the years (67). The cell line grows adherently as a monolayer. All procedures were performed in an aseptic environment.

3.2.1 Cell thawing

It is critical to thaw cells correctly to maintain the culture's viability and allow it to recover faster. As a result, the thawing of the HeLa cell culture was initiated first.

Cell lines vials were taken out of liquid nitrogen and put in a heating bath with aluminium beads heated at 37°C for one minute. Immediate resuspension of the cells in Dulbecco's modified eagle medium (DMEM) with 10% foetal bovine serum (FBS) is crucial to ensure viability. The cells were frozen in 10% dimethyl sulfoxide (DMSO) in DMEM, which is toxic above 4°C. Therefore, cultures must be thawed quickly and diluted with DMEM to minimize the toxic effects. Thawed cells in DMEM were transferred into a 10 cm culture dish, and medium was spread onto them before being kept in a cell incubator at 37°C, 5% CO_2 overnight.

The following day cells were inspected under a light microscope to check growth progress, viability, and confluency. The medium was replaced with fresh low glucose DMEM with 10% FBS, 1% glutamine, and 1% antimycotic/antibiotic.

3.2.2 Cell seeding

Firstly, the medium was aspirated from the culture dish, and cells were washed with PBS. To detach the adherent cells from the surface, 2mL of 0,25% Trypsin was used, and the dish was put in the incubator for four minutes at 37°C, with 5% CO_2. Then, 5mL of DMEM was pipetted to the dish several times in a movement up and down to ensure the deactivation of Trypsin and resuspension of the cells.

A 10 µl sample was pipetted into a Neubauer counting chamber to count the cells. Then, the appropriate volume of cells and medium needed to reach the quantity of 1.10^5 cells/well was calculated. Meanwhile, the 12-well plates were prepared by inserting a glass coverslip in each

well. Finally, once the dilution of cells is done, 1ml is pipetted into each well, and plates are left in the 37°C, 5% CO2 incubator overnight.

3.2.3 Transfection

Transfection is a molecular biology method of introducing nucleic acid into eukaryotic cells. The protocol used in this study was the PolyJet™ *In Vitro* DNA Transfection Reagent, which ensures an effective and reproducible transfection.

PolyJet™ is a cationic lipid with the unique feature of degrading faster than other polymers after transfection and complete endocytosis, making it much less cytotoxic. For that reason, PolyJet has been used by various research groups due to its expected improvements in transfection over other conventional reagents. In this case, according to the manufacturer (SignaGen® Laboratories), there is an 88% efficiency in transfection with HeLa cells using PolyJet [68].

Plasmid vectors containing A3A (pA3A), A3B (pA3B), and A3G (pA3G) used in these experiments were previously prepared by the Department of Clinical Biochemistry in the Faculty of Pharmacy, University of Ljubljana.

For transfection, HeLa cells attached to a glass coverslip inserted in the 12-well plate were used. According to the manufacturer's protocol, an optimal ratio of 3:1 PolyJet™ (µL): DNA (µg) was used, so 500ng of plasmid was added to 50 µL of serum-free DMEM for each well.

In parallel, tubes were prepared accordingly to the protein of interest that would be studied later. In one tube, we dilute PolyJet™ reagent into serum-free DMEM with High Glucose and proceed to vortex gently. In the other tubes, DNA of interest was diluted into serum-free DMEM with High Glucose and vortexed gently. The diluted PolyJet™ reagent was immediately added to the diluted DNA solutions all at once, always in this specific order. The tubes were incubated at room temperature for about 15 minutes (never longer than 20 minutes) to allow PolyJet™/DNA complexes to form.

Finally, 100µL of PolyJet™/DNA mixture was added to the medium in each well, and the 12-well plate was put in the 37°C, 5% CO2 incubator for 24 hours.

Several transfections were performed using this protocol, and plasmids such as pA3A, pA3B, and pA3G.

The vector that encoded for the Green Fluorescence Protein (GFP), originally derived from the Jellyfish *Aequorea victoria*, was also transfected since it is a crucial tool in biological imaging due to its capacity to report conditions in a cellular environment (69).

3.3 Immunostaining

Immunostaining techniques consist of using specific antibodies to detect a single target protein. This method involves exposure of fixed cells or tissues to primary antibodies directed against one or more proteins of interest. A secondary antibody conjugated with either a fluorescent or enzymatic tag is used to bind to the invariant portion of the primary antibody. The presence of the marker can thereby be visualised and/or quantified through the use of a fluorescence microscope or by the addition of a coloured substrate. These methodologies are used to study protein localisation, co-localisation, expression, and distribution at different cellular levels.

For the immunostaining of the studied proteins, the following protocol was employed. Firstly, the growth medium was removed from the transfection of the previous day, and cells were washed with PBS once for five minutes. Secondly, we proceeded to fixate the cells with 500 µl 4% paraformaldehyde (PFA) in PBS solution and let it incubate for 15 minutes at room temperature. Cells were washed three times with PBS for five minutes each time, and we began with the permeabilisation of the cells using 200µL of 3% BSA and 0,2% Tween in PBS and incubated for 5 minutes at room temperature. We continued by rewashing the cells with PBS two times for 5 minutes. To prevent unspecific binding of antibodies to non-target cell structures, 200µL of 3% BSA was added to each well and incubated for 30 minutes at room temperature. The cells were washed with PBS twice for 5 minutes. The next step was de addition of primary antibodies in a concentration of 1(pAb): 200 (3% BSA in PBS). After, plates were sealed with parafilm and left to incubate at 4°C overnight.

The following day, cells were washed with PBS twice for 5 minutes, and the addition of secondary antibodies was performed. The optimal ratio was 1(sAb): 1000 (3% BSA). The secondary antibodies are fluorescently labelled and thus light sensitive. For that reason, the sample was kept in dim light throughout the entire process as well as in the following steps.

For the first round of experiments, this protocol was used for staining the proteins of interest, but moreover, for the second and third rounds of experiments, we used a different yet similar protocol.

The second protocol is immunocytochemistry (ICC) – immunofluorescence (IF) by Sigma-Aldrich. According to the manufacturer's protocol, the growth medium from the transfection of the previous day was first removed, and the cells were washed with PBS. Then, the cells were fixed in ice-cold 4% PFA supplemented with 10% foetal bovine serum (FBS). Permeabilization was performed by adding 0,1% Triton X-100 in PBS 3 times for 5 minutes each. The next step was a short washing with PBS, proceeding with the addition of primary antibodies in a concentration of 1:200 in PBS supplemented with 4% FBS. After, the plates were sealed with parafilm and aluminium foil and left to incubate at 4ºC overnight. The following day, four PBS washings of seven minutes each were performed, and later the cells were incubated for 1.5 hours at room temperature with secondary antibodies in PBS supplemented with 4% FBS with a concentration of 1:500.

Table 1. List of Primary antibodies (pAb) and Secondary Antibodies (sAb) used according to the protein of interest.

Investigated Protein	Primary Antibody (1:200)	Secondary Antibody (1:500)
APOBEC3A	Anti-APOBEC3A antibody produced in rabbit (HPA 043237, Sigma-Aldrich)	Anti-rabbit IgG Alexa® Fluor 488 (A11008, Invitrogen Molecular Probes)
APOBEC3B	Anti-APOBEC3B antibody produced in rabbit (HPA 066719, Sigma-Aldrich)	
	Anti-HA antibody, mouse monoclonal (H3663, Sigma-Aldrich)	Anti-mouse IgG Alexa® Fluor 555 (A31570, Invitrogen Molecular Probes)
APOBEC3G	Anti-cMyc produced in goat (NB600-335, Novus Biologicals)	Anti-rabbit IgG Alexa® Fluor 647 (A27040 Thermo Fisher)

3.3.1 Immunofluorescence microscopy

After immunostaining, we prepared the cells for visualisation on the microscope.

Firstly, cells were washed with PBS 4 times for 7 minutes each. Next, the coverslips were prepared, and on each, a drop of ProLong^{TM} Gold Antifade Mounting reagent with DAPI was added in order to fix the samples and prevent future dehydration. The coverslips were taken out of the wells and carefully placed on the drop of the mounting reagent in a way that the side with cells was facing the drop of the mounting reagent. The coverslips were slightly pressed to ensure no air bubbles were underneath them. The microscope glasses were left overnight in a dark box in the fridge for the mountant to dry properly.

On the next day, the coverslips containing the samples were ready to be visualised through immunofluorescence microscopy. The images were acquired using the EVOS® FL Cell Imaging System from Thermo Fisher Scientific. It allows magnification from 4x to 40x and displays three fluorescent channels- DAPI, GFP, and RFP. The visualization of the nuclei is provided by the DAPI channel (blue), while GFP (green) and RFP (red) show the fluorescence corresponding to the bound proteins of interest.

In the end, coverslips were sealed with collarless nail polish to prevent further drying and to preserve the samples for future research.

4 Results

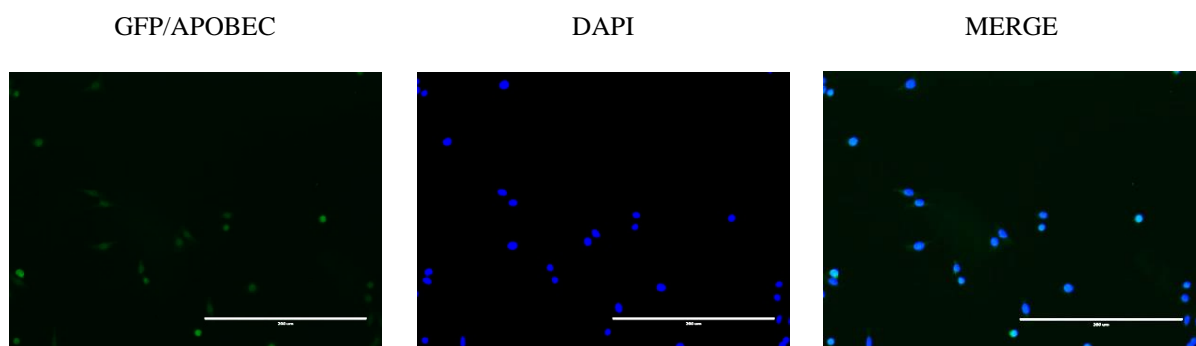## 4.1 Immunofluorescent Microscopy Results

As previously stated, A3 proteins localise differently in the cell. A3B is concentrated only in the nucleus due to an active imported mechanism, whereas A3G is found only in the cytoplasm, and A3A has been identified in both the nucleus and cytoplasm of transiently transfected cells. To analyse the subcellular localisation of A3 proteins, immunofluorescence microscopy was performed using HeLa cells transfected with pA3A, pA3B, and pA3G, as described in 2.1.3.

4.1.1 Subcellular localisation of APOBEC3A in HeLa Cells

Cotransfection of pGFP and pA3A was performed to evaluate the specificity and effectiveness of the signal transmitted by the tagged A3A protein.

HeLa cells transfected with the plasmid were fixed and probed with rabbit anti-A3A antibodies. The cells were then stained with a rabbit-specific secondary antibody directly conjugated to Alexa® Fluor 488 dye. Cell nuclei were stained with DAPI, and the samples were examined by confocal microscopy.

Immunofluorescence images obtained revealed a strong and clear signal from the nucleus compartment as well as from the A3A protein. Signal co-localisation was confirmed in the merged image.

Figure 4. Subcellular localisation of APOBEC3A in HeLa Cells.
Representative immunofluorescent microscopy images of GFP (green) as a control for nuclear permeabilisation and staining procedures. Nuclear DNA is stained blue with DAPI for compartment identification. Cells were imaged at 20x magnification; the length of the white bar is 200 µm.
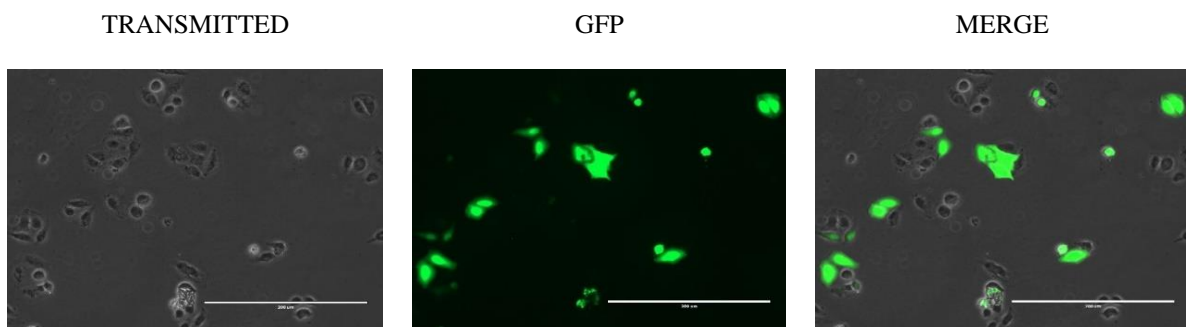
4.1.2 Subcellular localisation of APOBEC3A and APOBEC3B

The efficiency of the transfection was checked by the green fluorescence signal of the GFP protein successfully transfected to the cells (Figure 5).

In two wells of the 12-well plate, we studied A3A protein localisation by transfecting the plasmid A3A to only one of the two wells. This way, we could compare the signal of the A3A protein in transfected cells and non-transfected cells. Stainings of both wells were performed with the anti-APOBEC3A antibody produced in rabbit as the primary antibody and respective secondary antibody, Anti-rabbit IgG Alexa® Fluor 488. Cells without the transfected plasmid showed an heterogenous population where only some cells exhibited signal from endogenous A3A protein. Nevertheless, cells with the transfected plasmid exhibited a much stronger signal in comparison to the non-transfected ones, validating our immunostaining procedure (Figure 6, A and B).

APOBEC3B was also subjected to investigation. Endogenous A3B was detected by staining it with anti-APOBEC3B antibody produced in rabbit in concentration 1:200 and later stained with Anti-rabbit IgG Alexa® Fluor 488 as the secondary antibody in concentration 1:500. The results showed a strong signal mainly from the nuclear region, with a small portion of the protein located in the cytoplasm (Figure 6, C). Signal co-localisation was confirmed in the merged image.

The specificity of secondary antibody (sAb) was investigated by staining cells only with sAb in concentration 1:500, without previous incubation with pAb. Only a weak signal was observed (Figure 6, D).

TRANSMITTED	GFP	MERGE

Figure 5. Transfection efficiency of HeLa cells assessed by transfection of GFP.
The transfection efficiency was checked by the green fluorescence of the GFP protein. Cells were imaged at 20x magnification; the length of the white bar is 200 µm.
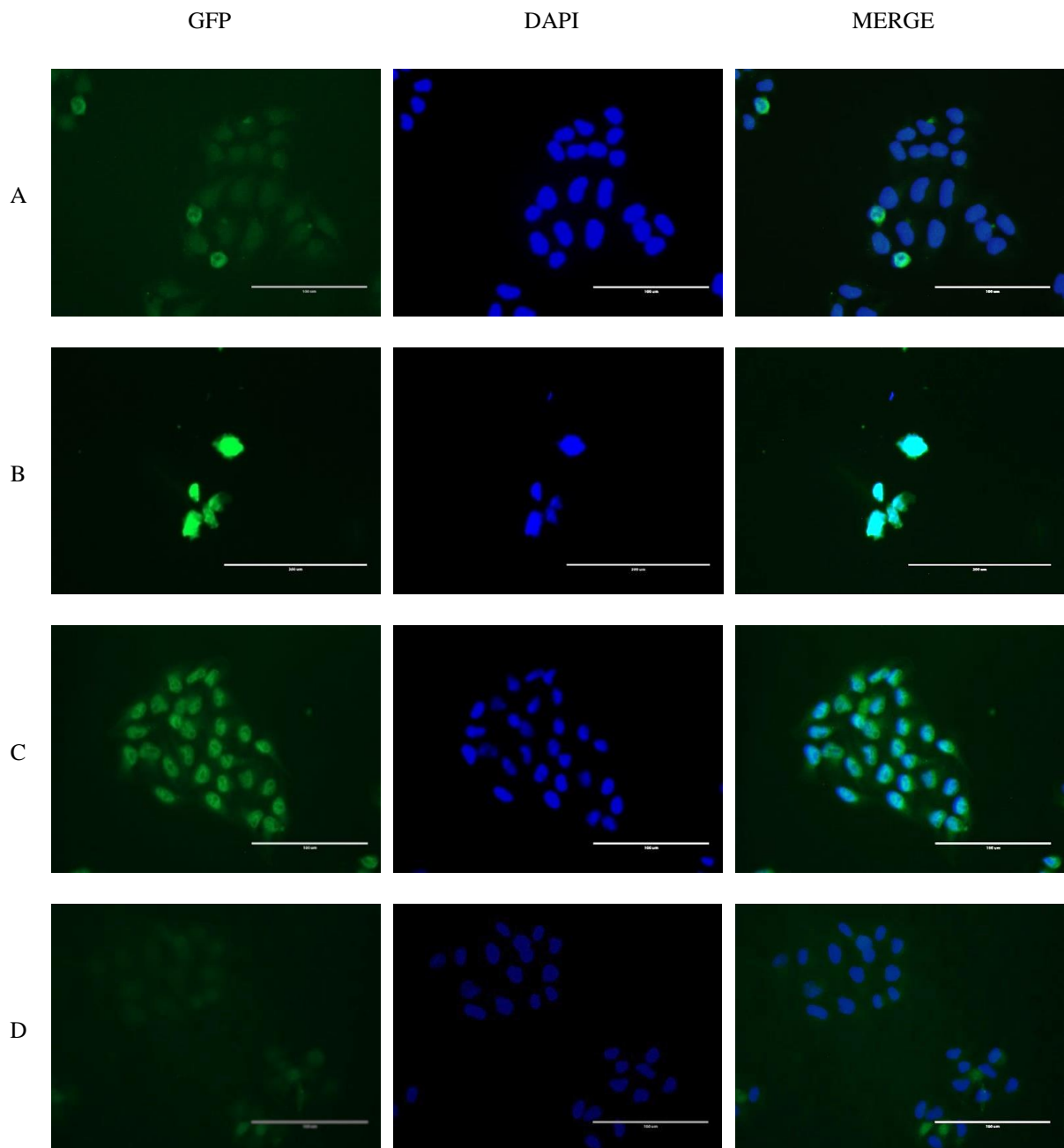
Figure 6. Subcellular localisation of APOBEC3A and APOBEC3B.
A) representative immunofluorescent microscopy images of endogenous A3A in non-transfected HeLa Cells. B) representative immunofluorescent microscopy images of A3A in transfected HeLa Cells. C) representative immunofluorescent microscopy images of endogenous A3B in non-transfected HeLa Cells. D) representative immunofluorescent microscopy images of HeLa Cells stained with just the secondary antibody (Anti-rabbit IgG Alexa Fluor 488). Cells were imaged at 40x magnification; the length of the white bar is 100 µm.

4.1.3 Subcellular localisation of APOBEC3A and APOBEC3G proteins in HeLa Cells exposed to normal and stress conditions
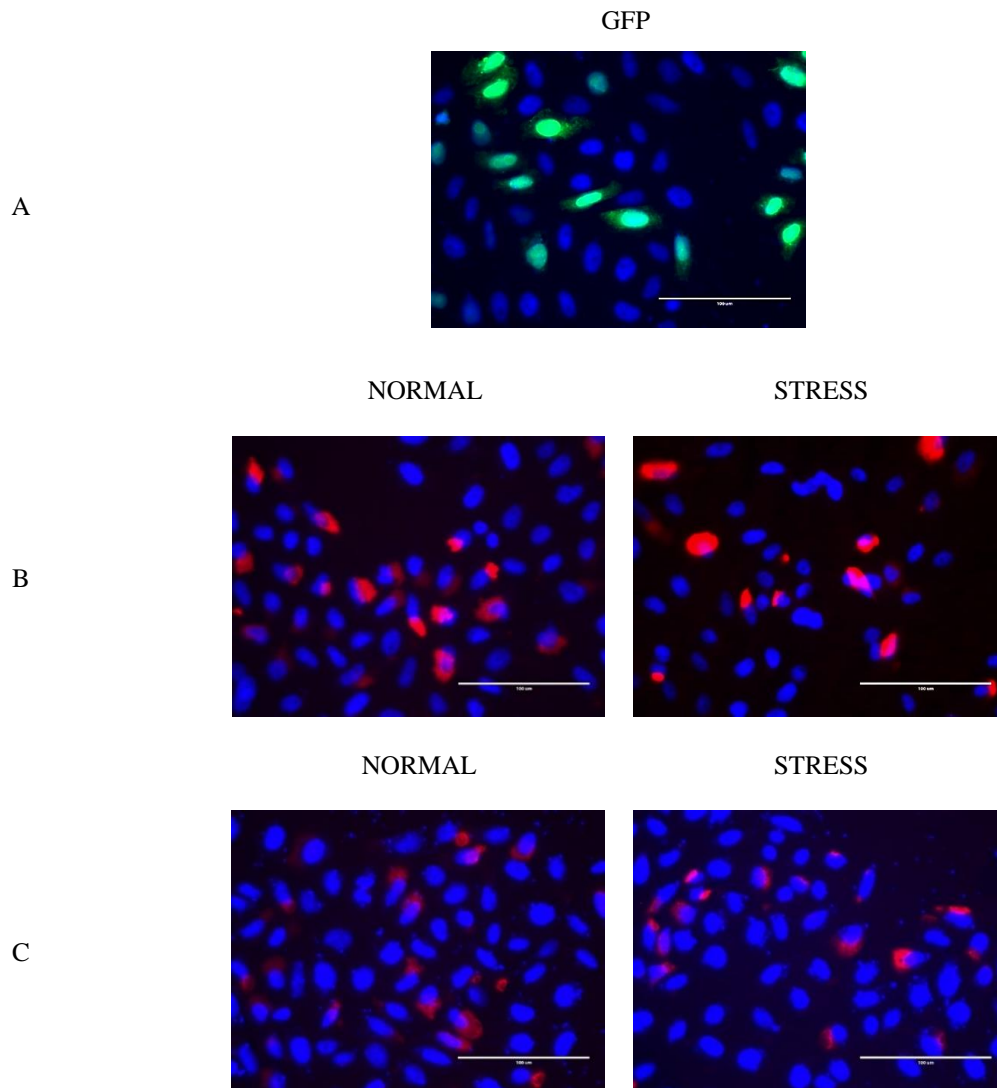
Immunofluorescence of APOBEC3A and APOBEC3G was tested in both stress and normal conditions to see if it had any influence in the subcellular localisation of the proteins. To address this, we used the same double-label microscopy strategy described above, except that the transfected HeLa cells were subjected to heat shock at 45°C for 30 min prior to fixation and examination to simulate stress conditions.

Firstly, the transfection efficiency of both plasmids (pA3A and pA3B) was checked by the green fluorescence of the GFP protein successfully incorporated into the host genome. No antibodies were added to this control preparation since its purpose was to observe basal fluorescence (Figure 7, A).

HeLa Cells transfected with A3A were incubated with the Anti-APOBEC3A antibody produced in rabbit and later with conjugated Anti-rabbit IgG Alexa® Fluor 647 as the secondary antibody. The signal was detected in both the cytosol and nucleus compartment (Figure 7, B).

APOBEC3G protein was also transfected and stained with anti-cMyc produced in goat, followed by the secondary antibody Anti-rabbit IgG Alexa® Fluor 647. The signal obtained was almost exclusively to the cytoplasm (Figure 7, C).

The two stainings offered similar results in both conditions showing a prominent red fluorescence signal of the proteins on the RFP channel and a solid blue nuclear fluorescence signal of the cells on the DAPI channel. Signal co-localisation was confirmed in the merged images. Overexpression of both proteins in the cells was visualised.

GFP

A

NORMAL   STRESS

B

NORMAL   STRESS

C

Figure 7. Subcellular localisation of APOBEC3A and APOBEC3G proteins in HeLa Cells exposed to normal and stress conditions.
A) representative immunofluorescent microscopy images of GFP (green) as a control for transfection, nuclear permeabilization and staining procedures. B) representative immunofluorescent microscopy images of endogenous A3A in HeLa Cells in both normal and stress conditions. Merged images of DAPI (blue) and RFP (red) channel. C) representative immunofluorescent microscopy images of endogenous A3G in HeLa Cells in both normal and stress conditions. Merged images of DAPI (blue) and RFP (red) channel. Cells were imaged at 40x magnification; the length of the white bar is 100 µm.
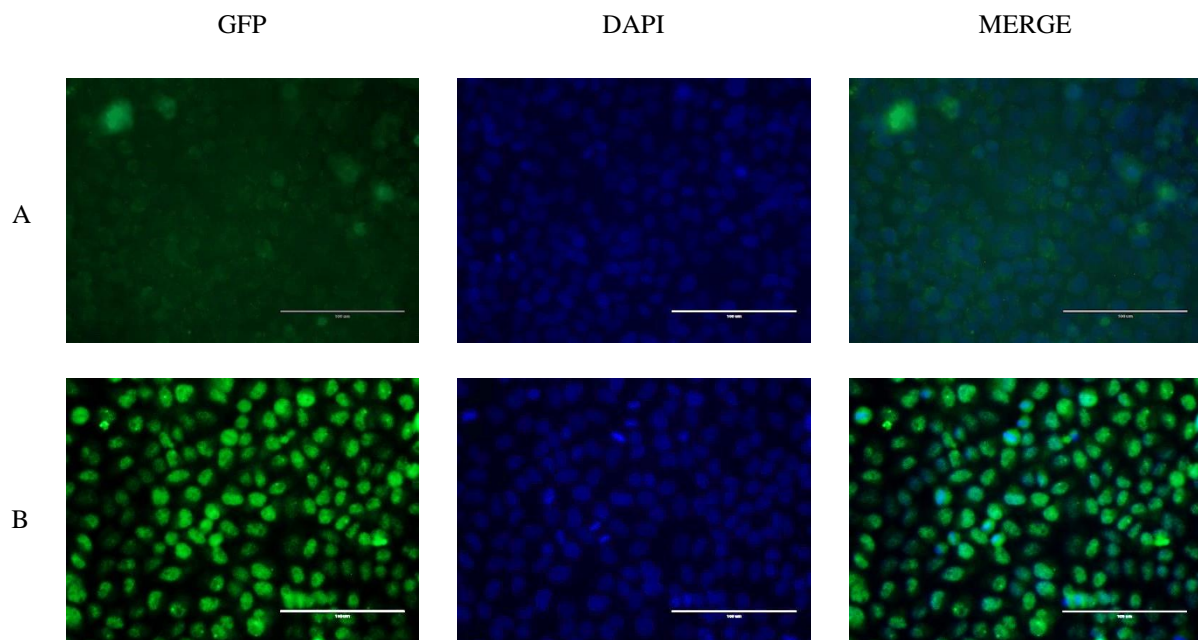
4.1.4 Subcellular localisation of APOBEC3B in transfected and non-transfected HeLa Cells

In this round of experiments, APOBEC3B was studied in transfected and non-transfected cells to evaluate the specificity of the signal transmitted by the A3B protein.

Two preparations, one submitted to transfection with pA3B and the other one using non-transfected cells, were stained with anti-APOBEC3B antibody produced in rabbit as the primary antibody and anti-rabbit IgG Alexa® Fluor 488 as the secondary antibody.

The resulting images revealed a poor signal from the non-transfected with just a slight signal representative of endogenous A3B (Figure 8, A). Transfected cells showed overexpression of A3B protein as well as endogenous A3B (Figure 8, B). The majority of the A3B signal in both preparations was obtained in the nucleus compartment.

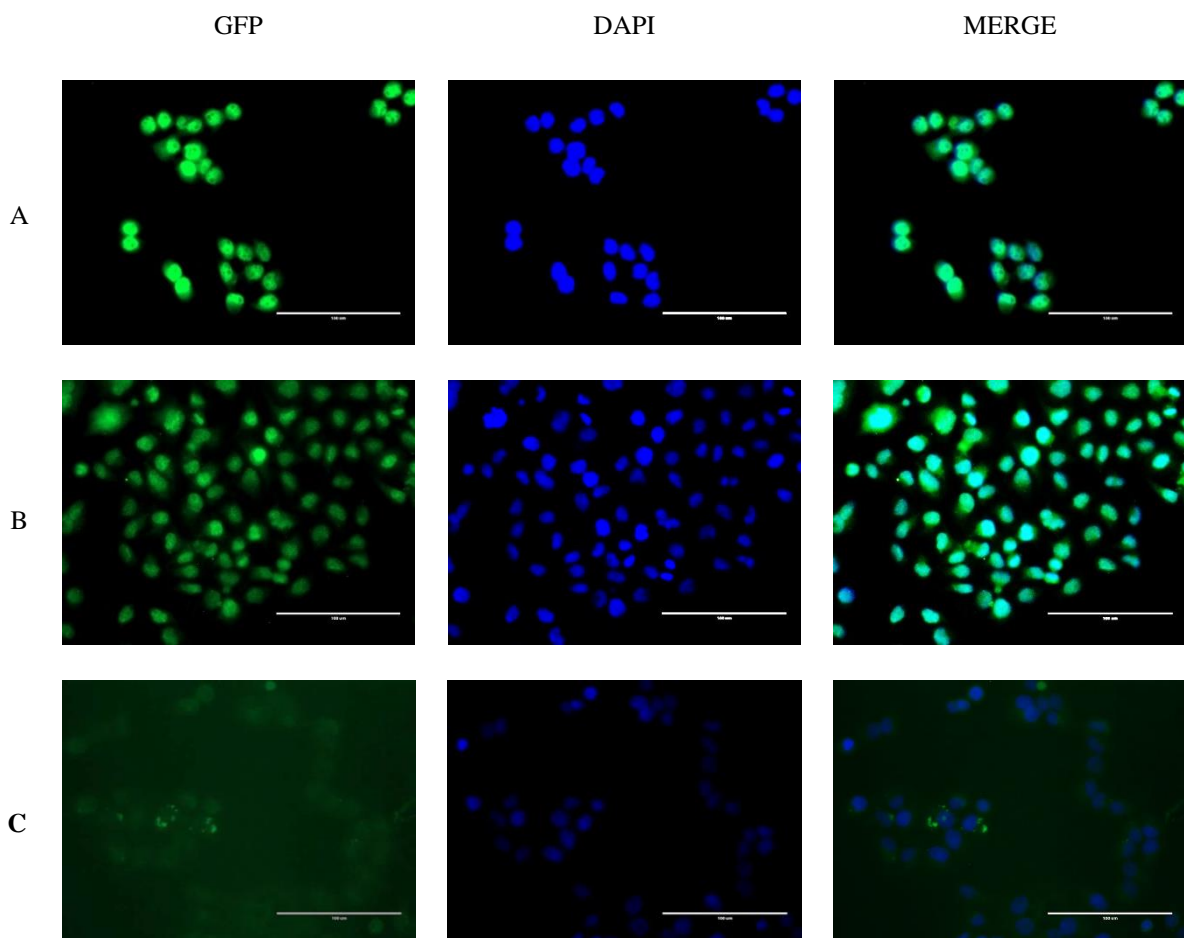**Figure 8. Subcellular localisation of APOBEC3B in transfected and non-transfected HeLa Cells.**
A) representative immunofluorescent microscopy images of non-transfected HeLa Cells. B) representative immunofluorescent microscopy images of HeLa Cells transfected with pA3B. Cells were imaged at 40x magnification; the length of the white bar is 100 µm.

4.1.5 Subcellular localisation of APOBEC3B in wildtype and knockout HeLa Cells

In this round of experiments, A3B knockout (KO) and wildtype (WT) HeLa cells were transfected and subjected to IF microscopy. A3B KO cell culture was prepared by the Department of Clinical Biochemistry in the Faculty of Pharmacy, University of Ljubljana.

A3B-depleted and control cells were stained with Anti-APOBEC3B antibody produced in rabbit as the primary antibody and Anti-rabbit IgG Alexa® Fluor 488 as the secondary antibody. Similar results were obtained in knockout and wildtype cells showing a strong nuclear signal in both preparations (Figure 9, A and B). Signal co-localisation was confirmed in the merged images.

To ascertain specificity, the last round of transfected cells was stained with only the secondary antibody (sAb) in concentration 1:1000, without first incubating with pAb. The immunofluorescence signal in this preparation showed to be weaker (Figure 9, C).

Figure 9. Subcellular localisation of APOBEC3B in wildtype and knockout HeLa Cells.

A) representative immunofluorescent microscopy images of A3B knockout cell lines stained with Anti-APOBEC3B antibody produced in rabbit. B) representative immunofluorescent microscopy images of A3B wildtype cell lines stained with Anti-APOBEC3B antibody produced in rabbit. C) representative immunofluorescent microscopy images of HeLa Cells stained with just secondary antibody. Cells were imaged at 40x magnification; the length of the white bar is 100 µm.

In order to further investigate the subcellular localisation of the A3B protein, we decided to select a different set of antibodies for the immunostaining procedure in this final round of experiments. Moreover, we opted to visualise the cells in a different microscopic channel.

The hemagglutinin (HA) tag is derived from the human influenza virus HA protein and is considered a successful marker for immunostaining procedures. Therefore, the anti-HA monoclonal mouse antibody was used as the primary antibody and conjugated with anti-mouse IgG Alexa® Fluor 555 as the secondary antibody. The preparation was subjected to confocal microscopy and the cultures were visualised in the RFP channel as well as in the DAPI channel.

The resulting images showed a strong red signal corresponding to the expressed A3B protein and a robust blue signal representant of the nucleus compartment of the transfected cells (Figure 10).

Signal co-localisation was confirmed in the merged image with stainings overlayed from the nucleus compartment and a slight staining from the cytoplasm.
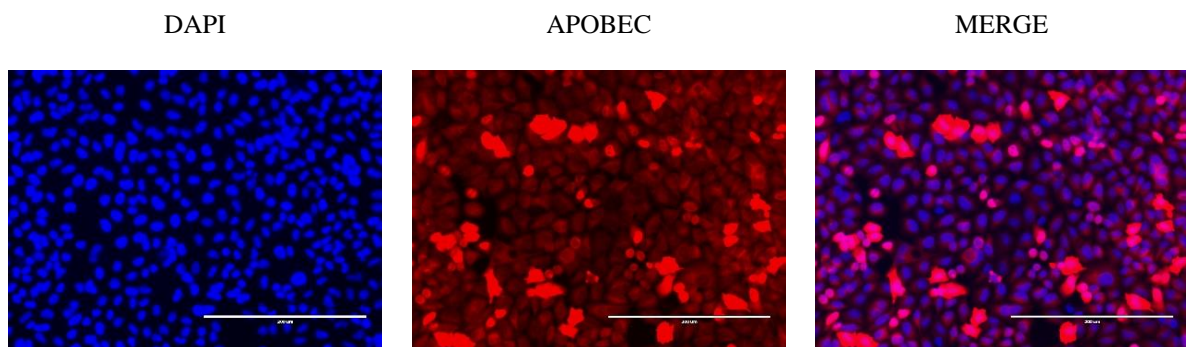
Figure 10. Subcellular localisation of APOBEC3B in transfected cells stained with ani-HA.
HeLa Cells transfected with pA3B were stained with anti-HA primary antibody. Nuclear DNA is stained blue with DAPI for compartment identification. Protein localisation is shown through red fluorescence of RFP channel. Cells were imaged at 20x magnification; the length of the white bar is 200 µm.

5 Discussion

APOBEC proteins have become an emerging research topic since they have been identified in many diseases and biological processes. This family of cytidine deaminases is comprised of an activation-induced deaminase (AID) and four subfamilies - APOBEC1, APOBEC2, APOBEC3, and APOBEC4. The focus of this study will be on the proteins of APOBEC3 subfamily (2).

APOBEC3 proteins are a part of our innate immune system and underpin a variety of immune defences (2). However, dysregulation of their functions and/or expression can cause undesired genomic mutations and RNA modification, leading to viral replication and carcinogenesis. As so, APOBEC3 enzymes are tempting targets for the development of chemical probes and therapeutic molecules to harness mutational processes in human disease. Therefore, a more holistic understanding will help improve the design of therapeutically relevant programmable base editors and lead to a more profound knowledge of the mechanisms behind carcinogenesis and viral replication of some viruses (18).

To begin to address the potential regulatory mechanisms for controlling APOBEC3 functions, we first need to analyse its basic characteristics and structure in cells. Hence, the scope of this study was to investigate the subcellular localisation of APOBEC3 proteins in HeLa cells, through immunofluorescence methods. The proteins investigated for this purpose were APOBEC3A, APOBEC3B, and APOBEC3G.

APOBEC3A is a potent DNA cytosine deaminase that has been identified as an interferon-inducible restrictor of foreign DNA, including plasmid DNA (34,35), retrotransposons (26,27), and several viruses such as HBV (8,28), HPV (9,29), and herpesviruses (32,33). A3A subcellular localisation was reported to be in both the nuclear compartment and dispersed through the cytoplasm (10,22,57). Our first hypothesis was to investigate A3A subcellular localisation and see whether it would be in conformity with previous research.

APOBEC3A subcellular localisation was successfully established in HeLa cells through an immunofluorescence optimized protocol. Tests on both non-transfected and transfected cells with pA3A were conducted to evaluate the specificity and effectiveness of the signal transmitted, having they all shown a more prominent signal on the cells cotransfected with GFP-pA3A in contrast to the non-transfected ones (Figure 6). Immunostaining of A3A proteins was performed using an optimised concentration of 1:200 of the primary antibody - Anti-

APOBEC3A antibody produced in rabbit - and a concentration of 1:500 of the secondary antibody - Anti-rabbit IgG Alexa® Fluor 488 - as described in 3. 3.. Finally, indirect immunofluorescence analyses of endogenous A3A, as well as ectopically expressed protein, revealed pronounced accumulation in the nucleus and more-diffuse localisation throughout the cytoplasm, being the results in line with previous reports. As seen in Figure 4 and Figure 6, the more prominent nuclear accumulation of A3A may explain its reported effects on retrotransposition. In addition, its access to the nucleus and, consequently, to genomic DNA makes it possible for this protein to bind and mutate DNA, being this the primary mechanism by which A3A functions. Thus, the protein localisation within the cell is critical for its role as a host restriction factor and a genomic DNA mutator.

Furthermore, heat shock was performed to evaluate if cellular stress had any influence on protein localisation. Two 12-well plates were prepared using the same immunofluorescence protocol, except one of the plates was subjected to heat shock at 45°C for 30 minutes prior to fixation. Both preparations were examined by confocal microscopy, and the resulting images were compared. Analysis of the results showed no differences in the localisation of A3A protein on cells exposed to stress conditions in contrast to the unstressed cells. Interestingly, the heated preparation showed a slight overexpression of A3A protein, suggesting that heat triggers the upregulation of A3A (Figure 7). Such speculation requires formal proof but is consistent with recent data (70). Nevertheless, further experiments are needed to determine whether heat shock plays a clear role in the expression of A3A protein.

APOBEC3G protein localisation was also put under investigation. In accordance with a previous study (71) done by Gallois-Montbrun and her colleagues, A3G localises almost exclusively throughout the cytoplasm in living cells. In addition, their findings also suggested that heat shock trigger dramatic changes in A3G localisation such that much of the protein redistributes towards other ribonucleoprotein-rich cytoplasmic microdomains termed stress granules (SGs). This hypothesis was evaluated by exposing one of the A3G preparations to stress conditions and the other to unstress conditions, as was done in one of the A3A rounds of experiments (4.1.3). Immunostaining of the two preparations transfected with pA3G was performed using a different set of antibodies: Anti-cMyc produced in goat as primary antibody, followed by the secondary antibody Anti-rabbit IgG Alexa® Fluor 647. Prior heat shock, both preparations were examined by immunofluorescence microscopy. For unstressed conditions, we favour a model where A3G staining is diffused primarily throughout the cytoplasm. Following the onset of cellular stress, where significant levels of A3G are redistributed to SGs,

which would reflect in small, strong-signal bordered granules located throughout the cytoplasm. However, the current findings were somewhat at variance with those of Gallois-Montbrun and colleagues. Although A3G localisation was successfully established - immunofluorescence images showed A3G localised throughout the cytoplasm (Figure 7) - SGs were never visualised, and no significant changes were observed between both preparations. Later we realised that the primary antibody used in the immunostaining was incorrect. It was used the Anti-cMyc produced in goat as the primary antibody, while the respective antibody to be used in order to conjugate correctly with the secondary antibody should have been Anti-cMyc produced in rabbit. Therefore, it compromises the whole experiment. As so, we can substantiate that this round of experiments failed, and consequently, the localisation of A3G proteins into stress granules couldn't be assessed, nor its accurate localisation in HeLa cells. Nevertheless, such analyses are important to identify the cellular machinery responsible for cytoplasmic localisation and A3G regulation. Further work is then needed to prove the overall hypothesis.

We next considered the double domain APOBEC3B. This protein is twice the size of the single domain proteins (~50 kDa native or ~75 kDa with GFP) and, in accordance with previous reports (22,38,57), harbours a predominantly nuclear location in the cell. Because of its size, A3B is actively shuttled to the nuclear compartment through a non-canonical N-terminal NLS (38). Holding exclusive access to genomic DNA might be the major benefactor behind its genomic mutation signatures in DNA and contribution to carcinogenesis. A3B has not been as extensively studied because it is specific to primates (there are no exact homologs of any specific APOBEC3 in mice (72)), and it is more challenging to work with (it is lethal to E. coli (73)). Nevertheless, several experiments were conducted in order to visualise A3B proteins in the cell. The same protocol was applied. For the immunostaining procedure, Anti-APOBEC3B antibody produced in rabbit was used as the primary antibody and Anti-rabbit IgG Alexa® Fluor 488 as the secondary antibody. The non-transfected cells exhibited a weak signal in contrast to the transfected cells, which showed a strong signal validating the immunostaining procedure (Figure 8). Immunofluorescence images obtained revealed the pattern of A3B distribution predominantly nuclear with a slight cytoplasmic display being in line with the literature, and therefore, verifying our hypothesis. Moreover, the protocol was also conducted in wildtype and knockout cells for A3B protein. Both cultures were prepared by the Department of Clinical Biochemistry in the Faculty of Pharmacy, University of Ljubljana. The preparation of the knockout cells was expected to show no signal compared to wild-type cell preparation; however, both experiments revealed a significant signal of A3B transmission (Figure 9). This

could have occurred due to poor performance during the genetic engineering of the knockout cell culture. This round of experiments failed, and consequently, the localisation of A3B proteins could not be assessed. To further examine its location in the cell, another round of experiments was performed using a different primary antibody and opting to visualise the protein in a different channel-RFP, which exhibits a red fluorescence signal corresponding to the bound of the proteins of interest. The HA (hemagglutinin) tag is known to be a well-characterised and extensively used as a general antibody epitope tag. Therefore, the anti-HA monoclonal mouse antibody was used as the primary antibody in the hope of obtaining a better outcome. The anti-mouse IgG Alexa® Fluor 555 was used as its conjugated secondary antibody. To test the hypothesis that the A3B protein has a nuclear localisation, we compared the immunofluorescence images from the RFP channel with the ones obtained from de DAPI channel, which would reveal the blue signal from the nucleus compartment of the transfected cells. Both images were overlayed and analysed. The merged image displayed an evident nuclear staining pattern of the A3B protein as well as a slight signal from the cytoplasm meaning that A3B was detected mainly in the nucleus of the cells, with a small portion of the protein located in the cytoplasm (Figure 10). Therefore, this round of experiment was considered successful, and the data obtained is in accordance with the literature.

Moreover, there are several aspects of this study that could lead to its shortcoming. Although proven to have an acceptable natural expression of APOBEC3 proteins, HeLa Cell line, like other cells, tends to change and degrade over time with each passage. Consequently, the high number of passages used in this study might have contributed to a wrong protein expression level, misleading to a wrong result and interpretation. The evaluation of subcellular localisation might depend on the cell cycle phase (57). Since we have not used cells with synchronised cell cycles, it is plausible that our microscopic images are being seen in different cell cycle phases and therefore do not illustrate accurate results. In addition, the resolution of fluorescence microscope images also limits subcellular localisation. To improve the resolution of images, switching to a confocal laser high-resolution microscopy could yield better results. Hence, immunofluorescence images were acquired focusing on a specific region of each coverslip; there is a possibility that the acquired images do not represent the true situation in the cell, as the process of selection is subjective. Introducing an unbiased observer to choose the regions to acquire images might bring more objectivity to it. To confirm the results we acquired, subsequent experiments using different cell lines are needed. Furthermore, additional methods,

such as gene silencing, are advisable to optimise the immunostaining specificity of APOBEC3 proteins and, consequently, clearly confirm its localisation in the cell.

6 Conclusion

In summary, the findings of this report indicate that the APOBEC3A is localised in both the nuclear compartment as well as in the cytoplasm, APOBC3B appears to have a predominantly nuclear localisation, and APOBEC3G has a more-diffuse localisation throughout the cytoplasm.

We have partially confirmed starting hypothesis, and the work can be perceived as successful. We successfully transfected plasmids with the desired proteins (A3A, A3B, and A3G) into HeLa Cells culture; subsequently, an immunostaining protocol was performed by incubating the cells with specific antibodies optimised to the proper concentration of 1:200 for primary and 1:500 for secondary. Ultimately, subcellular localisation was confirmed through immunofluorescence microscopy of the preparations.

Even though studies over the past decades have revealed significant advances in our understanding of APOBEC3 proteins biology, there remained a considerable lack of detail concerning the subcellular context in which APOBEC3A, APOBEC3B, and APOBEC3G function. Understanding the regulation of APOBEC3 proteins, including its subcellular localisation, is essential for knowing how the cell regulates APOBEC3's cytosine deaminase activity - either for its benefit or detriment.

7 References

1. Harris RS, Dudley JP. APOBECs and Virus Restriction. Virology. 2015;131–45.

2. Salter JD, Bennett RP, Smith HC. The APOBEC Protein Family: United by Structure, Divergent in Function. Trends Biochem Sci. 2016;41(7):578–94.

3. Giorgio S di, Martignano F, Torcia MG, Mattiuz G, Conticello SG. Evidence for RNA editing in the transcriptome of 2019 Novel Coronavirus. Sci Adv. 2020;6:1–8.

4. Ratcliff J, Simmonds P. Potential APOBEC-mediated RNA editing of the genomes of SARS-CoV-2 and other coronaviruses and its impact on their longer term evolution. Virology. 2021;556:62–72.

5. Cheng AZ, Yockteng-Melgar J, Jarvis MC, Malik-Soni N, Borozan I, Carpenter MA, et al. Epstein-Barr virus BORF2 inhibits cellular APOBEC3B to preserve viral genome integrity HHS Public Access. Nature Microbioly. 2019;4(1):78–88.

6. Chen Y, Hu J, Cai X, Huang Y, Zhou X, Tu Z, et al. APOBEC3B edits HBV DNA and inhibits HBV replication during reverse transcription. Antiviral Res. 2018;149:16–25.

7. Chen Y, Shen B, Zheng X, Long Q, Xia J, Huang Y, et al. DHX9 interacts with APOBEC3B and attenuates the anti-HBV effect of APOBEC3B. Emerg Microbes Infect. 2020;9(1):366–77.

8. Wang Y, Li Y, Zai W, Hu K, Zhu Y, Deng Q, et al. HBV covalently closed circular DNA minichromosomes in distinct epigenetic transcriptional states differ in their vulnerability to damage. Hepatology. 2022;75(5):1275–88.

9. Warren CJ, Westrich JA, Doorslaer K van, Pyeon D, Mcbride AA, Munger K. Roles of APOBEC3A and APOBEC3B in Human Papillomavirus Infection and Disease Progression. Viruses. 2017;9:1–20.

10. Lovin N, Peterlin BM. APOBEC3 Proteins Inhibit LINE-1 Retrotransposition in the Absence of ORF1p Binding. Ann N Y Acad Sci. 2009;1178:1–11.

11. Brown WL, Law EK, Argyris PP, Carpenter MA, Levin-Klein R, Ranum AN, et al. A Rabbit Monoclonal Antibody against the Antiviral and Cancer Genomic DNA Mutating Enzyme APOBEC3B. Antibodies (Basel). 2019;8(3):1–2.

12. Petljak M, Maciejowski J. Molecular origins of APOBEC-associated mutations in cancer. DNA Repair (Amst). 2020;94:1–3.

13. Swanton C, Mcgranahan N, Starrett GJ, Harris RS. APOBEC enzymes: mutagenic fuel for cancer evolution and heterogeneity The importance of cancer diversity Europe PMC Funders Group. Cancer Discov. 2015;5(7):704–12.

14. Vlachostergios PJ, Faltas BM. Treatment resistance in urothelial carcinoma: an evolutionary perspective. Nat Rev Clin Oncol. 2018;15(8):495–509.

15. Chen XS, Melikian GB. Insights into the Structures and Multimeric Status of APOBEC Proteins Involved in Viral Restriction and Other Cellular Functions. Viruses. 2021;13:1–25.

16.   Harjes S, Solomon WC, Li M, Chen KM, Harjes E, Harris RS, et al. Impact of H216 on the DNA Binding and Catalytic Activities of the HIV Restriction Factor APOBEC3G. J Virol. 2013;87(12):7008–14.

17.   Nabel CS, Lee JW, Wang LC, Kohli RM. Nucleic acid determinants for selective deamination of DNA over RNA by activation-induced deaminase. Proc Natl Acad Sci U S A. 2013;110(35):14225–30.

18.   Olson ME, Harris RS, Harki DA. APOBEC ENZYMES AS TARGETS FOR VIRUS AND CANCER THERAPY. Cell Chem Biol. 2018;25(1):36–49.

19.   Vieira VC, Soares MA. The Role of Cytidine Deaminases on Innate Immune Responses against Human Viral Infections. Biomed Res Int. 2013;2013.

20.   Lackey L, Law EK, Brown WL, Harris RS. Subcellular localization of the APOBEC3 proteins during mitosis and implications for genomic DNA deamination. Cell Cycle. 2013;12(5):762.

21.   Pecori R, Giorgio S, Paulo Lorenzo J, Nina Papavasiliou F. Functions and consequences of AID/APOBEC-mediated DNA and RNA deamination. Nat Rev Genet. 2022;23:505–18.

22.   Covino DA, Gauzzi MC, Fantuzzi L. Understanding the regulation of APOBEC3 expression: Current evidence and much to learn. Journal od Leukocyte Biology. 2017;103:433–44.

23.   Sadeghpour S, Khodaee S, Rahnama M, Rahimi H, Ebrahimi D. Human APOBEC3 Variations and Viral Infection. Viruses. 2021;13(7).

24.   Venkatesan S, Angelova M, Puttick C, Zhai H, Caswell DR, Lu WT, et al. Induction of APOBEC3 exacerbates DNA replication stress and chromosomal instability in early breast and lung cancer evolution HHS Public Access. Cancer Discov. 2021;11(10):2456–73.

25.   Land AM, Law EK, Carpenter MA, Lackey L, Brown WL, Harris RS. Endogenous APOBEC3A DNA cytosine deaminase is cytoplasmic and nongenotoxic. J Biol Chem. 2013;288(24):17253–60.

26.   Chen H, Lilley CE, Yu Q, Lee D v., Chou J, Narvaiza I, et al. APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons. Curr Biol. 2006;16(5):480–5.

27.   Bogerd HP, Wiegand HL, Hulme AE, Garcia-Perez JL, O'Shea KS, Moran J v., et al. Cellular inhibitors of long interspersed element 1 and Alu retrotransposition. Proc Natl Acad Sci U S A. 2006;103(23):8780–5.

28.   Lucifora J, #1 YX, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and Nonhepatotoxic Degradation of Nuclear Hepatitis B Virus cccDNA HHS Public Access. Science (1979). 2014;343(6176):1221–8.

29.   Warren CJ, Xu T, Guo K, Griffin LM, Westrich JA, Lee D, et al. APOBEC3A Functions as a Restriction Factor of Human Papillomavirus. J Virol. 2015;89(1):688.

30.   Nakaya Y, Stavrou S, Blouch K, Tattersall P, Ross SR. In Vivo Examination of Mouse APOBEC3- and Human APOBEC3A- and APOBEC3G-Mediated Restriction of Parvovirus and Herpesvirus Infection in Mouse Models . J Virol. 2016;90(17):8005–12.

31. Narvaiza I, Linfesty DC, Greener BN, Hakata Y, Pintel DJ, Logue E, et al. Deaminase-Independent Inhibition of Parvoviruses by the APOBEC3A Cytidine Deaminase. PLoS Pathog. 2009;5(5):1000439.

32. Cheng AZ, Moraes SN, Shaban NM, Fanunza E, Bierle CJ, Southern PJ, et al. viruses APOBECs and Herpesviruses. Viruses. 2021;13:1–14.

33. Cheng AZ, Moraes SN, Attarian C, Yockteng-Melgar J, Jarvis MC, Biolatti M, et al. A Conserved Mechanism of APOBEC3 Relocalization by Herpesviral Ribonucleotide Reductase Large Subunits. J Virol. 2019;93(23):1–17.

34. Suspène R, Aynaud MM, Guétard D, Henry M, Eckhoff G, Marchio A, et al. Somatic hypermutation of human mitochondrial and nuclear DNA by APOBEC3 cytidine deaminases, a pathway for DNA catabolism. Proc Natl Acad Sci U S A. 2011;108(12):4858–63.

35. Mussil B, Suspène R, Aynaud MM, Gauvrit A, Vartanian JP, Wain-Hobson S. Human APOBEC3A Isoforms Translocate to the Nucleus and Induce DNA Double Strand Breaks Leading to Cell Stress and Death. PLoS One. 2013;8(8):1–17.

36. Cortez LM, Brown AL, Dennis MA, Collins CD, Brown AJ, Mitchell D, et al. APOBEC3A is a prominent cytidine deaminase in breast cancer. PLoS Genet. 2019;15(12):1–32.

37. Gansmo LB, Sofiyeva N, Bjørnslett M, Romundstad P, Hveem K, Vatten L, et al. Impact of the APOBEC3A/B deletion polymorphism on risk of ovarian cancer. Sci Rep. 2021;11(1):1–8.

38. Lackey L, Demorest ZL, Land AM, Hultquist JF, Brown WL, Harris RS. APOBEC3B and AID have similar nuclear import mechanisms. J Mol Biol. 2012;419(5):301–14.

39. Caval V, Suspène R, Shapira M, Vartanian JP, Wain-Hobson S. A prevalent cancer susceptibility APOBEC3A hybrid allele bearing APOBEC3B 3′UTR enhances chromosomal DNA damage. Nature Communications . 2014;5(1):1–7.

40. Chan K, Roberts SA, Klimczak LJ, Sterling JF, Saini N, Malc EP, et al. An APOBEC3A hypermutation signature is distinguishable from the signature of background mutagenesis by APOBEC3B in human cancers. Nat Genet. 2015;47(9):1067–72.

41. Caval V, Bouzidi MS, Suspène R, Laude H, Dumargne MC, Bashamboo A, et al. Molecular basis of the attenuated phenotype of human APOBEC3B DNA mutator enzyme. Nucleic Acids Res. 2015;43(19):9340–9.

42. Burns MB, Temiz NA, Harris RS. Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat Genet. 2013;45(9):977–83.

43. Mao Y, Lv M, Zhang Y, Nie G, Cui J, Wang Y, et al. APOBEC3B expression and its prognostic potential in breast cancer. Oncol Lett. 2020;19(4):3205–14.

44. Yan S, He F, Gao B, Wu H, Li M, Huang L, et al. Increased APOBEC3B Predicts Worse Outcomes in Lung Cancer: A Comprehensive Retrospective Study. J Cancer. 2016;7(6):618–25.

45. Swanton C, McGranahan N, Starrett GJ, Harris RS. APOBEC Enzymes: Mutagenic Fuel for Cancer Evolution and Heterogeneity. Cancer Discov. 2015;5(7):704–12.

46. Xu L, Chang Y, An H, Zhu Y, Yang Y, Xu J. High APOBEC3B expression is a predictor of recurrence in patients with low-risk clear cell renal cell carcinoma. Urol Oncol. 2015;33(8):340.e1-340.e8.

47. Brown WL, Law EK, Argyris PP, Carpenter MA, Levin-Klein R, Ranum AN, et al. A Rabbit Monoclonal Antibody against the Antiviral and Cancer Genomic DNA Mutating Enzyme APOBEC3B. Antibodies (Basel). 2019;8(3):1–17.

48. Shaban NM, Yan R, Shi K, Moraes SN, Cheng AZ, Carpenter MA, et al. Cryo-EM structure of the EBV ribonucleotide reductase BORF2 and mechanism of APOBEC3B inhibition. Sci Adv. 2022;8(17):1–13.

49. Cheng AZ, Yockteng-Melgar J, Jarvis MC, Malik-Soni N, Borozan I, Carpenter MA, et al. Epstein-Barr virus BORF2 inhibits cellular APOBEC3B to preserve viral genome integrity HHS Public Access. Nature Microbioly. 2019;4(1):78–88.

50. Salamango DJ, Harris RS. Demystifying Cell Cycle Arrest by HIV-1 Vif. Trends Microbiol. 2021;29(5):381–4.

51. Stupfler B, Verriez C, Gallois-Montbrun S, Marquet R, Paillart JC. Degradation-Independent Inhibition of APOBEC3G by the HIV-1 Vif Protein. Viruses. 2021;13(4):1–17.

52. Albin JS, Harris RS. Interactions of host APOBEC3 restriction factors with HIV-1 in vivo: implications for therapeutics. Expert Rev Mol Med. 2010;12:1–29.

53. Mohamadkhani A, Pourdadash A, Tayebi S, Estakhri A, Nazem H, Sotoudeh M, et al. The potential role of APOBEC3G in limiting replication of hepatitis B virus. Arab J Gastroenterol. 2012;13(4):170–3.

54. Ni L, Li C, Li Y. Correlation of APOBEC3G expression with liver function indexes of patients with chronic hepatitis B and comparison in chronic hepatitis B, liver cirrhosis and liver cancer. Oncol Lett. 2020;19(3):2562.

55. Sasada A, Takaori-Kondo A, Shirakawa K, Kobayashi M, Abudu A, Hishizawa M, et al. APOBEC3G targets human T-cell leukemia virus type 1. Retrovirology. 2005;2:32.

56. Koyama T, Arias JF, Iwabu Y, Yokoyama M, Fujita H, Sato H, et al. APOBEC3G Oligomerization Is Associated with the Inhibition of Both Alu and LINE-1 Retrotransposition. PLoS One. 2013;8(12).

57. Lackey L, Law EK, Brown WL, Harris RS. Subcellular localization of the APOBEC3 proteins during mitosis and implications for genomic DNA deamination. Cell Cycle. 2013;12(5):762–72.

58. Revathidevi S, Murugan AK, Nakaoka H, Inoue I, Munirajan AK. APOBEC: A molecular driver in cervical cancer pathogenesis. Cancer Lett. 2021;496:104–16.

59. Bergstrom EN, Luebeck J, Petljak M, Khandekar A, Barnes M, Zhang T, et al. Mapping clustered mutations in cancer reveals APOBEC3 mutagenesis of ecDNA. Nature. 2022;602(7897):510–7.

60. Alexandrov LB, Kim J, Haradhvala NJ, Huang MN, Wei A, Ng T, et al. The repertoire of mutational signatures in human cancer. PCAWG Mutational Signatures Working Group. 1943;578:15.

61. Nik-Zainal S, Alexandrov LB, Wedge DC, van Loo P, Greenman CD, Raine K, et al. Mutational processes molding the genomes of 21 breast cancers. Cell. 2012;149(5):979–93.

62. Burns MB, Lackey L, Carpenter MA, Rathore A, Land AM, Leonard B, et al. APOBEC3B is an enzymatic source of mutation in breast cancer. Nature. 2013;494:366–70.

63. list of authors A, affiliations appears at the end of the paper their. Signatures of mutational processes in human cancer. 2013;

64. Langenbucher A, Bowen D, Sakhtemani R, Bournique E, Wise JF, Zou L, et al. An extended APOBEC3A mutation signature in cancer. Nat Commun. 2021;12:1–11.

65. Petljak M, Maciejowski J. Molecular origins of APOBEC-associated mutations in cancer. DNA Repair (Amst). 2020;94:1–10.

66. Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, et al. COSMIC: Somatic cancer genetics at high-resolution. Nucleic Acids Res. 2017 Jan 1;45(D1):D777–83.

67. The Johns Hopkins University TJHH and TJHHSC. Johns Hopkins Medicine [Internet]. The Legacy of Henrietta Lacks. 2022. Available from: https://www.hopkinsmedicine.org/henriettalacks/

68. SignaGen Laboratories. In Vitro DNA Transfection Reagents [Internet]. PolyJet^{TM} In Vitro DNA Transfection Reagent. 2022. Available from: https://signagen.com/In-Vitro-DNA-Transfection-Reagents/SL100688/PolyJet-DNA-In-Vitro-Transfection-Reagent

69. Kong J, Wang Y, Qi W, Huang M, Su R, He Z. Historical Perspective Green fluorescent protein inspired fluorophores. Adv Colloid Interface Sci. 2020;285:1–13.

70. Yang Y, Wang H, Zhang X, Huo W, Qi R, Gao Y, et al. Heat Increases the Editing Efficiency of Human Papillomavirus E2 Gene by Inducing Upregulation of APOBEC3A and 3G. Journal of Investigative Dermatology. 2017 Apr 1;137(4):810–8.

71. Gallois-Montbrun S, Kramer B, Swanson CM, Byers H, Lynham S, Ward M, et al. Antiviral Protein APOBEC3G Localizes to Ribonucleoprotein Complexes Found in P Bodies and Stress Granules. J Virol. 2007;81(5):2165–78.

72. LaRue RS, Andrésdóttir V, Blanchard Y, Conticello SG, Derse D, Emerman M, et al. Guidelines for Naming Nonprimate APOBEC3 Genes and Proteins. J Virol. 2009 Jan 15;83(2):494–7.

73. Stenglein MD, Harris RS. APOBEC3B and APOBEC3F inhibit L1 retrotransposition by a DNA deamination-independent mechanism. Journal of Biological Chemistry. 2006 Jun 23;281(25):16837–41.