REGULATION OF mRNA TRANSLATION BY eIF5B IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

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DEDICATION

To my beloved family and friends,

My inspiration, my parents Jayant Muley and Ujwal Muley

Mine dearly loved brother Rohit Muley

My late Grandparents Purushottam Muley and Vijaya Muley

I am grateful to have these wonderful people in my life… With their good wishes, teachings, support, and love I have been able to achieve success and happiness in life.

ABSTRACT

Studies have demonstrated that eIF5B promotes cap-independent translation of the anti-apoptotic factor, XIAP, under stress conditions. Also, eIF5B depletion results in the decreased levels of several pro-survival proteins, thus sensitizing glioblastoma cells to TRAIL-induced apoptosis. Here, we have shown high *EIF5B* mRNA expression in HNSCC was associated with poor patients' survival. Further, to assess the impact of eIF5B on HNSCC biology, we depleted eIF5B in three HNSCC cell lines. We also show that eIF5B depletion enhanced the sensitivity of Cal-33 and UM-SCC-29 cells to TRAIL-induced apoptosis. Further, we show the levels of anti-apoptotic proteins Bcl-xL, cIAP1, cFLIPs, and XAIP were decreased upon eIF5B depletion in Cal-33 suggesting the role of eIF5B in the translation of these proteins which was further supported by our polysome profiling data. Altogether our findings suggest that eIF5B may play an important role in the translation of mRNAs encoding key anti-apoptotic proteins that evade apoptosis.

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4E-BP	eIF4E-binding protein
Apaf1	Apoptotic protease activating factor 1
Bcl-xL	B-cell lymphoma extra-large
BIR	Baculovirus IAP repeat
c-FLIP	FADD-like IL-1β-converting enzyme-inhibitory protein
cIAP1	Cellular inhibitor of apoptosis 1
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DR4/5	Death receptor 4/5
dsDNA	Double-stranded deoxyribonucleic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FADD	Fas-associated protein with death domain
FasR	Fas-receptor
GTP	Guanosine triphosphate
hnRNP	Heterogenous ribonucleoprotein
HuR	Human antigen R
IAP	Inhibitor of apoptosis protein
tRNAi	initiator transfer RNA
IRES	Internal ribosome entry site
MDM2	Murine double minute 2
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween 20
PDCD4	Programmed cell death 4
PERK	PKR-like ER kinase
PI3K	Phosphatidylinositol 3-kinase
PKR	Protein kinase R
PMSF	Phenylmethane sulfonyl fluoride
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SDS	Sodium dodecyl sulfate
siRNA	Small interfering ribonucleic acid
Smac	Second mitochondria-derived activator of caspases
TNFα	Tumor necrosis factor-alpha
TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R	Tumor necrosis factor-related apoptosis-inducing ligand-receptor
UTR	Untranslated region

XIAP	X-linked inhibitor of apoptosis
HNSCC	Head and Neck Squamous Cell Carcinoma
OSCC	Oral Squamous Cell Carcinoma
TCGA	The Cancer Genome Atlas
UM-SCC-59	University of Michigan-Squamous Cell Carcinoma-59
UM-SCC-29	University of Michigan-Squamous Cell Carcinoma-29
Cal-33	Centre Antoine Lacassagne-33
CARD	caspase activation recruitment domain
TMZ	temozolomide

CHAPTER 1

Introduction

This chapter gives a detailed account of translation initiation mechanisms in eukaryotes. It talks about the cis-acting factor and their regulatory role in translation initiation when the cell encounters stress conditions. It also provides a brief overview of Head and Neck Squamous Cell Carcinoma (HNSCC) as I have defined the role of eukaryotic initiation factor 5B (eIF5B) in HNSCC cell survival. Finally, this chapter concludes with the hypothesis that is proposed to investigate mRNA translation regulation in HNSCC.

1.1 Overview of eukaryotic translation initiation

The genetic information needed for an organism to develop, survive, and reproduce is carried by (deoxyribose nucleic acid) DNA. This information, however, needs to be processed into a message in the form of mRNA by the process of transcription which is executed by an enzyme RNA polymerase.[1] The mRNA formed is then modified at the ends where its 5' end is capped by the addition of m^7G methyl guanosine cap and its 3' terminal is polyadenylated. mRNA is further spliced to remove intron resulting in the formation of mature mRNA which can be translated into proteins by the process of translation.[2] The ultimate stage of gene expression is the process of translation where proteins are synthesized from mRNA. The process of mRNA translation is divided into three stages: initiation, elongation, and termination. Translation initiation is tightly regulated and rate-limiting step during protein synthesis. It involves at least 12 eukaryotic initiation factors, mature mRNA, initiator tRNA (tRNAi), ribosomal subunits, and some auxiliary protein

factors. Translation initiation proceeds by; (a) formation of 43S pre-initiation complex (b) formation of 48S pre-initiation complex and (c) 80S initiation complex. Once the 80S initiation complex is formed, the ribosome continues with the translation elongation.[3] Translation initiation is classified as cap-dependent/canonical translation, which requires a 5' cap on mRNA to recruit ribosome to initiate translation. In most cases of non-canonical translation initiation, the ribosome is recruited onto the mRNA without the involvement of the 5' cap structure.[4]

1.1.1 Cap-dependent/canonical translation initiation

Cap-dependent translation initiation is widely used by eukaryotes for the translation of most of their mRNAs. Among the several eukaryotic initiation factors involved, eukaryotic initiation factor 2 (eIF2) is one of the important factors that is involved in the rate-limiting step during the mRNA translation and its mechanism is explained in detail in the latter part of this chapter. eIF2 has three subunits; eIF2α, eIF2β, and eIF2γ which are divided based on their increasing molecular masses.[5] eIF2 is involved in the formation of the ternary complex where it is known to interact with GTP and met-tRNAi. Out of the three eIF2 subunits, eIF2γ interacts with GTP whereas eIF2β and eIF2γ interact with met-tRNAi. eIF2α is less remarkable as far as binding with the GTP is concerned.[5] Eukaryotic initiation factor 4F (eIF4F) is a complex comprising of eukaryotic initiation factor 4E (eIF4E), eukaryotic initiation factor 4G (eIF4G), and eukaryotic initiation factor 4A (eIF4A) that assembles onto the 5'cap of mRNA to initiate the process of translation. eIF4E also known as a cap-binding protein that captures the methylated 5' m^7G cap of the mRNA and interact with the multidomain scaffold protein eIF4G along with the mRNA helicase

eIF4A.[6] eIF4A is ATP dependent helicase that causes the unwinding of the secondary structure in the 5'untranslated region of mRNA during 40S ribosome subunit scanning.[7] Once this eIFs assemble onto the mRNA, the ternary complex that comprises of eIF2-GTP-met-tRNAi interacts with the 40S ribosomal subunit and is recruited onto the 5' end of mRNA.[7] Although the helicase activity of eIF4A is weak, it is stimulated by eIF4G and eIF4B. eIF4B is known to guide the 40S ribosomal subunit and promotes its recruitment onto the mRNA by interacting with the 18S ribosomal RNA (rRNA).[8] eIF4G is a critical link between eukaryotic initiation factor 3 (eIF3) and eIF4A as it possesses binding sites for both eIFs. However, the most important role of eIF4G is it links the mRNA cap and the 40S ribosomal subunit via eIF4E and eIF3 respectively.[9,10] The 40S ribosomal subunit is recruited onto mRNA along with eIF2-GTP-met-tRNAi ternary complex together forms 43S pre-initiation complex. eIF4A and eIF4B help 43S pre-initiation complex to scan the mRNA in 5'to 3'direction to locate the initiation codon.[11] Once the initiation codon AUG is recognized, the ribosome stops scanning the mRNA and binds to the AUG start codon to form a 48S pre-initiation complex. Further, eIF5 binds to 48S pre-initiation complex and induces GTPase activity of eIF2 which releases all the bound initiation factors. The eIF2-GDP form after the GTPase activity of eIF2 is also released which is then recycled for the next round of initiation.[12] Further, eIF5B joins the 60S ribosomal subunit with the 40S to form 80S, which then proceeds for elongation step.[3,12] All the components of the translation machinery are recycled at the end of the process to start the initiation. The process of mRNA translation utilizes a lot of energy (as much as ~30% of the cellular energy), and hence it is important to control this process at the initiation stage.

1.1.2 Cap-independent/non-canonical initiation

Cap-independent translation does not involve a cap structure for ribosome recruitment onto the mRNA. Typically, stress conditions like hypoxia, nutrient deprivation, viral infections, or endoplasmic reticulum stress result in the modification of the important eIFs. This results in the impairment of the cap-dependent translation and cell switches to the cap-independent translation initiation mode. Cells express certain cytoprotective proteins to overcome these stress conditions and survive.[13] In cap-independent translation initiation mode, the ribosome is recruited on the mRNA escaping the 5'cap via some specialized RNA structures and *cis*-acting elements. These *cis*-elements specifically include internal ribosomal entry sites (IRESs) and cap-independent translation enhancers (CITEs).[14-16] Among these *cis*-elements, my study focuses on the translation of IRES-containing mRNAs when the eIF5B levels in the cells are decreased.

1.1.2.1 Importance of cap-independent translation initiation during apoptosis

The global protein synthesis is downregulated when cells are exposed to different kinds of stress like hypoxia, heat shock, ER stress, DNA damage, starvation, and apoptosis.[17-20] For cells to cope with these conditions and survive, they must express proteins that can provide cytoprotection. Generally, during such conditions cells expresses both pro-survival and pro-death proteins. If the pro-survival proteins levels exceed the level of pro-death proteins, the cell can survive. However, if the pro-death proteins are predominantly expressed, cells commence to a self-destructive pathway known as apoptosis. Thus, depending on the expression of pro-death or pro-survival proteins during stress conditions, the fate of the cell is decided. When cells are under severe stress the cap-

dependent mechanism is attenuated in several ways. Phosphorylation of eIF2α, sequestration of eIF4E by 4E binding proteins (4E-BP), and cleavage of eIF4G are the major regulatory steps in translation initiation.[21,22] There are four kinases namely heme-regulated inhibitor (HRI), protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK) and general control non-depressible 2 (GCN2) phosphorylate eIF2 under various stress conditions.[23] Phosphorylation of eIF2α inhibits the formation of ternary complex thereby inhibiting the global cap-dependent translation. The endoplasmic reticulum stress generated as a result of unfolded protein response activates PKR-like ER kinase (PERK) which is also known to cause eIF2α phosphorylation.[24] Another mechanism that interferes with the cap-dependent initiation is by 4E-BPs. The mammalian target of rapamycin (mTOR) is activated in response to growth signals. 4E-BPs have an affinity for eIF4E, and it competes for the same binding site with eIF4G on eIF4E.[25,26] mTOR phosphorylates 4E-BPs that dissociate from eIF4E thereby making eIF4E available to bind eIF4G and proceed with the canonical translation. In stress conditions, when mTOR is inhibited, 4E-BPs are hypophosphorylated and they bind eIF4E thereby repressing the canonical translation.[3] Apart from these proteins, a class of proteins called caspases are activated on the induction of programmed cell death.[27] Caspase-3 particularly is known to cleave eIF4G that ultimately inhibits the cap-dependent translation.[28] During these stress conditions when the global translation is attenuated, cells synthesize some stress response proteins by the selective cap-independent mechanisms.[29] Apoptotic protease activating factor-1 (APAF-1), which is involved in the apoptosis pathway, is expressed by the cap-independent mechanism. This protein is involved in an apoptotic pathway in response to DNA damage and can also get activated by extrinsic apoptotic stimuli *via* caspase-8.[30] Apaf-1 is present

in the cytoplasm as a monomer[31,32] but the apoptotic signals result in its oligomerization. Intrinsic apoptotic stimuli result in the release of cytochrome C that binds to dATP and stimulates Apaf-1 oligomerization to form an apoptosome. The formation of apoptosome triggers the caspase 9 activation that activates the caspase signaling cascade and executes apoptosis.[33] Apaf-1 is expressed predominantly during stress conditions in a cap-independent way utilizing an IRES-mediated translation.[34] Another example is of the tumor suppressor protein p53 which is activated as a response to DNA damage.[35] Normally, during physiological conditions, the p53 activity is maintained by mouse double minute 2 protein (Mdm2) by its proteasomal degradation via E3 ubiquitin ligase.[36] However, in response to DNA damage, p53 levels in the cells increases; Mdm2 levels decreases, and results in the cell cycle arrest.[36] This results in the recruitment of DNA repair machinery to repair the DNA damage and failure of this pushes cells towards apoptosis due to the expression of proteins like Bax and PUMA.[36] Thus, based on the levels of the proteins expressed in the cell fate is decided. There are two known isoforms of p53 that are expressed in the cells by IRES-mediated translation.[37] c-Jun protein which is involved in stimulating the transcription of components of cell cycle and repressing the transcription of a tumor suppressor is expressed by IRES-mediated translation.[38] Many anti-apoptotic proteins like X-linked inhibitor of apoptosis (XIAP), a cellular inhibitor of apoptosis 1 (cIAP-1), and B-cell lymphoma-extra large (Bcl-xL), that are known to play role in cell survival, are also expressed via non-canonical translation initiation.[39-41] The above findings shed light on the importance of cap-independent translation initiation with respect to cell physiology or survival, particularly during stress conditions.

1.1.2.2 IRES elements and their discovery

IRESes were first discovered in 1988 in RNA viruses (picornaviridae) where the RNA of the virus doesn't bear any cap structure.[42] Viruses belonging to this family encode the protease 2A[43] which cleaves an important initiation factor eIF4G that is involved in cap-dependent translation initiation.[44] These viruses also increase the expression of 4E-BP proteins upon infection and thereby interferes with the eIF4F complex formation.[45] The absence of the eIF4F complex shuts down the translation of the host cells but not of the viruses. This resulted in the discovery of the IRES-mediated translation by viruses where they utilize host cell translation machinery without the involvement of a 5' cap.[42] The discovery of IRESes was not just limited to the viruses. Later, it was found that some of the eukaryotic mRNAs bear these secondary structures in their 5' untranslated region (UTR) which helps in translation when the global protein synthesis is impaired.[46] The first cellular IRES was discovered in the mRNA that codes for immunoglobulin heavy chain binding protein (BiP).[47,48] It is estimated that nearly 10% of the cellular mRNAs contain IRES elements that were discovered using the technique like bicistronic reporter assays.[49] Interestingly, some of the cellular mRNA bearing IRESes are expressed as a response to severe stress conditions, development, and diseases like cancer.

Although viral IRESes share structural and functional similarities, they differ in the primary sequence and the requirements of the *trans*-acting factors.[50] Based on this, IRESes are classified into four distinct types; Type I and type II IRESes that utilize eIF4A and eIF4G for ribosome recruitment.[51] These classes do not require eIF4E and include poliovirus and enterovirus 71 and encephalomyocarditis virus respectively. Type III IRESes are also known as HCV like IRESes that do not depend on eIFs like eIF4F, eIF4B,

eIF1, and eIF1A. Instead, they can directly recruit ribosomes onto the mRNA. Dicistroviruses belong to the type IV class of IRESes that do not require any eIFs or initiator tRNA (eg: cricket paralysis virus).[3,47]

Unlike viral IRESes, cellular IRESes do not have any conserved primary or secondary structures.[52,53] However, both viral and cellular IRESes are controlled by a class of proteins known as IRES *trans*-acting factors (ITAFs). ITAFs bind to specific sequences in the 5'-UTR of the mRNA and introduces conformational changes in the structure which promotes easy recruitment of ribosomes onto the mRNA.[54,55] The entire process of IRES-mediated translation involves very few eIFs along with specific trans-acting factors like polypyrimidine tract binding proteins, La autoantigen, human antigen R (HuR), program cell death 4 protein (PDCD4) and heterogeneous ribonucleoproteins (hnRNPs).[14,47] Thus, eIFs and ITAFs levels drive the IERS-mediated translation and determine the fate of the cell during stress.

1.2 Apoptosis

Apoptosis is a distinct type of cell death mechanism utilized by cells. It is also known as programmed cell death where the dying cells do not release the content of the cells in the surrounding and prevent the anti-inflammatory response.[56] Apoptosis is a normal phenomenon that occurs in the cells during development and aging to maintain the cell population in the tissue. For example, in a normal physiological state of the body apoptosis plays a role during gametes maturation.[57] This process is very important and serves as a "guardian" during gametes maturation to remove any abnormal cells that have undergone mutation or DNA damage. Apoptosis also plays a role during an immune response or

certain complex diseases (eg: Alzheimer disease or cancer).[58] The cell morphology in apoptosis is characterized by cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation ultimately resulting in the formation of "apoptotic bodies".[59] These apoptotic bodies formed are then removed by a process called phagocytosis where immune cells like macrophages engulf these bodies without triggering the immune response.[60]

Apoptosis is a complex process that involves an ATP driven cascade of several molecular events.[27] There are two major pathways by which the apoptotic process is executed; the Extrinsic or death receptor pathway and the Intrinsic or mitochondrial apoptotic pathway. Both these pathways crosstalk *via* common molecule Bid and ultimately, they converge to trigger a caspase signaling cascade. Bid is activated upon receptor-mediated signaling pathway by caspase-8 which then results in the activation of a mitochondrial apoptotic pathway that amplifies the entire process of apoptosis.[61] Caspases are the class of proteolytic enzymes that are expressed in an inactive proenzyme form (pro-caspases).[62] Upon activation, they induce protease cascade in which one caspase activates the other to amplify the overall apoptotic signaling.[27] There are total 10 major caspases that are classified into three different classes; Caspases-2,-8,-9,-10 are the initiator or apical caspases; Caspase-3,-6,-7 are the executioner or effector caspases and Caspases-1,-4,-5 are the inflammatory caspases.[63] The initiator procaspases which exist as monomers get activated to form dimers which on its autocatalytic cleavage forms active initiator caspases.[62] On the other hand, the executioner caspases exist as a homodimer with less activity. However, when initiator caspases cleaved them their activity is enhanced.[62]

1.2.1 Extrinsic or death receptor pathway

The interaction of the death ligand with its corresponding receptor, which is a transmembrane protein, induces a signaling cascade. These death receptors with the cysteine-rich extracellular domain belong to the tumor necrotic factor (TNF) family.[64] The cytoplasmic domain of these receptors is called the "death domain" and is critical for inducing the downstream death signals. There are several death ligands for the corresponding extra-cellular death receptors like; FASL/FASR, TNFα/TNFR1, Apo2L/DR, Apo2L/DR5, and Apo3L/DR3.[65-69] Upon extrinsic stimulation especially when a death ligand like FAS binds to its receptor FASR1 it causes receptor clustering. This results in the recruitment of adapter protein Fas-associated protein with death domain (FADD) and receptor-interacting protein (RIP) on the cytoplasmic side of the FASR1 which then forms death-inducing signaling complex DISC.[27] Once the DISC complex is formed, it oligomerizes and autocatalysis the activation of procaspase 8/10. The active initiator caspase 8/10 then cleaves procaspase 3/7 to form active executioner caspases 3/7 that execute the apoptosis.[70,71]

1.2.2 Intrinsic/ Mitochondrial apoptosis pathway

Intrinsic signaling pathway also known as mitochondrial apoptosis pathway initiates the receptor-independent process of apoptosis. The intrinsic apoptotic pathway is induced mainly due to DNA damage.[27] This generates a stimulus that causes the alteration of an inner mitochondrial membrane which results in pore formation and loss of mitochondrial transmembrane potential.[72] As a result, some pro-death factors like cytochrome c and second mitochondria-derived activator of caspase (smac) that triggers the signaling cascade of apoptosis are released.[73] The cytochrome c then interact with

apoptotic protease activating factor 1 (Apaf1) and procaspase 9 to form apoptosome complex. This results in the activation of caspase 9 which further activates the effector caspases 3/6/7 that commences apoptosis.[74] As mentioned earlier, Smac inhibits XIAP and cIAP1/2 and as a result, the caspases are not inhibited and are available to execute apoptosis.[75]

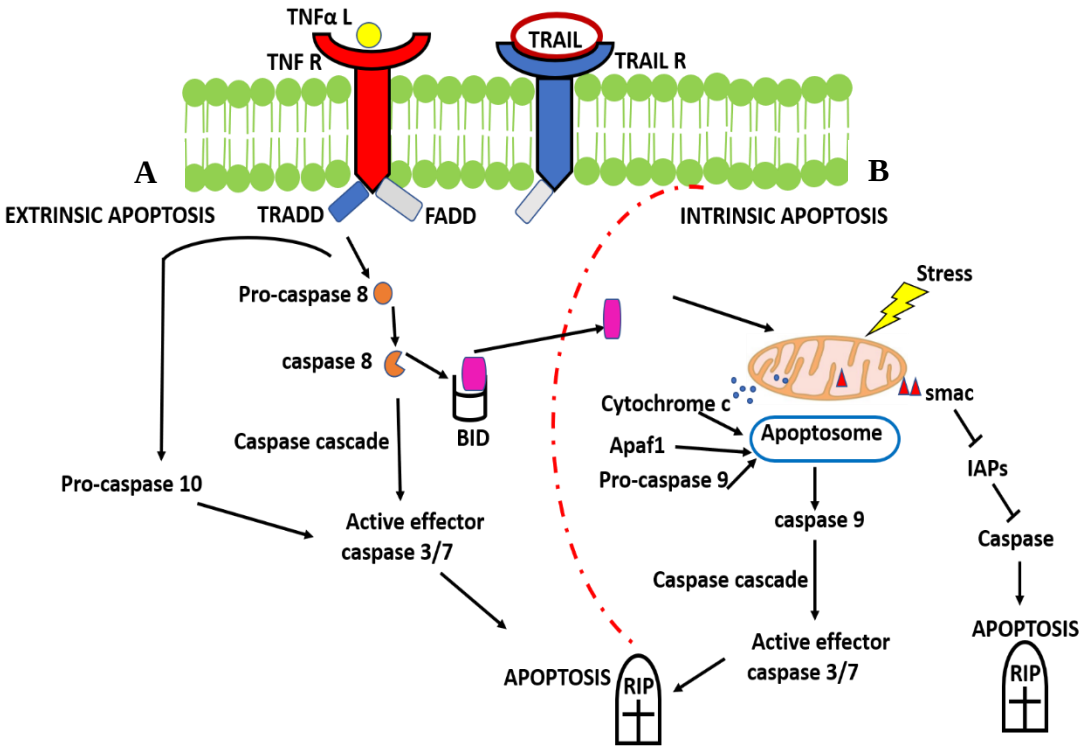

Figure 1.1: **Schematic representation of extrinsic and intrinsic apoptosis pathways.** (A) Extrinsic pathway: Binding of external ligand induces downstream signalling cascade via TRADD and FADD forming DISC complex that activate pro-caspase 8/10. Active caspase 8/10 further induces caspase cascade to activate effector caspase 3/7 which execute apoptosis. (B) Intrinsic pathway: Induced upon DNA damage due to different stress inducers which causes disruption of mitochondrial membrane to release Cytochrome C. Cytochrome C along with Apaf1 and pro-caspase 9 forms apoptosome which further activates Caspase 9. Active caspase 9 then trigger the caspase cascade ultimately activating effector caspases 3/6/7 and apoptosis is executed.

1.2.3 Anti- and pro-apoptotic proteins

The process of apoptosis is mostly bypassed due to the expression of anti-apoptotic proteins. For example, Bcl-xL is a member of the Bcl-2 family and is known to inhibit the intrinsic apoptotic pathway by protecting the integrity of the mitochondrial membrane.[76] It interacts with pro-apoptotic proteins BAX and BAK and impairs the pore formation on the mitochondrial membrane.[77] This inhibits the release of cytochrome c from mitochondria which then restrict the apoptosome formation and procaspase 9 activations.[78] As a result, the downstream executioner caspases eg: Caspase-3,-6, and -7 are not activated, which are important players for both intrinsic and extrinsic apoptotic pathways. I have discussed in the extrinsic apoptosis section above that FADD is involved in the DISC complex formation when death-ligand binds to a death receptor. Thus FADD plays an important role in the activation of procaspase 8/10 which are important upstream mediators of the extrinsic apoptotic pathway. Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) is another anti-apoptotic protein that interacts with FADD and impairs the activation of caspase 8/10. This results in the inhibition of the extrinsic apoptotic pathway.[79] There are multiple splice variants of cFLIP like c-$FLIP_L$, c-FLIPs, and c-FLIPR[80], and they are dysregulated in several malignancies.[81] IAPs I mentioned earlier, are characterized by the presence of the baculovirus IAP repeat (BIR) domain which is a large zinc-binding domain that is implicated in the protein-protein interactions with caspases.[82] All IAPs should contain the BIR domain but all BIR domain-containing proteins are not categorize under IAP. BIR domains are known to interact with the caspases, IAP-antagonists like Smac/Diablo, TNF receptor (TNFR) associated factor (TRAF1/2), and TAB1 which is an upstream adaptor of the transforming growth factor-β activated kinase 1 (TAK1). IAPs also

bear ubiquitin associated domain (UBA), a really interesting new gene (RING) domain that contains E3 ubiquitin ligase activity and caspase activation recruitment (CARD) domain.[83,84] cIAP1, cIAP2, survivin, XIAP, BRUCE/Apollon, and Livin are some of the classic examples of IAPs that are important and well characterized.[84,85] XIAP is known to inhibit apoptosis by targetting executioner caspase 3 and 7 and also inhibit apical caspase 9.[86,87] It contains three BIR domains and one RING domain. BIR2 domain of caspase binds in a specific pocket above the caspase-3 and -7 which interferes with the substrate interaction with the active site and inhibits caspases.[88] However, the inhibition of caspase-9 by XIAP is different than its inhibition of the executioner caspases. In the latter case, the BIR3 domain of XIAP binds to the homodimerization surface of the caspase-9 and impairs its activation. The RING domain of caspase is involved in the ubiquitination of caspase-7 and its autoubiquitination.[89-91] Apart from XIAP, cIAP1 and cIAP2 are involved in alleviated intrinsic and extrinsic death signaling.[92] Altogether, IAPs play an important role in inhibiting two major apoptotic pathways i.e. intrinsic and extrinsic pathways.

Unlike anti-apoptotic proteins, the pro-apoptotic proteins favor the process of apoptosis. They belong to the B-cell lymphoma 2 (Bcl-2) family and can be categorized into two types; (i) pro-apoptotic pore-forming proteins which include BAX, BAK, BOK, and (ii) pro-apoptotic BH3 only proteins like BAD, BID, BIK, BIM, NOXA, PUMA, etc. The expression of pro-apoptotic BH3 only proteins like Bim, Bid, Bam, NOXA, and PUMA enhances intrinsic apoptotic signals which then bind to the anti-apoptotic proteins like Bcl2, Bcl-xL, and inhibit apoptosis.[93] Among all these BH3 only proteins, BID serves as a critical link between intrinsic and extrinsic apoptotic pathways. When death-ligand like TNF-α binds onto its receptor, the downstream signaling cascade is activated that

causes caspase-8 to proteolytically cleave BID and activate it.[94] BID then gets cleaved to tBID that activates BAX to get recruited onto the mitochondrial outer membrane. Recruitment of these pore-forming proteins makes outer mitochondrial membrane permeable which then results in the release of cytochrome c. [95,96] Similarly, tBID can also be activated by the FAS or granzyme B pathway that enhances the release of cytochrome c.[97] Hence the fate of the cell whether to commence apoptosis or not is determined by the ratio of pro- and anti-apoptotic protein levels. Most of these anti-apoptotic proteins are expressed during stress conditions to protect the cell from immediate apoptosis.[98] However, when their expression is highly dysregulated, cells become resistant to apoptosis, which is a hallmark of cancer. The expression of these anti-apoptotic proteins is mediated by eIF5B in a cap-independent manner [40,41] which is discussed in the later section.

1.3 eIF5B and its importance in cap-independent translation

eIF5B is a universally conserved eukaryotic translation initiation factor that plays an important role during both cap-dependent and cap-independent translation initiation.[99] This 175 kDa protein is a translational GTPase and mediates the 60S and 40S ribosomal subunit joining during cap-dependent translation.[100] There is evidence that shows that eIF5B play role in IRES-mediated translation.[41] eIF5B is a bacterial homolog of IF2[101] that is shown to bind met-tRNAi in the ribosomal P site during IRES-mediated translation in viruses.[102] Thakor et al., have shown that eIF5B promotes IRES-mediated translation of one of the anti-apoptotic protein XIAP[41] eIF5B and provides critical cell survival switch under eIF2α phosphorylation condition.[41]

1.3.1 Role of eIF5B in the regulation of apoptosis in glioblastoma multiform

As discussed above, eIF5B plays an important role in cap-independent translation initiation. Previously, our lab defined the role of eIF5B in the regulation of apoptosis of glioblastoma multiform (GBM). Ross *et al*. have shown that eIF5B depletion sensitized U343 glioblastoma multiform cells to TRAIL-induced apoptosis *via* a pathway that involves Caspases-8, -9, and -7.[40] They also identified that eIF5B promoted the non-canonical translation of several IRES-containing mRNAs like XIAP, Bcl-xL, cIAP1, and cFLIPs to bypass apoptosis. Further, it was suggested that eIF5B depletion resulted in decreased Nrf2 translation thereby upregulating apoptosis due to enhanced reactive oxygen species. Furthermore, these findings suggest the regulatory role of eIF5B to evade apoptosis in the cancer cells.[40] Similar kind of study was performed in the patient-derived brain tumor stem cells (BTSCs) where it was shown that depletion of eIF5B in BTSC lines BT25 and BT48 sensitized these cells to temozolomide (TMZ).[103] Particularly, eIF5B-depleted BT48 cells were shown to have enhanced TMZ-mediated caspase-3 activation leading to apoptosis along with the substantial decrease in anti-apoptotic proteins.[103] Looking at the fundamental role of eIF5B in regulating apoptosis, I wanted to explore the mechanistic role of eIF5B in another type of cancer. The work in this thesis explains the role of eIF5B in Head and Neck Squamous Cell Carcinoma survival.

1.4 Head and Neck Squamous Cell Carcinoma (HNSCC)

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer in the world and causes 350,000 cancer death annually.[104] Among all types of Head and Neck malignancies, the most common (90%) is squamous cell carcinoma (SCC). It

involves the cancer of the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, paranasal sinuses, and salivary glands out of which the cancer of oral cavity and oropharynx are the most common. Although there are several advances made in the treatment of HNSCC with respect to surgery and radiation therapy, the 5-year survival rate of the patients with HNSCC is modestly increased along with the recurrence rate of the disease.[105] HNSCC is the highly malignant tumors that show extensive invasive phenotype in the surrounding tissues along with the ability of distant metastasis.[106,107] My research here is focused on understanding the mechanistic aspects of non-canonical translation initiation during HNSCC progression that can help in possible targetted therapies. The aggressiveness of HNSCC is not only associated with the biology of the tumor cell but also associated with the extracellular microenvironment. The hypoxic condition experienced by cancer cells results in the aggressive phenotype of HNSCC and is thought to be associated with a high rate of metastasis and recurrence.[108] HNSCC tumors show a very high rate of genetic heterogeneity.[109] As a result of a loss of function mutation in the tumor suppressor genes like p53 and $p16^{INK4a}$ and gain of function mutation of oncogenes like the epidermal growth factor receptor (EGFR), [110] and PIK3CA[111] is very common in HNSCC. EGFR, NOTCH, and MET signaling are regularly altered in HNSCC.[112,113] As a result, downstream RAS/RAF/ERK, PI3K, and JAK/STAT pathways get dysregulated and hence promote proliferation, migration, and cell survival in HNSCC.[112] Nearly 80% of the Human Papilloma Virus (HPV)-negative HNSCC have p53 mutation[114,115] and mutation in both *TP53* and Retinoblastoma (Rb) triggers cancer cell replication.[116] Cancer stem cell trait is maintained by NOTCH1 signaling that is responsible for recurrence and metastasis through Wnt signalling[117] in HNSCC. Overall, HNSCC has diverse biology that hinders the

development of targetted therapies. Hence it is important to expand our knowledge in understanding the molecular mechanism in HNSCC biology in more depth to overcome these challenges.

Studies by Yao.Z and *et.al* (2017) have shown that eIF5B interacts with EGFR.[118] Recent studies by Ross *et al* with glioblastoma cell lines (for example, U343 and U251N) shed light on the EGFR regulation via eIF5B. The knockdown studies of eIF5B showed a decrease in the phosphorylation status of EGFR suggesting the role of eIF5B in EGFR activation.[40] It is evident from the literature that EGFR activation is linked to NF-κB-dependent transcription[119] and that NF-κB is involved in enhanced expression of pro-and anti-apoptotic genes like XIAP,cIAP1/2, and cFLIP. Further, our lab findings also showed a decrease in activation of NF-κB upon eIF5B depletion with in cellulo studies. Altogether, these findings suggest that eIF5B is indirectly involved in regulating these multiple pathways which play an important role in cancer cell survival and progression. Therefore, in my study, I intend to understand the role of eIF5B in the survival of HNSCC cells.

1.5 Hypothesis and Objectives

Certain mRNAs are translated *via* IRES-mediated translation by a cap-independent translation initiation mechanism. As mentioned earlier, eIF5B positively regulates IRES-mediated translation during stress conditions.[99] Recently published studies demonstrate that the non-canonical translation of several IRES bearing mRNAs that encodes anti-apoptotic proteins like XIAP, Bcl-xL, cIAP1, and cFLIPs is regulated by eIF5B in GBM.[40] Besides the role of eIF5B in IRES-mediated translation, it was shown that eIF5B depletion sensitizes GBM cells to TRAIL-induced apoptosis. Previously

published data also demonstrate that eIF5B acts as a regulatory node for cells to evade apoptosis as it majorly promotes IRES-mediated translation of mRNA encoding stress response proteins. I hypothesize that eIF5B plays a critical role in the survival of HNSCC cells *via* regulating the translation of mRNA encoding anti-apoptotic proteins. In this regard, I aim 1) to determine if the depletion of eIF5B enhance the sensitivity of HNSCC cells for TRAIL-induced apoptosis; 2) to determine if the levels of anti-apoptotic proteins are decreased in eIF5B-depleted HNSCC cells, and 3) to determine if the depletion of eIF5B affects the translation of mRNAs encoding anti-apoptotic proteins.

CHAPTER 2

Materials and methods

2.1. Cell culture and reagents

In this project, three different types of oral squamous cell carcinoma (OSCC) cell lines like Centre Antoine Lacassagne-33 (Cal-33), University of Michigan-Squamous Cell Carcinoma-59 (UM-SCC-59) and University of Michigan-Squamous Cell Carcinoma-29 (UM-SCC-29) were used. These cell lines were obtained from Dr. Pinaki Bose, Departments of Biochemistry and Molecular Biology and Oncology, Cumming School of Medicine, University of Calgary.

2.2 Culturing/maintenance of OSCC and transfection

Three different types of mammalian OSCC cell lines; Cal-33, UM-SCC-59, and UM-SCC-29 were revived in Dulbecco's Modified Eagle Medium (DMEM; HyClone) with 4 mM L-glutamine, 4500 mg/L glucose, and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) in 10 cm dish. The cell lines were supplemented with extra 1 mL serum during their revival. Further, cells were incubated at 37 ºC in 5% CO_2. Once cells reached 70% confluency, trypsin (Sigma) was used to collect cells and 300,000 cells were passaged to a new 10 cm plate for further use. 70-75% confluent plates for Cal-33, UM-SCC-29, and UM-SCC-59 were used for siRNA transfection to perform knockdown in these cell lines. Non-specific control siRNA (siC) was obtained from Qiagen. Stealth RNAi^{TM} siRNAs targeting eIF5B (HSS114469/70/71) were obtained from Invitrogen. TRAIL was obtained from Millipore-

Sigma and Inc. z-VAD-fmk from Promega. Necrostatin-1 and calpain inhibitor III were purchased from Millipore-Sigma.

2.3 *In vitro* cell viability assay

Once the cells reached around 70-75% of confluency, they were trypsinized, and 3,000 cells/well of Cal-33; 7500 cells/well of UM-SCC-59 and 2500 cells/well of UM-SCC-29 were seeded in 96 well plates for transfection. Reverse transfection using Lipofectamine RNAiMAX (Invitrogen) was carried out by adding corresponding siRNAs and Opti-MEM according to the manufacturer's protocol. This mixture was incubated at room temperature for approximately 25-30 min. Further, upon the addition of the transfection mix, the plate was incubated at 37 ºC in 5% CO_2 for 48 hours for the cells to settle. After 48 hours, the OSCC cells were treated with a pro-death cytokine TRAIL (Millipore-Sigma) or with vehicle control (DMSO) and incubated at 37 ºC to see the additional effect on cell viability after eIF5B knockdown. Cal-33 was treated with inhibitors as stated, for 2 hours with z-VAD-fmk (Promega), Necrostatin-1 (Millipore-Sigma.), Calpain Inhibitor III (Millipore-Sigma.), before adding DMSO or TRAIL. After a further 72 hours of incubation, cell viability was determined by alamarBlue assay (Resazurin sodium salt; Sigma-Aldrich). Fluorescence (excitation, 560 nm; emission, 590 nm), measured in a Cytation 5 plate imager (BioTek) and data were normalized to vehicle treatment.

2.4 Bright-field and fluorescence microscopy

200,000 cells/well of Cal-33 cells were seeded and reverse-transfected in 6-well plates. After 72 hours of incubation, cells were treated with vehicle control (DMEM) or

TRAIL (100 ng/mL) for a further 4 hours. Next, cells were rinsed with PBS, and thereafter general DNA stain (1 µg/mL Hoechst 33342; Thermo Scientific), was added onto the cells in PBS. Finally, cells were imaged at 20x magnification in a Cytation 5 plate imager. For fluorescence microscopy, cells were imaged using a DAPI filter to analyze Hoechst-stained nuclear DNA.

2.5 Western blotting

Cal-33 cells were seeded at 100,000 cells/well; UM-SCC-59 were seeded at 300,000 cells/well; UM-SCC-29 were seeded at 100,000 cells/well and reverse transfection was performed in 6-well plates. After 96 hours of incubation, the cells were harvested in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 0.05% SDS, protease inhibitors and phosphatase inhibitors). The cells were then lysed on ice for 30 min, vortexing after every 10 min. The lysate was then subjected to centrifugation for 10 min at 10,000g, 4^0C to remove any cell debris. The Bradford assay was then used to determine the concentration of the protein in the lysate. Further, an equal amount of protein (20 µg per well) was loaded and resolved using SDS-PAGE and transferred onto nitrocellulose membranes (GE healthcare). The membrane was then subjected to blocking with 10% skimmed milk in 1X PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 1% tween 20) for 1 hour. After blocking, the membrane was incubated with primary antibody overnight at 4^oC. Each protein was detected separately by immunoblotting with different antibodies specific to eIF5B, XIAP, Bcl-xL, cIAP1, cFLIP, and β-actin (Table 2. 1). The next day the blot was incubated with the secondary antibody for 1 hour at room temperature and the primary antibodies were

detected with anti-rabbit HRP conjugate (Abcam) in an AI600 imager (GE) and densitometry performed using the AI600 analysis software.

Table 2.1: List of Antibodies target, company, dilution used in the study and catalogue number.

Antibody Target	Company	Catalogue number	Dilution
Secondary: Goat anti-rabbit-HRP conjugate	Abcam	ab97051	1:5000
eIF5B	ProteinTech	13527-1-AP	1:1000
β-actin (hFAB Rhodamine)	Bio-Rad	12004163	1:1000
cIAP1	Abcam	ab108361	1:1000
Bcl-xL	Cell Signalling Tech.	2762	1:1000
c-FLIP$_{L/S}$	Cell Signalling Tech.	56343	1:1000
XIAP	Cell Signalling Tech	2045	1:1000

2.6 Polysome profiling

Cal -33 cells were seeded at 250,000 cells per well of 6 well plate and reverse transfected in two 6 well plates per condition. After 96 hours, the control or eIF5B depleted cells were pooled, lysed in RNA lysis buffer (1.1 mL basic solution, 110 µL 20% Triton X100, 2 µL SUPERase-In, 5.5 µL cycloheximide.[120] The lysate was then transferred to pre-chilled 1.5 mL tubes, and incubated on ice for 30 min, vortexing every ~10 min. The lysate was centrifuged at 2,000 x g, 4 °C, 5 min, and the supernatant was collected to a fresh pre-chilled 1.5 mL tubes. Centrifuge again at 17,000 x g, 4 °C, 5 min to pellet remaining cell debris. The supernatant (~550 µL) was collected to a fresh set of pre-chilled tubes which is the RNA lysate. Further, these samples were subjected to polysome profiling in 10-15% sucrose density gradients.[120] Gradients were fractionated using a BR-188 density gradient fractionation system (BRANDEL). The samples were then subjected to ultracentrifugation at 39,000 rpm (260,343 x g) for 1.5 hours at 4 °C.

Total RNA was isolated using New England Biolabs Monarch Total RNA Miniprep kit following the manufacturer's instructions.

2.7 qPCR

RNA was isolated using a New England Biolabs Monarch Total RNA Miniprep kit (#T2010S), and cDNA was generated from equal volumes of RNA using the qScript cDNA synthesis kit (Quanta Biosciences). Quantitative PCR was performed in a CFX-96 real-time thermocycler (Bio-Rad) with PerfeCTa SYBR Green SuperMix (Quanta Biosciences) according to the manufacturer's instructions. RT-qPCR was performed in technical duplicates for the negative control without template DNA (primer control) as well as each cDNA using the following cycle conditions: 1 cycle at 95℃ for 3 min followed by 45 cycles of 95°C for 15s, the annealing temperature (Table 2.2) for 35s, and 72°C for 1 min. A melting curve step was added to check the purity of the PCR product. This step consisted of a ramp of the temperature from 65 to 95°C at an increment of 0.5°C and a hold for 5 seconds at each step. Expression levels of mRNAs encoding XIAP, Bcl-xL, cFLIPs were normalized to relative to β-actin mRNA and were determined using the ΔCt method.

Table 2.2: List of different primers used for qPCR studies with their annealing temperatures and sources

Target	Primer	Sequences (5' to 3')	Annealing Temp (^{0}C)	Reference or Source
Bcl-xL	-	QuantiTect primer assay, QT00236712	55	Qiagen
XIAP	-	QuantiTect primer assay, QT00042854	55	
cFLIPs	Fwd Rev	GATGTTGCTATAGATGTGGTTC ATTTCCAAGAATTTTCAGATCAGGA	47	121
β-actin	Fwd Rev	TCACCCACACTGTGCCCATCTACGA TGAGGTAGTCAGTCAGGTCCC	55	122

2.8 Statistical analyses

Unless otherwise specified, all quantitative data represent the mean ± standard error on the mean (SEM) for at least 3 or max 6 independent biological replicates. Statistical significance was determined by an unpaired, two-tailed t-test without assuming equal variance. The significance level was set at a p-value of 0.05. Data were analyzed using GraphPad Prism, version 7.

2.9 Bioinformatics analysis

Kaplan-Meier survival plot showing HNSCC patient survival against time concerning *EIF5B* mRNA levels was obtained from our collaborator Dr. Pinaki Bose, Assistant professor, from Departments of Oncology, Biochemistry and Molecular Biology at the University of Calgary. *EIF5B* mRNA levels across 17 cancer types were examined in The Cancer 127 Genome Atlas (TCGA) cBioportal (http://www.cbioportal.org/).[123]

CHAPTER 3

Results

3.1 Higher levels of eIF5B mRNA correlate with the worst patient's outcome in HNSCC.

To understand the role of eIF5B in HNSCC survival, I first wanted to check if higher eIF5B mRNA levels correlate with the poor patient's survival in HNSCC patients. To accomplish this task I secured help from Dr.Pinaki Bose, Assistant professor, from Departments of Oncology, Biochemistry, and Molecular Biology at the University of Calgary with some bioinformatics analysis. Using cbioportal *EIF5B* mRNA levels were examined across 17 cancer types in The Cancer 126 Genome Atlas (TCGA). This data suggest that eIF5B is expressed at the higher levels in all the different cancers including HNSCC (Figure 3.1**)**. Based on this fact, we further assessed the survival probability of HNSCC patients with above-median and below-median *EIF5B* mRNA expression using a Kaplan-Meier plot. In figure 3.2, the Y-axis indicates the probability of HNSCC patients surviving and the X-axis indicates the survival time in days. The survival probability of the group expressing above-median *EIF5B* and below-median *EIF5B* at the time zero is 1.00 or 100%. However, the survival curve dropping towards zero represents poor survival. It is clear from the graph that the group with the below-median *EIF5B* expression shows a better survival probability in comparison to the group expressing the above-median EIF5B. This data reveals that the higher *EIF5B* mRNA expression correlates with poor patient survival in HNSCC. Therefore to further extend my understanding of the role of *EIF5B* in translation regulation, I chose to work with oral squamous cell carcinoma cell lines that fall under the HNSCC category.

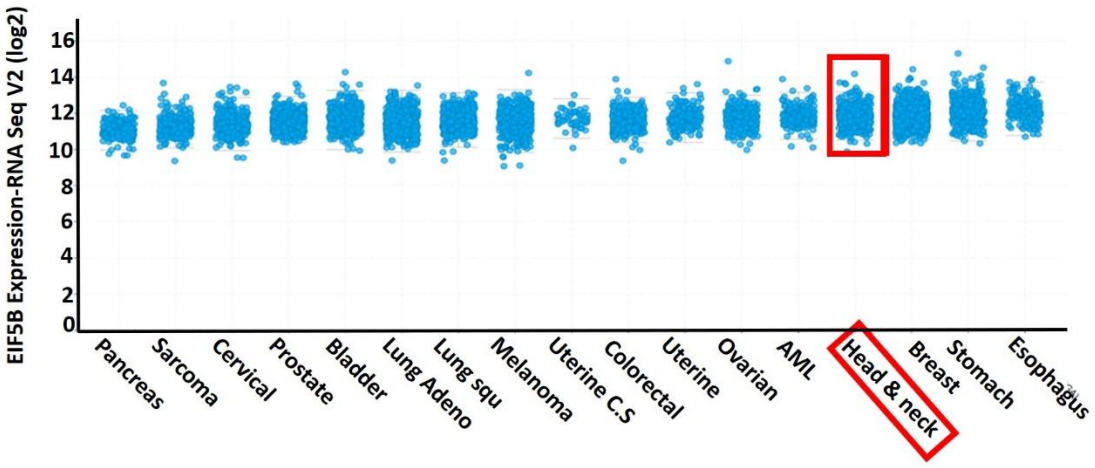

Figure 3.1: Median expression of *EIF5B* mRNA in different cancer types. Increasing median expression of *EIF5B* mRNA in 17 different cancer types in The Cancer 126 Genome Atlas (TCGA) using cbioportal, suggesting that EIF5B is not lost in HNSCC.

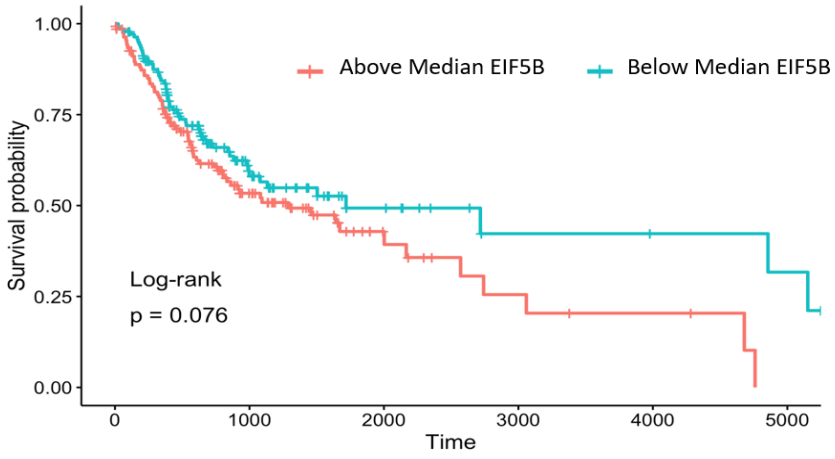

Figure 3.2: Kaplan-Meier plot representing patient survival with respect to *EIF5B* mRNA levels. Kaplan-Meier survival curves portraying survival probability of the HNSCC patients with higher *EIF5B* mRNA levels with respect to time (in days) corelates with the poor patient survival probability in HNSCC over the time.

3.2 eIF5B depletion enhanced the sensitivity of Cal-33 cells to TRAIL-induced apoptosis.

It is known that eIF5B plays a pro-survival role by mediating the non-canonical translation of mRNA of several anti-apoptotic proteins in GBM.[40] To examine the effect of eIF5B depletion on the viability of OSCC cells, I knocked down eIF5B in Cal-33, UM-SCC-29, and UM-SCC-59 cells. Knockdown conditions for a particular protein (in this case its eIF5B) can vary from cell line to cell line and hence require proper optimization of time. Therefore, to optimize the time for an efficient knockdown, I seeded 3,000 cells/well of Cal-33 and 2,500 cells/well of UM-SCC-29 in 96 well plates. eIF5B knockdown in UM-SCC-59 cells was checked by seeding 300,000 cells/well in 6 well plates at different time points. UM-SCC-29 and Cal-33 cells were each harvested at 24 hrs, 48 hrs, 72 hrs, and 96 hrs and it was observed that the knockdown peaks at 48 hrs (Figure 3.3 A and B). UM-SCC-59 cells were harvested at 48 hrs, 72 hrs, and 96 hrs because UM-SCC-59 cells showed successful knockdown only after 48 hrs (Figure 3.3C). Western blot analysis with the lysates from all the three OSCC cell lines confirmed efficient eIF5B knockdown after 48 hours. (Figure 3.3 A, B and C). Hence, this knockdown time was used to perform all the further cell viability experiments in these cell lines for my work.

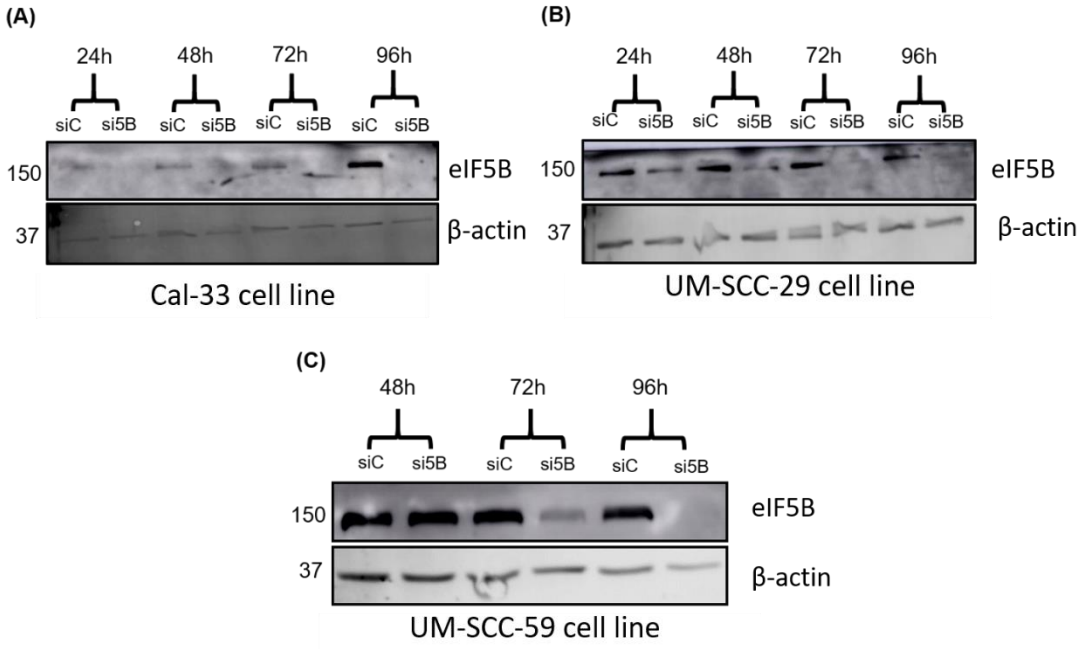

Figure 3.3: Optimization time to knockdown eIF5B in OSCC cells. As mentioned in section 2.3, OSCC cell lines were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B). Cal-33 cells (a) and UM-SCC-29 cells (b) were incubated at different time points like 24 hours, 48 hours, 72 hours and 96 hours in 96-well plate. However, UM-SCC-59 cells were incubated for 48 hours, 72 hours and 96 hours in 6-well plate (c). Further, cells were harvested in RIPA lysis buffer, and 20 µg of total protein resolved by SDS-PAGE before performing immunoblotting.

Next, to investigate if eIF5B knockdown affects the OSCC cell viability, I performed alamarBlue assay. I depleted eIF5B from Cal-33, UM-SCC-59, and UM-SCC-29 cells as mentioned in section 2.3. The alamarBlue assay revealed that the silencing of eIF5B alone in the absence of TNF-related apoptosis-inducing ligand (TRAIL) treatment did not cause a significant effect on the cell viability (Figure 3.4 A). The previously optimized conditions were used for the viability experiment to knockdown eIF5B and cell lysate from the

respective cell lines were used for Western blotting to confirm successful eIF5B knockdown (Figure 3.4 B). Further, I wanted to test if the silencing of eIF5B would sensitize the OSCC cell line upon treatment with a pro-death compound. To this end, I treated the control and eIF5B-depleted OSCC cells with TRAIL and determined OSCC cell viability (Figure 3.5). For this experiment, I seeded and reverse-transfected OSCC cells separately as per the protocol in section 2.3. After 48 hours, the eIF5B-depleted OSCC cells were treated with TRAIL and were incubated for another 72 hours following alamarBlue addition. All three OSCC cell lines were sensitive to TRAIL treatment alone without eIF5B removal. However, the depletion of eIF5B from Cal-33 cells further enhanced their sensitivity to TRAIL treatment (Figure 3.5 A). Similarily, UM-SCC-29 cells showed increased sensitivity to TRAIL treatment upon eIF5B depletion (Figure 3.5 B). Interestingly, the cell viability of eIF5B-depleted UM-SCC-59 cells did not decrease upon TRAIL treatment as compared to si-Control with TRAIL (Figure 3.5 C). Overall, this data suggests that the sensitivity of eIF5B-depleted Cal-33 and UM-SCC-29 cells to TRAIL was enhanced similar to what was observed in the GMB cells.[40]

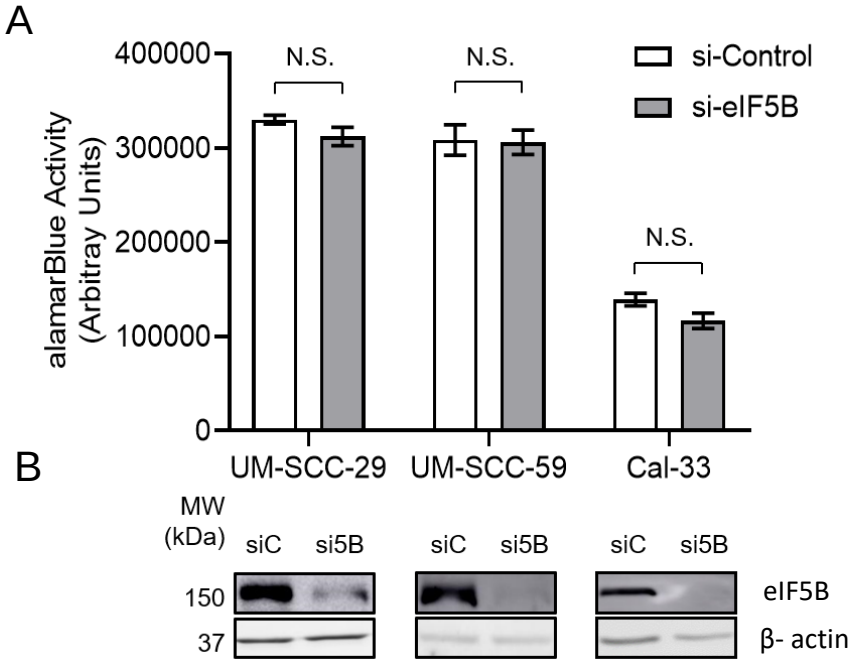

Figure 3.4: eIF5B depletion from OSCC cells does not affect the viability. (A) OSCC cell line UM-SCC-29, UM-SCC-59 and Cal-33 were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B), incubated 96 hours and alamarBlue assay was performed. No significant decrease in cell viability was observed in OSCC cells indicating that eIF5B depletion did not affect cell viability (A). OSCC cells were treated similarily as in panel (A) however after 96 hours of incubation, cells were harvested in RIPA lysis buffer and 20 µg of total protein resolved by SDS-PAGE before performing immunoblotting. Panel (B) Represents images of immunoblots probed for eIF5B in all the three OSCC lines with β-actin as a loading control.

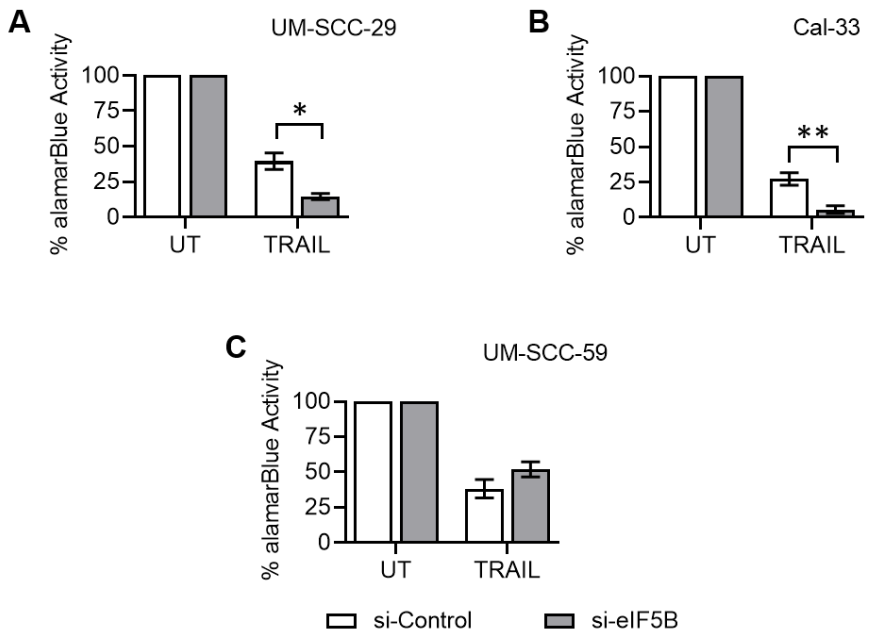

Figure 3.5: eIF5B depletion enhanced sensitivity of two cell lines to TRAIL-induced cell death. UM-SCC-29 cells as mentioned in section 2.3 were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B) and percent viability was measured using alamarBlue assay (A). UM-SCC-29 cells showed further decrease in cell viability on TRAIL treatment in eIF5B knockdown condition. Decrease in cell viability of Cal-33 cells was enhanced upon eIF5B knockdown and TRAIL treatment (B). UM-SCC-59 cells did not show enhancement in sensitivity to TRAIL under eIF5B depletion condition (C). The data here are expressed as mean ± SEM for at least 3 independent biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

3.3 eIF5B-depletion from OSCC cells did not enhance their sensitization to Cisplatin treatment

It is known that radiation therapy and chemotherapy are administered in combination to obtain a high cure rate in advanced HNSCC.[124] Studies have shown that the combination of chemotherapeutic agent cisplatin and fluorouracil (PF) in HNSCC patients have proved significantly beneficial for reducing the distant metastasis.[125] Cisplatin is a DNA damaging agent that crosslinks with the purine bases by binding to N7 reactive purines thereby blocking cell division. Cisplatin also interferes with the repair mechanism of DNA, ultimately resulting in tumor cell death.[126] Ross *et al*. have shown that eIF5B depletion sensitizes brain tumor stem cells (BTSC) to frontline therapeutic agent temozolomide (TMZ).[103] TMZ is a chemotherapeutic agent that induces intrinsic apoptotic cell death in the cells. TMZ is an alkylating agent that delivers methyl group to purine bases in DNA (O6-guanine) that activates mismatch repair thereby introducing DNA breaks that are lethal for the cell.[127] The similarity between both cisplatin and TMZ is that they activate the intrinsic apoptotic pathway in the cells. Since cisplatin is used in the treatment of HNSCC, I wanted to check if eIF5B depletion sensitizes OSCC cells to cisplatin treatment. Hence, OSCC cells were transfected to deplete eIF5B with control and eIF5B siRNAs (section 2.3). Further, the cells were treated with different concentrations of cisplatin for 72 hours and the cell viability was measured. Unlike eIF5B-depleted BTSCs, eIF5B-depleted UM-SCC-29 and UM-SCC-59 cells did not show enhanced sensitization of these cells to a frontline chemotherapeutic agent. When treated with the different concentrations of cisplatin, the IC50 value of eIF5B-depleted UM-SCC-29 and UM-SCC-59 was higher as compared to control. This data reveals that the depletion of eIF5B did not increase the

sensitivity of UM-SCC-29 and UM-SCC-59 cells upon treatment with cisplatin (Figure 3.6). However, the IC50 value of eIF5B-depleted Cal-33 cells decreased under cisplatin treatment compared to si-Control. This data suggests the enhanced sensitization of eIF5B-depleted Cal-33 cells to cisplatin however, the further validation of this data is still required. Together this data indicates that although OSCC cells are sensitive to cisplatin treatment, unlike Cal-33 cells, their sensitivity was not enhanced further under eIF5B depletion condition for UM-SCC-29 and UM-SCC-59 cell lines.

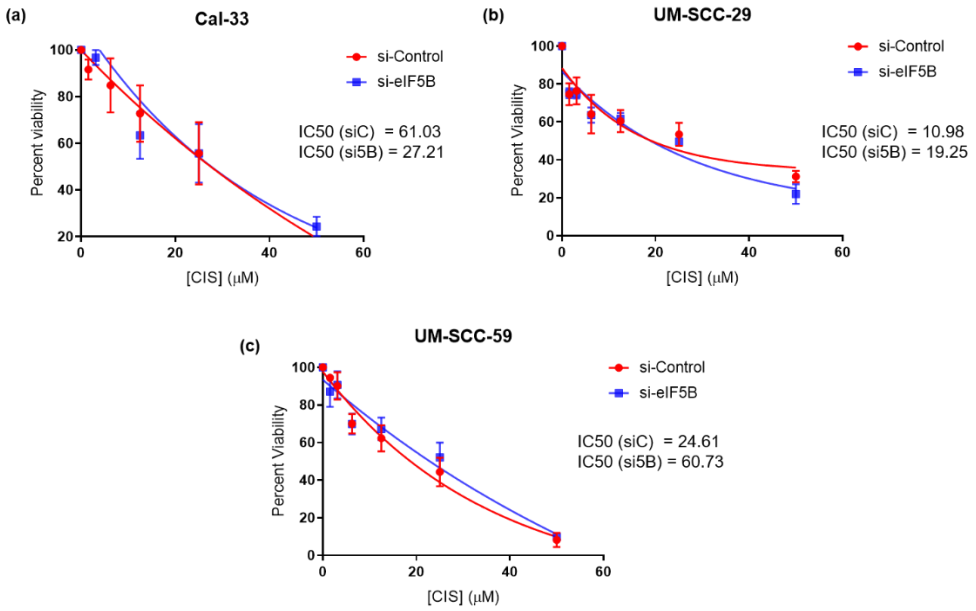

Figure 3.6: Depletion of eIF5B enhanced the sensitization of Cal-33 cells but did not enhanced the sensitivity of UM-SCC-29 and UM-SCC-59 cells for cisplatin. OSCC cell lines were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B) and percent viability was measured using alamarBlue assay. OSCC cell lines were treated with cisplatin in varying concentration of 20uM, 40uM and 60uM for 72 hours. eIF5B-depleted Cal-33 cells were further sensitized to cisplatin treatment (a). However, eIF5B-depleted UM-SCC-29 and UM-SCC-59 cells were not further sensitized to Cisplatin treatment (b and c). Data are expressed as mean ± SEM for at least 3 of each (a and b) and mean ± SEM for at least 2 for UM-SCC-59 (c) independent biological replicates.

3.4 TRAIL-mediated apoptosis upon eIF5B depletion in Cal-33 is caspase-dependent.

My experiments so far with OSCC cell lines have shown that at least two cell lines; Cal-33 and UM-SCC-29 get further sensitized with eIF5B knockdown upon TRAIL treatment (Figure 3.5 A and B). Next, I wanted to narrow down my studies to decipher the mechanism of the decrease in cell viability in these cell lines. To this end, I chose the Cal-33 cell line to accomplish my aim to understand the cell death mechanism in these cells on eIF5B depletion in combination with TRAIL treatment. Previously published literature has shown that eIF5B depletion in GBM cells sensitizes them to caspase-8/-9 dependent apoptotic pathway.[40] However, it is known from the literature that TRAIL utilizes other mechanisms like necroptosis and autophagy apart from apoptosis to induce cell death.[128,129] Therefore, I wanted to investigate if the cell death phenotype that was observed in the Cal-33 cells was due to apoptosis or the other parallel cell death mechanisms that TRAIL can induce. To confirm the cell death phenotype, first, a bright-field microscopy experiment was conducted. Membrane blebbing and cell shrinkage that are the characteristic features of apoptosis were observed in the bright field microscopy (Figure 3.7 A). Further, TRAIL-treated+ eIF5B depleted Cal-33 cells showed an increased nuclear fragmentation when they were subjected to Hoechst live-cell nuclear staining (Figure 3.7 B). The microscopy data suggested that the viability phenotype observed in Cal-33 cells is due to apoptosis.

To further confirm the cell death phenotype, I performed alamrBlue assay by pre-treating eIF5B-depleted Cal-33 cells with different cell death pathway inhibitors. After 48 hours of eIF5B knockdown, the Cal-33 cells were treated with different cell death pathway inhibitors like z-VAD-fmk which is a pan-caspase inhibitor, RIP1 kinase inhibitor

Necrostatin 1 and calpain inhibitor III for 2 hours. Following this, the cells were treated with TRAIL and further incubated for 72 hours. The cell viability result revealed that eIF5B depleted +TRAIL treated Cal-33 cells reverted the cell death phenotype in the presence of z-VAD-fmk relative to vehicle control. This confirmed that eIF5B depletion enhances TRAIL-induced apoptosis in Cal-33 cells (Figure 3.8). Conversely, the cell death phenotype was not abrogated in the eIF5B-depleted Cal-33 cells in the presence of Necrostatin 1, or calpain inhibitor III. Overall this data suggests that eIF5B depletion in Cal-33 cells enhanced their sensitization to TRAIL-induced apoptosis.

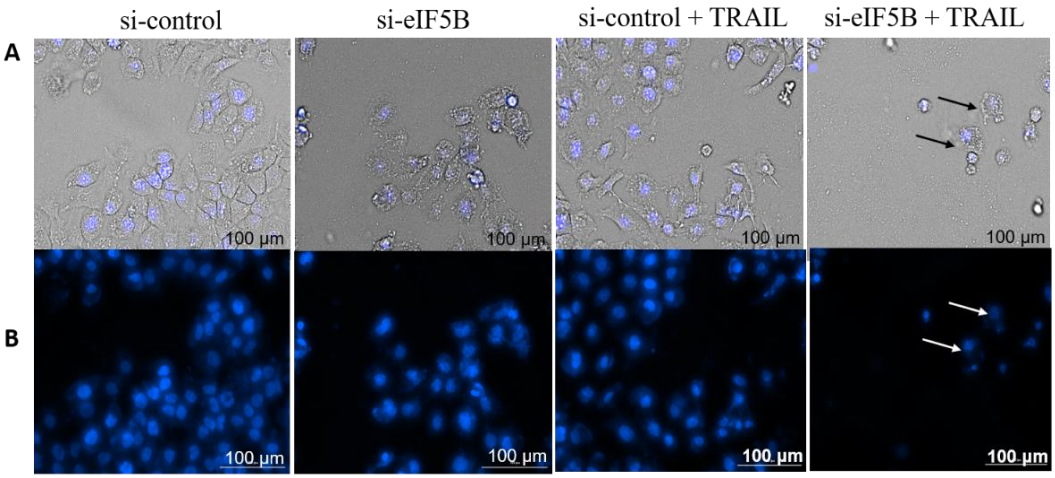

Figure 3.7: Cal-33 cells exhibit TRAIL-induced apoptosis morphology upon EIF5B depletion. Cal-33 cells as mentioned in section 2.3 were reverse-transfected with a non-specific control siRNA (siC), EIF5B-specific siRNA pool (si5B) in 6-well plate. EIF5B-depleted Cal-33 cells were treated with TRAIL for 4 hours and subjected to microscopy. Cal-33 cells were imaged at 20 x magnification by bright field microscopy showed characteristic features of apoptosis (A). Cal-33 cells were imaged for Hoechst-stained nuclear DNA (blue) analysis by fluorescence microscopy (B).

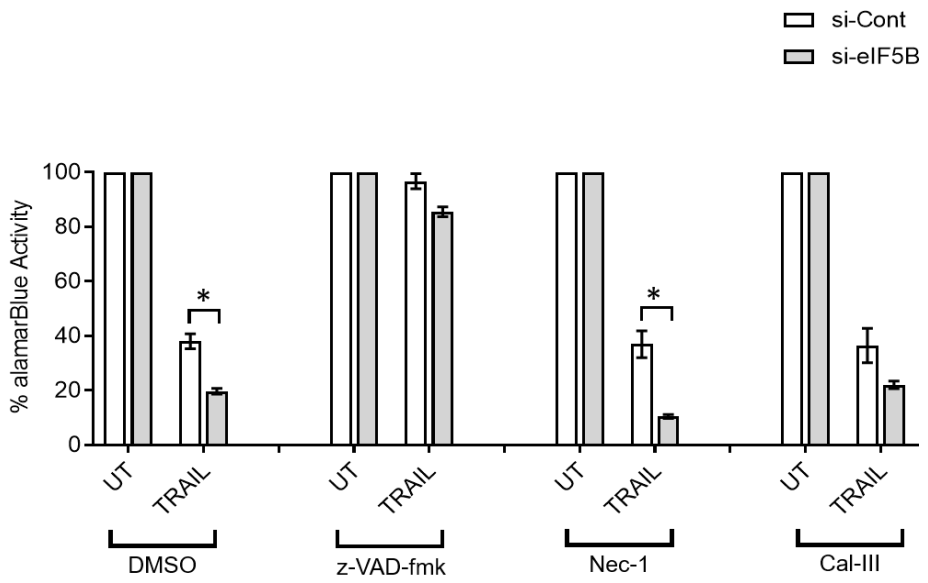

Figure 3.8: EIF5B depletion enhanced the sensitization of Cal-33 cells to TRAIL-induced apoptosis. EIF5B-depleted Cal-33 cells were reverse transfected using a pool of three EIF5B-specific siRNAs and a non-specific control siRNA. After 48 hours of knockdown, cells were treated for 2 hrs with the cell death pathway inhibitors like z-VAD-fmk; Nec-1 and Calp III as shown above. Further, cells were treated with TRAIL following the inhibitors treatment, and were incubated for 72 hrs. Post 72 hrs, alamarBlue was added for 16 hrs and cell viability was checked. Caspase specific z-VAD-fmk inhibitors abrogated the cell death phenotype in EIF5B/TRAIL treated Cal-33 cells. Data expressed as mean ± SEM for at least 2 independent biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

3.5 Levels of Bcl-xL, XIAP, and cFLIP$_L$ decrease in eIF5B-depleted Cal-33 cells but not in UM-SCC-59 cells.

During stress conditions, IRES-mediated translation of certain mRNAs is eIF5B dependent.[40,41] One of the classic examples I have discussed above is the IRES-mediated

translation of anti-apoptotic protein XIAP in HEK293T cells.[41] Previously published data also revealed that the levels of anti-apoptotic proteins XIAP, Bcl-xL, $cFLIP_{L,}$ and cFLIPs, cIAP1 decreased in eIF5B-depleted U343 cells. Here, I have shown that the levels of anti-apoptotic proteins Bcl-xL, cFLIPL, and XIAP decrease upon eIF5B-depletion in Cal-33 cells (Figure 3.9). Some of these anti-apoptotic proteins like cIAP1 and XIAP are the IAPs whose mRNA bears the IRES element which helps in their translation when a global translation is attenuated.[41] To investigate the effect of eIF5B depletion on the anti-apoptotic protein levels in OSCC, I used cell lysates from UM-SCC-59 and Cal-33 cell lines to performed Western blotting. The Western blot analysis showed a robust decrease in the levels of anti-apoptotic proteins Bcl-xL and $cFLIP_L$ upon eIF5B depletion in Cal-33 cell lines. The levels of XIAP and cFLIPs were moderately decreased. However, the levels of cIAP1 did not change significantly upon eIF5B-depletion (Figure 3.9). This variation in the decreased levels of different anti-apoptotic proteins upon eIF5B depletion is plausible because the IRES-mediated translation may be cell line-specific. However, similar to cell viability data, eIF5B depletion in UM-SCC-59 cell line did not show any decrease in the levels of anti-apoptotic proteins Bcl-xL, cFLIP, cIAP1 and XIAP (Figure 3.10). These findings suggest that depletion of eIF5B affects the levels of anti-apoptotic proteins Bcl-xL, cFLIP, and XIAP in Cal-33 cells.

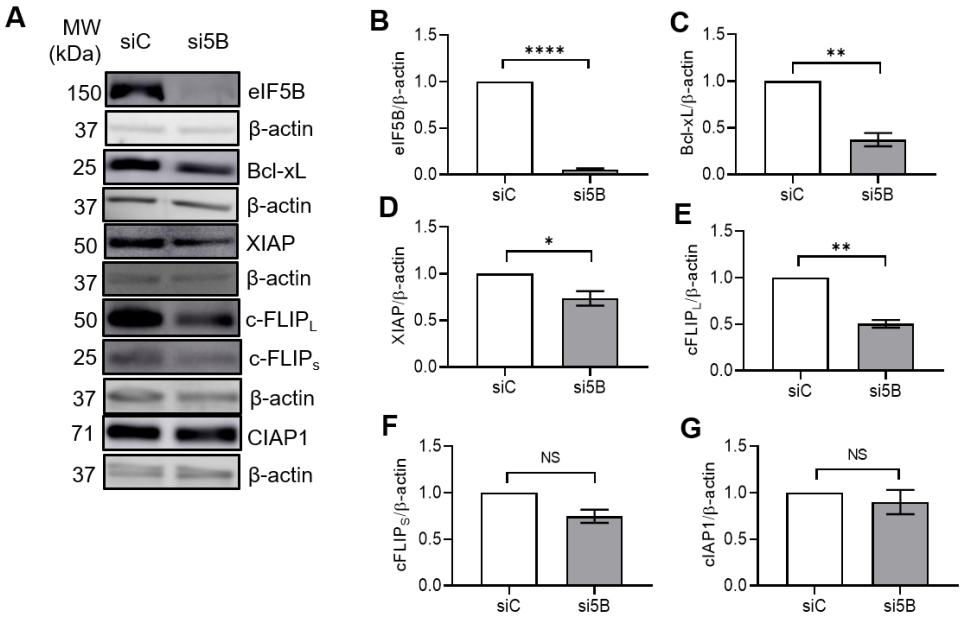

Figure 3.9: Depletion of eIF5B leads to decreased levels of certain anti-apoptotic proteins like Bcl-xL, cFLIP$_L$ and XIAP. Cal-33 cells were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B). The cells were then incubated for 96 hours and harvested in RIPA lysis buffer. From the cell lysate, 20 µg of total protein was resolved by SDS-PAGE which was further subjected to immunoblotting Representative images of Western blots probed for eIF5B and β-actin (internal control) (A). Western blot data indicate more than 95% knockdown of eIF5B. Quantitation of EIF5B (B) Bcl-xL (C), XIAP (D), cFLIP$_L$ (E), cFLIPs (F), cIAP1 (G), normalized to β-actin. Data expressed as mean ± SEM for at least 3 independent biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

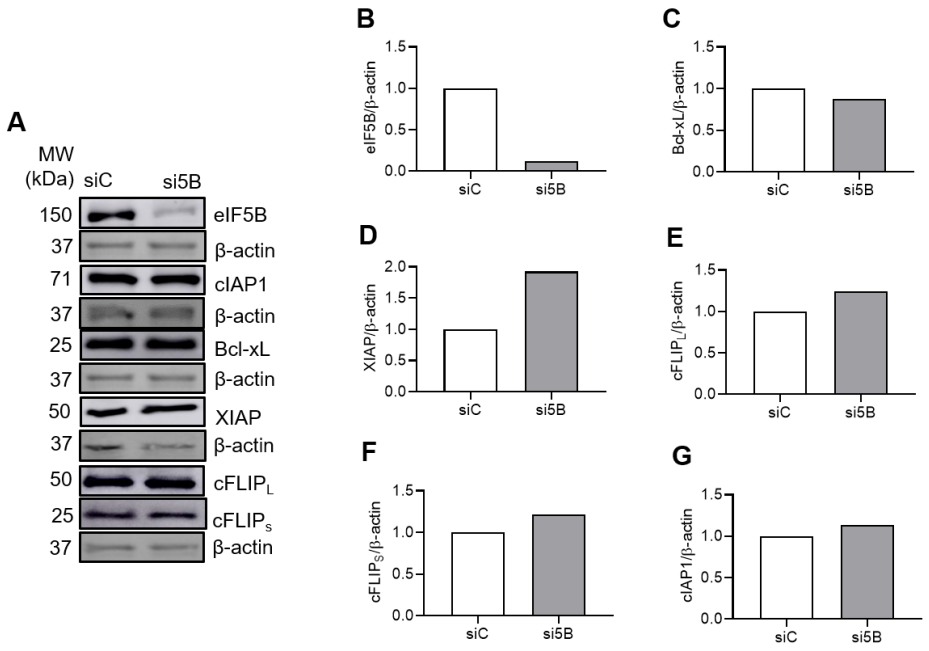

Figure 3.10: Depletion of eIF5B in UM-SCC-59 cells did not decrease the levels of anti-apoptotic proteins Bcl-xL, cFLIPs/L, XIAP and cIAP1. UM-SCC-59 cells were reverse-transfected with a non-specific control siRNA (siC) or an eIF5B-specific siRNA pool (si5B). The cells were then incubated for 96 hours and harvested in RIPA lysis buffer. From the cell lysate, 20 µg of total protein was resolved by SDS-PAGE which was further subjected to immunoblotting (A) Representative images of Western blots probed for eIF5B and β-actin (internal control). Western blot data indicate more than 90% knockdown of eIF5B. However, the levels of anti-apoptotic proteins were not affected in eIF5B-depleted UM-SCC-59 cells. Quantitation of eIF5B (B) Bcl-xL (C), XIAP (D), $cFLIP_L$ (E), cFLIPs (F), cIAP1 (G), normalized to β-actin, from UM-SCC-59 cells.

3.6 eIF5B regulates the translation of mRNAs encoding Bcl-xL, cFLIPs, and XIAP.

It is shown in the previous section 3.4 that the levels of anti-apoptotic proteins Bcl-xL, cFLIPs, and XIAP decrease in eIF5B-depleted Cal 33 cells. However, it does not explain if the decrease in these anti-apoptotic proteins levels is due to the decrease in the

levels of steady-state mRNA levels or due to the decrease in the translation. Therefore, I performed RT-qPCR analysis to check the steady-state levels of the mRNA encoding XIAP, Bcl-xL, and cFLIPs in Cal-33 cells. The RT-qPCR results show that the steady-state levels of these mRNAs were not affected by eIF5B depletion in Cal-33 cells (Figure 3.11 A). Therefore, to confirm that eIF5B plays a role in the translation of these mRNAs, I conducted polysome profiling followed by RT-qPCR assays to check the association of these mRNAs with multiple ribosomes (polyribosomes). In this process, Cal-33 cells were lysed, and the lysate was fractionated by ultracentrifugation on a sucrose density gradient. This procedure separates the monosomes from polysomes on the wide range of the sucrose gradient. Further, the RNA was isolated from each fraction and was subjected to RT-qPCR to measure the association of mRNA of interest with each fraction. Next, the ratio of mRNA of interest relative to the actin mRNA was calculated in each fraction. The earlier fractions from a fraction (1-3) represent monosomes and the later fractions from (4-10) represent polysomes (Figure 3.11 B, C, and D). The proportion of Bcl-xL, cFLIPs, and XIAP mRNAs associated with polysomes versus monosomes is decreased in eIF5B depleted conditions (Figure 3.11 B). Altogether this data suggests the direct role of eIF5B in the regulation of XIAP, Bcl-xL, and cFLIPs specifically at the translational level.

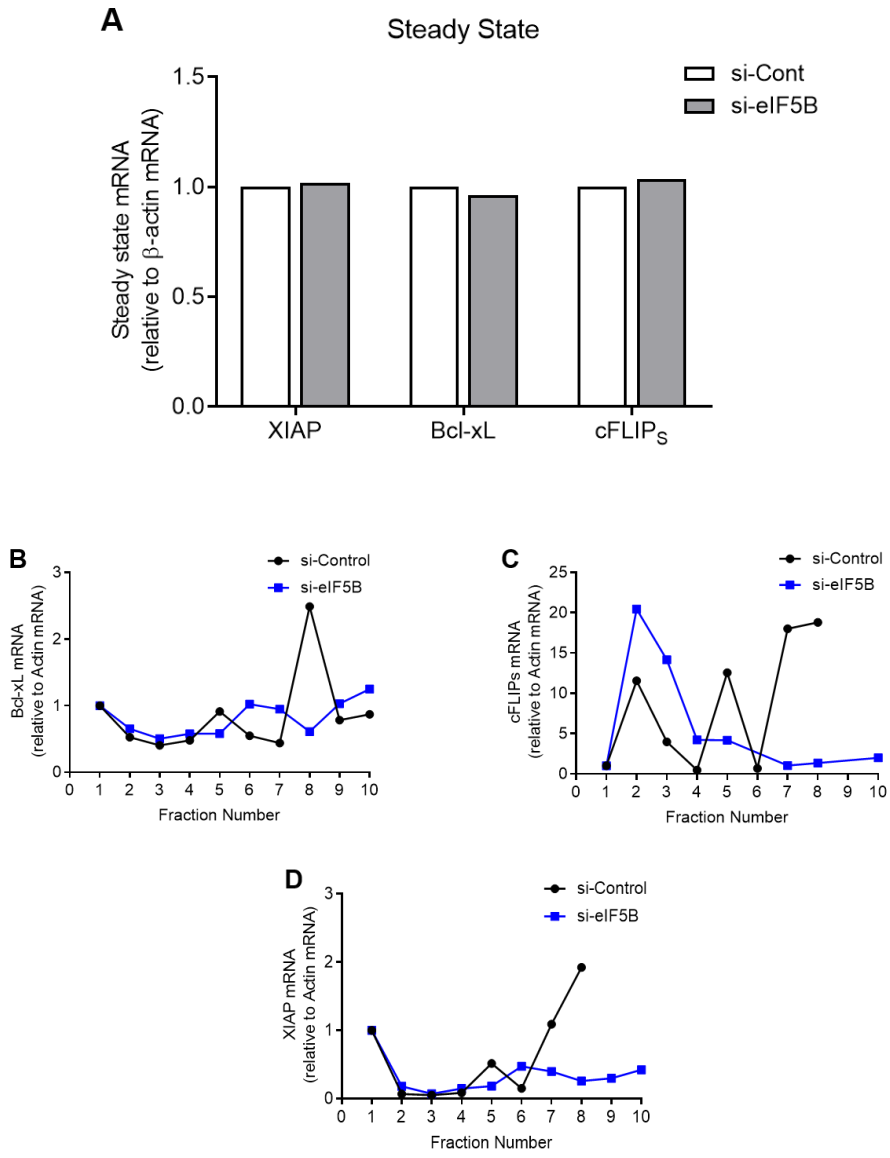

Figure 3.11: eIF5B promotes translation of XIAP, Bcl-xL and cFLIPs in Cal-33 cells. (A) Total RNA isolated from Cal-33 cells from control and eIF5B depleted cells, RT-qPCR was performed and steady-state levels of Bcl-xL, XIAP and cFLIPs was quantified relative to β-actin mRNA. (B) Polysome profiling analysis of the above-mentioned mRNAs was performed and the fractions were subjected to RT-qPCR. The proportion of each mRNA Bcl-xL, cFLIPs and XIAP (relative to β-actin) for each fraction is shown in Figure 8 B, C and D. Former Fractions 1–3, represents monosomes, whereas later fractions 4–10, represents polysomes in eIF5B depleted Cal-33 cells.

3.7 Interacting/functional partners of eIF5B regulate TRAIL-induced death in Cal-33.

Recent findings showed that eukaryotic initiation factor 1A (eIF1A) and eukaryotic initiation factor 5 (eIF5) compete for binding to eIF5B which suggests their involvement in translation initiation.[130] Besides this, another eukaryotic initiation factor 2A (eIF2A), which is an initiator tRNA carrier, and eIF5B were suggested to function synergistically in the eIF2-independent translation of IRES-containing mRNA.[131] My cell viability data in the eIF5B-depleted Cal-33 cells showed a robust decrease in their viability upon TRAIL treatment. Therefore, I wanted to test if any of the above-mentioned eIFs synergistically functions with eIF5B to affect the cell viability of Cal-33 under TRAIL treatment. To this end, I seeded and reverse transfected OSCC cells as per the protocol in section 2.3 and knockdown different eIFs using siRNA specific to eIF1A, eIF2A, eIF5, eIF5B and non-specific control (siC). Later, cells were treated with TRAIL and cell viability was monitored using alamarBlue assay. Cell viability in eIF5B-depleted Cal-33 cells reduced significantly under the TRAIL treatment condition as was observed previously. Interestingly, depletion of other initiation factors like eIF1A, eIF2A, and eIF5 also showed a remarkable decrease in the cell viability of Cal-33 cells upon TRAIL treatment (Figure 3.12 A). Further, I have also performed Western blotting to confirm the successful knockdown of all the eIFs used (Figure 3.12 B). Altogether, this data suggests that depleting eIF1A, eIF2A, and eIF5 sensitizes Cal-33 cells to TRAIL-induced apoptosis-like what was observed upon eIF5B depletion.

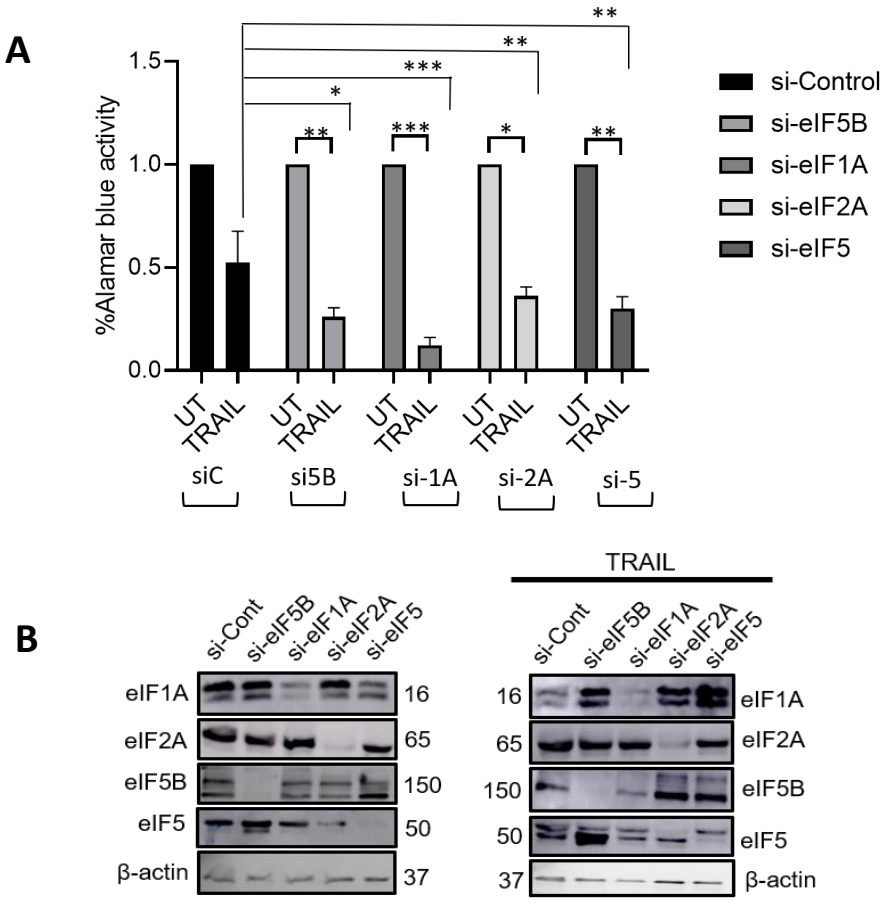

Figure 3.12: Interacting/functional partners of eIF5B regulate TRAIL-induced death in Cal-33 cells. Cal-33 cells as mentioned in section 2.3 were reverse-transfected with a non-specific control siRNA (siC), eIF5B-specific siRNA pool (si5B), siRNA specific to eIF1A, eIF2A and eIF5 and percent viability was measured using alamarBlue assay. eIF5B-depleted Cal-33 cells showed robust sensitization upon TRAIL treatment. This sensitization is also observed in eIF1A, eIF2A and eIF5 depleted Cal-33 cells upon TRAIL treatment (A). Representative Western blots showing successful knockdown of individual eIFs; eIF1A, eIF2A, eIF5 and eIF5B relative to internal control actin in 96-well plate (B). Data expressed in panel (A) as mean ± SEM for at least 3 technical replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

CHAPTER 4

4.1 Discussion

Since Crick proposed the central dogma of life in 1958, several key findings elucidated the role of various regulatory factors during eukaryotic gene expression. These finely orchestrated regulatory factors maintain the homeostasis and physiology of the cell(s) and therefore are directly responsible for the coordinated functioning of the whole organism. Any abnormality during gene expression can lead to malfunctioning of the cells and result in a disease condition. The process of eukaryotic mRNA translation plays a central role among the different gene expression processes during a cellular stress response. This work is focused on gaining better insights into the role of eukaryotic initiation factor eIF5B in the survival of head and neck squamous cell carcinoma progression (HNSCC). eIF5B which is a homolog of bacterial IF2 plays a critical role in regulating multiple pathways like pro-growth pathways[132] and pathway regulating central carbon metabolism.[133] Recently it has been shown that eIF5B is involved in the regulation of non-canonical translation mechanisms mediated by IRESes and uORFs.[40,41,134] eIF5B facilitates a switch from canonical to non-canonical translation during stress conditions.[41] Previously it was shown that eIF5B bind met-tRNAi in the ribosomal P site during IRES-mediated translation of viral mRNAs.[102] Recent studies have shown eIF5B as a pro-survival factor in providing resistance to TRAIL-induced apoptosis in GBM cells.[40] Therefore, I wanted to investigate if the pro-survival role of eIF5B is also unwavering in other cancer types. Here, I identified that eIF5B resist TRAIL-induced apoptosis by regulating the non-canonical IRES-mediated translation in oral squamous cell carcinoma OSCC cell lines.

Although eIF5B is a universally conserved initiation factor, it is not essential under normal cellular conditions in eukaryotes and has a negligible effect on cell viability and global translation.[41,135] The results of my study demonstrate that the depletion of eIF5B from OSCC cell lines Cal-33, UM-SCC-29, and UM-SCC-59 did not have any robust effect on the cell viability (figure 3.4). In glioblastoma multiform cells U343, it has been observed that EGFR phosphorylation decreases with eIF5B depletion and these cells are sensitized to TRAIL-induced apoptosis.[40] Interestingly, along with glioblastoma, lungs and breast cancers,[136,137] EGFR alteration is most commonly observed in HNSCC.[138] In some HNSCC cell lines, *PIK3CA* mutation is suggested to serve as a predictive biomarker for drug targeting EGFR/PI3K pathway.[139] It is known that the PI3K/Akt pathway out of several other signaling pathways is also activated by EGFR.[140] Interestingly, studies have shown that eIF5B interacts with EGFR.[118] Therefore, possibly, depleting eIF5B in OSCC cells might have decreased phospho EGFR levels like what was observed in GBM cells. EGFR is involved in the activation of NF-κB-dependent transcription[119] and depletion of eIF5B resulted in decreased activation of the pro-survival NF-κB pathway[40] in GBM cells. In cellulo studies on OSCC cell lines revealed that most of these cell lines are relatively resistant to TRAIL-induced apoptosis because of the highly active PI3K/Akt pathway.[141] Here, I have shown a decrease in the cell viability in the OSCC cells (Cal-33 and UM-SCC-29) under conditions of eIF5B depletion and TRAIL treatment (Figure 3.5 A and B) where eIF5B depletion has plausibly decreased EGFR activation thereby resulting in the decrease PI3k/Akt pathway. NF-κB being pro-survival pathway is known to enhance the expression of the pro-survival and anti-apoptotic genes like XIAP, cFLIP, cIAP1/2 upon its activation.[142-144] In the case of OSCC, primary OSCC specimens studied earlier were

shown to express DR4, DR5, or both at high levels.[145] Despite this, I observed that one of the OSCC cell lines UM-SCC-59 used in my work was not sensitized further under eIF5B depleted conditions upon TRAIL treatment.

The first option to treat recurrent and metastatic HNSCC is the chemotherapeutic treatment containing cisplatin. However, cisplatin resistance is the major hurdle faced in HNSCC treatment. One of the major reasons for cisplatin resistance in HNSCC is due to higher expression of ETS-1 via the SRC/ETS-1 signaling pathway.[146] Interestingly, there is a molecular link between SRC and MEK/ERK pathways which are the downstream target of EGFR signaling. Based on our previous assumptions, if eIF5B depletion decreases EGFR phosphorylation, it can plausibly affect the ETS-1 levels thereby increasing the sensitivity of OSCC cells to cisplatin. Also, in a separate study with brain tumor stem cells (BTSC), it was observed that temozolomide (TMZ), a chemotherapeutic agent, sensitizes BTSC under the condition of eIF5B depletion.[103] I hypothesized that eIF5B depletion might also increase the sensitivity of OSCC cells to cisplatin treatment. Therefore eIF5B-depleted OSCC cells Cal-33, UM-SCC-29, and UM-SCC-59 were subjected to cisplatin treatment. However, no enhanced sensitization of eIF5B depletion on UM-SCC-29 and UM-SCC-59 was seen towards cisplatin compared to control but eIF5B-depleted Cal-33 cells showed increased sensitization to cisplatin treatment (Figure 3.6).

As discussed above, we observed cell death phenotype in the eIF5B-depleted OSCC cells under TRAIL treatment which I attributed to apoptosis. TRAIL is known to induce receptor-mediated apoptosis via the formation of DISC and activation of upstream caspase-8.[147] Further, I used different cell death pathway inhibitors among which only z-VAD-fmk

showed reverted cell death phenotype in eIF5B depleted Cal-33 cells confirming apoptosis (Figure 3.8). IAPs are the key regulators of many biological processes and their role is very important in the field of cancer biology. They are known to inhibit apoptosis by directly binding and inhibiting certain caspases.[90,148] Studies have shown IAP as a therapeutic target for small molecules to overcome the resistance in malignant gliomas.[149] For instance, using proteasome inhibitor MG132 which reduces levels of FLIP, XIAP, and cIAP1.[150] Basically, inhibition of proteasome using proteasome inhibitors upregulates the pro-apoptotic factors like NOXA, p53, and Bax whereas decreases the levels of anti-apoptotic proteins.[151] p53 accumulation due to proteasome inhibition results in the activation of its downstream target genes like p21, Fas ligand, PUMA, and Bax which triggers the apoptosis process.[152] SMCs in combination with pro-death compounds like TRAIL is used to inhibit IAPs.[153] Smac mimetic compounds are also used to induce degradation of some of the IAPs like cIAP1 and cIAP2.[154] In a recent study, eIF5B was shown to play a role in the translation of pro-survival proteins which includes important IAPs like XIAP, cIAP1 and anti-apoptotic proteins like Bcl-xL, cFLIPs in GBM.[40] As eIF5B displays a positive effect on the translation of these pro-survival proteins in GBM, I wanted to check if it exhibits a similar effect in OSCC cell lines. Therefore, I depleted eIF5B from OSCC cell lines Cal-33 and UM-SCC-59. Notably, the levels of Bcl-xL, $cFLIP_{L,}$ and XIAP were decreased considerably under eIF5B depleted conditions in Cal-33 cells. cIAP1 and cFLIPs levels did not decrease significantly upon eIF5B depletion in Cal-33 (Figure 3.9). The decrease in the levels of Bcl-xL could be explained by the presence of putative IRES in the mRNA of this protein. As eIF5B can specifically regulate anti-apoptotic proteins by non-canonical mechanism[40] depletion of eIF5B resulted in decreased levels of these anti-apoptotic

proteins. There is a possibility that $cFLIP_L$ and cFLIPs levels are saturated in Cal-33 and UM-SCC-59 cells because these two proteins are known to be overexpressed in some HNSCC cell lines.[155] $FLIP_L$ levels decrease moderately in Cal-33 without much of a change in the level of cFLIPs whereas cFLIP levels did not decrease in UM-SCC-59. In the case of XIAP, its level is moderately decreased upon eIF5B depletion in Cal-33 cells possibly because XIAP is translated from two alternative transcripts.[156] Among the two transcripts, only one transcript bears the IRES, and eIF5B is known to play a role in the IRES-mediated translation of XIAP.[41] There was no decrease in the anti-apoptotic protein levels under eIF5B depleted conditions in the UM-SCC-59 cell line (Figure 3.8). There is a difference in the expression of these anti-apoptotic proteins on eIF5B depletion in OSCC cell lines possibly because the IRES-mediated translation could be genotype-specific. The decrease in Bcl-xL in OSCC cells would result in more robust mitochondrial-dependent apoptosis. Bcl-xL is known to bind to Bax which is a mitochondrial membrane permeabilization protein thereby inhibiting its activity. Therefore, a decrease in the levels of Bcl-xL, allows Bax to associate with Bak and actively form pore onto the mitochondrial membrane thereby releasing cytochrome c and triggering apoptosis.[157] It is clear from the above results that the levels of some of the anti-apoptotic proteins get affected by silencing eIF5B.(Figure 4.1) However, it was not clear whether this decrease in protein levels was at the transcriptional level or the translational level. Further, polysome profiling was performed using the eIF5B-depleted OSCC cell line Cal-33 as it showed considerable changes in anti-apoptotic protein levels. These results indicated that eIF5B depletion in Cal-33 resulted in a decrease in the translation of these anti-apoptotic proteins (Figure 3.9 B, C and D) whereas the steady-state data reveals that the mRNA levels of these proteins were

unaffected (Figure 3.9 A). This suggests that the translation of these mRNAs might be directly affected by eIF5B. These findings are consistent with the recent body of work from Dr. Thakor's lab on GBM cell lines. eIF5B was shown to have other interacting partners like eIF1A and eIF5 that competes for binding on eIF5B.[130] eIF2A and eIF5B were also involved in the translation of IRES-bearing mRNA.[131] This raises the possibility that these eIFs might synergistically function with eIF5B to produce the overall effect on cell viability on TRAIL treatment in the OSCC cell lines. Hence to further extend my understanding in this regard, I depleted eIF1A, eIF2A, eIF5, and eIF5B individually from Cal-33. This result revealed that the depletion of all these eIFs individually caused a robust decrease in the cell viability in Cal-33 cells upon TRAIL treatment. Altogether my work suggests that by targeting eIF5B the non-canonical translation of pro-survival proteins can be regulated which can plausibly help in overcoming the tendency of HNSCC cells to evade apoptosis.

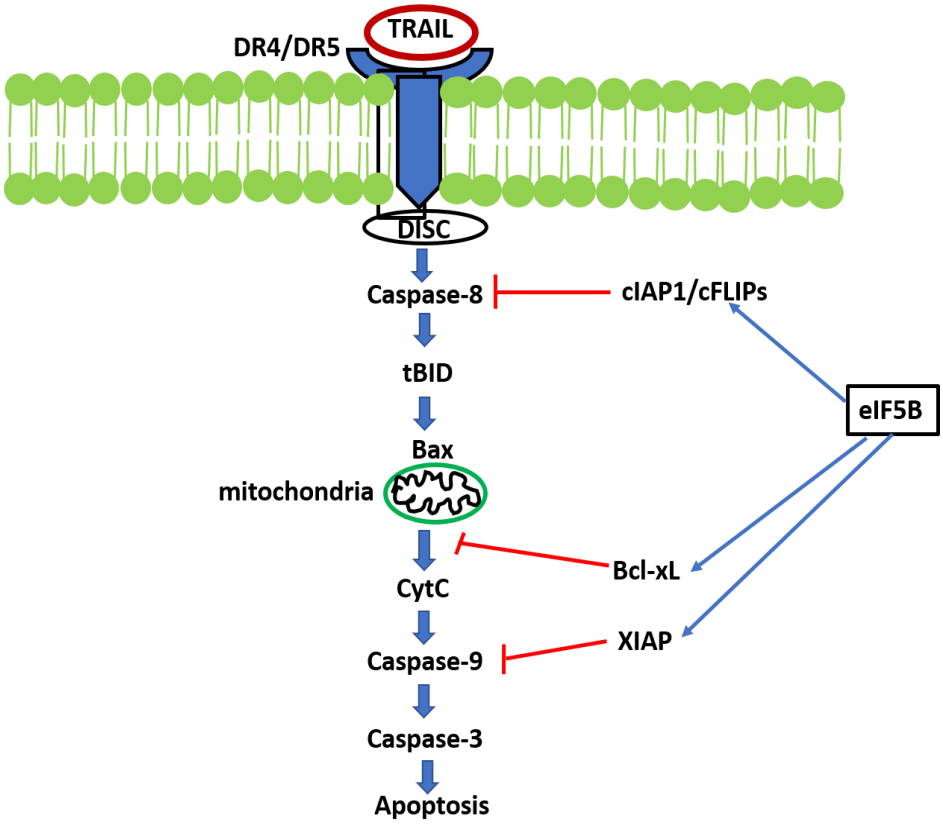

Figure 4.1: Schematic diagram showing TRAIL-mediated extrinsic apoptotic pathway and regulation of anti-apoptotic proteins *via* eIF5B. Binding of TRAIL induces extrinsic apoptosis pathway combined with role of eIF5B in the regulation of IRES-containing mRNAs XIAP, Bcl-xL, cFLIP and cIAP1.

4.2 Future directions

The major problem posed by cancer cells that contributes to therapeutic resistance is the over-expression of the inhibitors of apoptosis proteins (IAPs). The natural antagonist of IAPs which is endogenously expressed in the cells is the Second mitochondria-derived activator of caspase (Smac).[158] Smac on its release in the cytosol is known to interact with the IAPs *via* its N-terminal AVPI domain which then releases the inhibition over caspases by IAPs. Several small molecules are developed that mimic the AVPI domain of Smac which showed the anti-cancer effect when used in combination with other chemotherapeutic agents.[159-161] Since it is known that eIF5B is directly involved in the cap-independent translation of IAPs during stress conditions,[40,41] it would be interesting to study the effect of Smac mimetic compounds on the HNSCC cells under eIF5B-depleted conditions. Smac can mediate apoptosis in a particular class of agents like Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Tumor Necrosis factor-related Apoptosis-Inducing Ligand (TRAIL).[158,162] My current work shows that eIF5B depletion sensitizes OSCC cells to TRAIL-induced apoptosis. Therefore, treating the eIF5B-depleted OSCC cells with Smac mimetic compounds along with TRAIL might become a better option to further increase the sensitivity of OSCC cells to apoptosis.

Platinum-based chemotherapy in combination with radiation treatment is the frontline therapy used to treat HNSCC.[163] Despite all these advanced technologies, tumor resistance to platinum-based drugs remains a significant challenge for HNSCC. Therefore, different strategies are being developed to overcome this hurdle. Based on my current data, it will be interesting to check the effect of radiation treatment on the eIF5B-depleted OSCC

cells. A recent study has also shown to utilize immunotherapy as a tool to treat cancer. Pembrolizumab, which is a humanized antibody is known to stimulate the body's immune response to fight against cancer.[164] The treatment of OSCC cells with pembrolizumab in combination with eIF5B knockdown could give us some interesting and robust results in the treatment of HNSCC.

As mentioned before, eIF5B interact with different eIFs namely eIF1A, eIF2A and eIF5 and play role in regulating non-canonical translation. My work here showed that depleting these aforementioned eIFs individually sensitized OSCC cells to TRAIL-induced apoptosis. Therefore, it will be interesting to study the combined effect of depletion of eIF5B and its interacting partners to understand the mechanism by which the cell viability is affected in OSCC cells.

HNSCC is a highly malignant and hard-to-treat cancer which poses a challenge because of its high relapsed rate in the more advanced patients. The *in cellulo* studies in this project demonstrates the role of eIF5B in the survival of HNSCC. Specifically, eIF5B was shown to regulate the translation of a critical set of mRNAs for the proteins that are required for cell survival in oral squamous cell carcinoma. Therefore, it is important to develop different strategies to target the translation machinery to decrease the progression of the disease and eventually increase patient survival. As mentioned earlier, eIF5B depletion decreases phospho-EGFR levels in GBM.[40] Therefore, by targeting eIF5B in HNSCC we can potentially regulate the phosphorylation of EGFR. Phospho EGFR plays an important role in triggering angiogenesis *via* EGFR/HIF-1α/VEGF molecular pathway. Henceforth, eIF5B shows promise as a potential candidate for small-molecule inhibition as

a strategy to suppress the progression of HNSCC by targeting angiogenesis which is one of the important aspects of cancer.

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