UNIVERSITY OF ESSEX

SCHOOL OF LIFE SCIENCES

Zinc Finger Protein 36L1 (ZFP36L1): Gene Expression, Regulation and Interactions with Immune Receptors in Human Tumour Cells.

VEDA V HEGDE

(2020)

Acknowledgments

I would like to thank my supervisors, Prof. Nelson Fernandez, Dr. Raj Thaker for their continuous guidance and wonderful support throughout my experience in the School. They kept me motivated and determined and inspired me to do better every day.

I would like to acknowledge and thank the efforts of Bander Khayat for helping me hone my skills at several techniques and experiments. I would also like to thank the School of Life Sciences for the opportunity to work, collect and publish significant data. I would like to extend my appreciation to the lab technicians Julie Arvidson and Lynwen James for helping me with any difficulties I faced in the lab.

A special thanks to my friends and family for always reminding me that there is more to life than science**.**

TABLE OF CONTENTS

List of Figure Legends

List of Table Legends

List of Abbreviations

APS: Ammonium per sulphate 27

ARE: AU rich elements 11

ATCC: American type culture collection 23

Bcl-2: B cell lymphoma 2 14

BRF1: Butyrate response factor 1 9

BSA: Bovine serum albumin 35

CCR4-Not: Carbon catabolite repression- negative on TATA- less 13

CD44: Cluster of differentiation 44 18

COX 2: Cycloxygenase 2 14

CRISPR: Clustered regularly interspaced short palindromic repeats 24

DAPI: 4', 6-diamidino 2-phenylindole 35

DMSO: Dimethylsulphoxide 23

ECL: Enhanced chemiluminescence solution 30

ELISA: Enzyme linked immunosorbant assay 30

EMT: Epithelial mesenchymal transition 20

ERKs: Extracellular signal related kinases 21

ESRP1: Epithelial splicing regulatory protein 1 20

FBS: Foetal bovine serum 23

GM-CSF: Granulocyte macrophage- colony stimulating factor 14

HNSCC: Head and neck squamous cell carcinoma 18

HRP: Horseradish peroxidase 29

IEGs: Immediate early response genes 10

JNKs: cJunm NH2-terminal kinases 21

MAPK: Mitogen activated protein kinases 12

ABSTRACT

The Zinc finger protein 36 like 1 (ZFP36L1) or Butyrate response factor 1 (BRF1) is involved in the differentiation, proliferation, and angiogenesis of various cell types. ZFP36L1 can positively or negatively regulate cellular processes such as transcription and signalling, depending on external and internal stimulations. The basic mechanism with which this protein functions is via its binding to the AU- rich regions of the mRNA causing their degradation. However, its exact roles in cellular physiology and mRNA targets are poorly understood. We hypothesize that ZFP36L1 regulates expression of effectors and receptors linked to immunity and is therefore important in controlling cell proliferation and cancer. We investigated roles of ZFP36L1 in regulating expression of TNF α and VEGF A as well as of the adhesion receptor CD44 involved in lymphocyte activation, haematopoiesis and tumour metastasis. In this study we also report data on the detection and quantification of ZFP36L1 in several tumour cell lines such as THP1, Jurkat, B16, MCF7, and MC38. To study the function of ZFP36L1 a CRISPR-Cas9 system was used to generate ZFP36L1 knockout MC38 cell line. Our results show that ZFP36L1 limits production of TNF α and VEGF A. Our preliminary data also show co-localization of ZFP36L1 with CD44 upon cellular permeablization, which needs further investigation and future studies. In conclusion, we show that ZFP36L1 is expressed in various cancer cell lines including Jurkat immune cells and its expression regulates various effector molecules and cell growth.

CHAPTER 1

Introduction

Zinc finger protein 36 ring like 1 (ZFP36L1)

Zinc finger proteins (ZFPs) are a part of one of the largest families of transcription factors in the human genome (Ye et al., 2019), containing conserved zinc finger motifs. Recent studies have showed that ZFPs are closely associated with the different stages of cancer development such as tumorgenesis, invasion and metastasis (Ye et al., 2019). ZFPs function in several biological processes including development, differentiation, metabolism and apoptosis. Our ZFP of interest is the RNA binding protein ZFP36L1. The *zfp36l1* gene encoding for protein ZFP36L1 is a member of the immediate early genes (IEGs).

IEGs are usually activated in response to several types of cellular stimuli. The activation and transcription of IEGs takes place within minutes following stimulation and does not require the process of de novo protein synthesis (Bahrami and Drablos, 2016). Currently, there around 40 known early response genes which have been characterised (Baou et al., 2009), ZFP36L1 being one of them. ZFP36L1 is a 'putative' (post) transcription factor and is an mRNA-binding protein (Baou et al., 2009).

ZFP36L1/BRF 1 is an mRNA binding protein that regulates certain pathways related to the growth, development, differentiation and/or maturation of cells. The gene encoding the protein is a member of the TIS11 family of IEG's, consisting of 4 members; TIS11 (ZFP36), TIS11b (ZFP36L1/BRF 1), and TIS11d (ZFP36L2/BRF 2) (Baou et al., 2009). The fourth member ZFP36L3 was found in rodents, expressed in mouse placenta, but was not found in human placenta or any other human tissues. Our focus will be on ZFP36L1. This gene is well conserved across species and has a promoter that contains motifs seen in other

early-response genes. This putative nuclear transcription factor most likely functions in regulating the response to growth factors (Martinez-Calle et al., 2019).

Structure of ZFP36L1

Zfp36l1 gene was located on chromosome number 14q24.1 and was found to be around 6000 base pairs long. The gene consisted of two type Ⅱ restriction endonucleases, HINDⅢ and BamHⅢ (Fig 1). These restriction endonucleases are responsible for the recognition and cleavage of the AU rich regions. There has been no report of a pseudogene gene so far. The conserved tandem zinc finger domain (ZFD) (dotted box, Fig 1) directly binds to class Ⅱ AU-rich elements (ARE) in the 3'-untranslated region of mRNA.

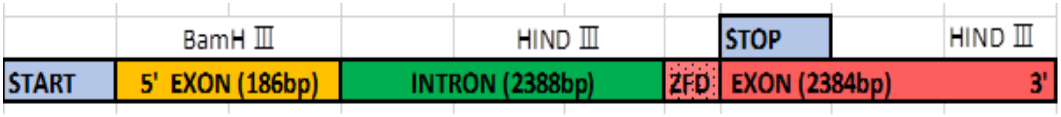

Figure 1: Diagram of genomic structure of human Zfp36l1 gene. The gene consists of two exon regions, one at 5' and second one at 3' end. A 186 base pair exon at 5' is followed by a large intron spanning 2388 base pairs. This intron is then followed by a 2834bp exon 2. A short ZFD (dotted box) exists after the intron region. This region is composed of tandem repeats. "START and STOP" regions represent the translation start and stop points. BamH Ⅲ and HINDⅢ are type Ⅱ restriction endonucleases. Adapted from, (Stumpo et al., 2010).

In humans, ZFP36L1 protein consisted of 338 amino acids and had an expected molecular weight of 36.3 kDa (Stumpo et al., 2010). The structure of ZFP36L1 consists of five domains each with a distinct role in the activity of the RNA binding. Two mRNA decay activation sites are present which are activated upon binding to an mRNA, leading to its degradation (Fig 2). Activation sites recruit factors that decay the mRNA strand such as de-capping enzymes and exonucleases (Lykke-Andersen and Wagner, 2005). Structurally, zinc fingers are motifs present in proteins that are characterised by one or more zinc ion co-ordinated to stabilize the fold. C_3H_2 type ZFDs have been known to interact with the 3' untranslated

region of mRNA. The ZFP36L1 protein consists of two such zinc finger domains that are around 20 to 50 amino acids in length. (According to PROSITE ProRule annotations) A small but significant domain exists composed of repeated homologous serine residues, about 9 amino acids long (Fig 2). At this poly serine region phosphorylation takes place, serine residues 92 and 203 respectively (Schmidlin et al., 2004). The structure has only been postulated based on predictions. Crystallization of the protein would provide a better understanding of the protein in future studies.

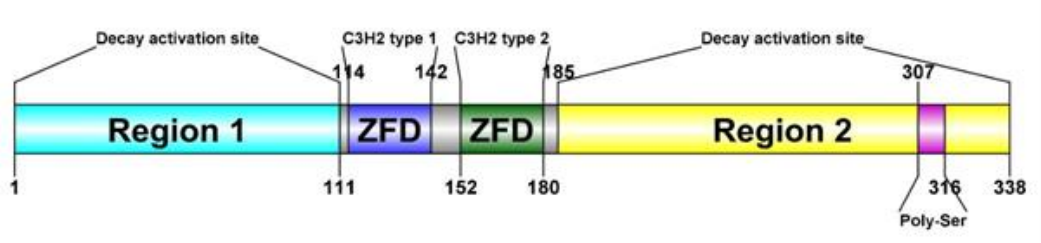

Figure 2: *Domain structure of human ZFP36L1*. Region 1 and Region 2: mRNA decay activation sites. Two CH2 zinc finger domains (ZFDs) that consists of a co-ordinated zinc metal ion. A short region consisting of poly serine amino acid residues. Constructed using (Ren et al., 2009).

Mechanism of action

ZFP36L1 protein regulates several cellular processes, including cell apoptosis, by binding to AU rich elements in the 3′ untranslated regions of sets of target mRNAs to promote their degradation (Schmidlin et al., 2004). Adenylate and uridylate rich element (ARE) motifs are elements present in the 3′ untranslated region of mRNA transcripts that encode several inflammation and cancer-associated genes (Schmidlin et al., 2004). These zinc finger proteins are known to regulate various pathways such as Mitogen activated protein kinases (MAPK), Tumour necrosis factor α (TNF α) and vascular endothelial growth factor A (VEGF A).

The mechanism of ZFP36L1 is a several step process involving the following (Fig 3): Protein binding to the ARE region of various mRNAs upon the stimulation of differentiation and proliferation (Fig 3A). Phosphorylation of the zinc finger protein

takes place giving it the ability to degrade the mRNA (Fig 3B). Phosphorylation takes place by inducing the Serine 92 and 203 on the poly serine region by Protein kinase B or MAPK phosphatase. Following phosphorylation, ZFP36L1 recruit exosomes, exonucleases and de-capping enzymes (Fig 3C). Thus the deadenylation of the poly (A) tail attenuates protein synthesis (Stoecklin et al., 2002). On the contrary there is evidence of degradation of ARE containing mRNA independent of, or in the absence of the poly (A) tail (Lai et al., 2002) (Fig 3D).The last step includes hydrolyse and destabilization of mRNA strand rendering it inactive.

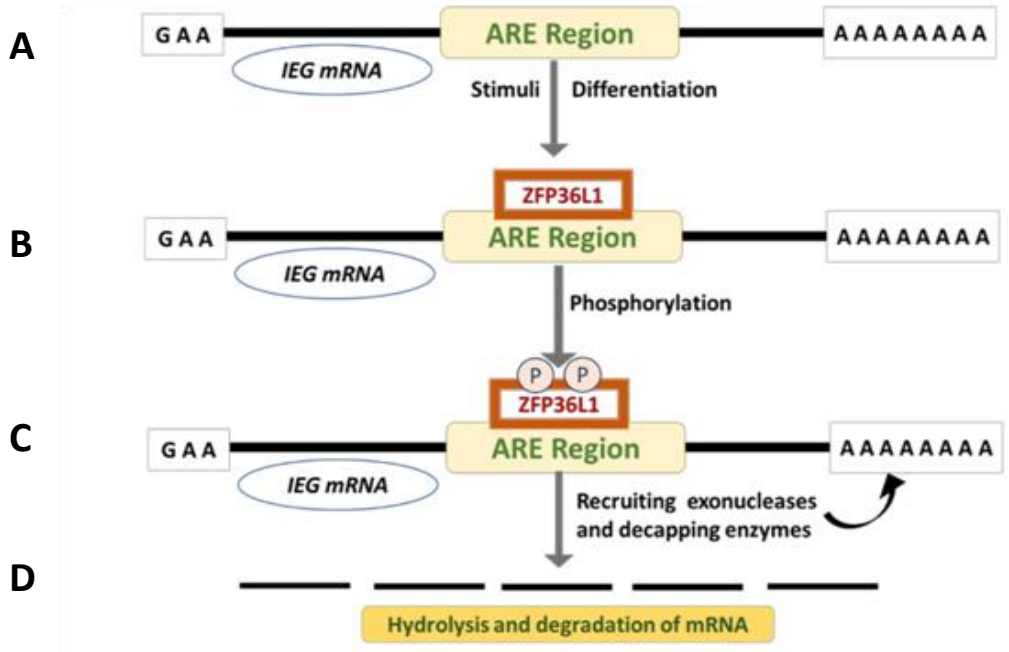

Figure 3: *Mechanism of ZFP36L1.* A) A direct stimulation such as differentiation initiates ZFP36L1 to bind. B) Binding to the 3' untranslated region, promotes phosphorylation of the protein. C) Phosphorylation propagates the hydrolysis process with the help of de-capping enzymes. D) Degradation of mRNA by hydrolysis.

Not only does ZFP36L1 have cytosolic mRNA decay functions, they also present nuclear mechanisms (Desroches-Castan et al., 2011). ZFP36L1 have also been shown to have post translational modifications for example, by using a proteomic method, it was demonstrated that the protein ZFP36L1 binds directly to the low density lipoprotein receptor 3'-untranslated region of the mRNA and activated the carbon catabolite repression- negative on TATA- less (CCR4-Not) deadenylase complex, leading to mRNA instability. Whereas on phosphorylation, the deadenylase enzyme complex dissociated from the protein and mRNA was stabilized. This shows that ZFP36L1 has important regulatory functions (Desroches-Castan et al., 2011).

Regulation of Cell signalling proteins

Negative Regulation

ZFP36L1 has been shown to negatively regulate some effector molecules such as, in knockout cell lines derived from mice, the macrophages show high TNF α mRNA stability and TNF α production due to the absence of ZFP36L1 (Suk et al., 2019). On the other hand, ex vivo studies have shown that, when ZFP36L1 was overexpressed, differentiation of adipocytes was suppressed (Tseng et al., 2017). Negative regulation can also be seen by pathways of Doxycycline (analogue of tetracycline) with granulocyte macrophage-colony stimulating factor (GM-CSF), VEGF, cyclooxygenase 2 (COX 2), Cyclin D and B cell lymphoma 2 (Bcl-2) (Fig 4) (Suk et al., 2019, Guo et al., 2019). A study clearly showing regulation, says that VEGF A production is enhanced in the absence of the respective zinc finger protein. When the ZFP36L1 gene was mutated, mice embryo died due to heart problems and vascular defects (Bell et al., 2006). On testing the mice it was discovered that there was an over production of VEGF A. ZFP36L1 was required to keep VEGF A under control and prevent vascular abnormalities; being a

negative regulator (Bell et al., 2006). Therefore, functions of ZFP36L1 are linked to inflammation, apoptosis, proliferation and angiogenesis.

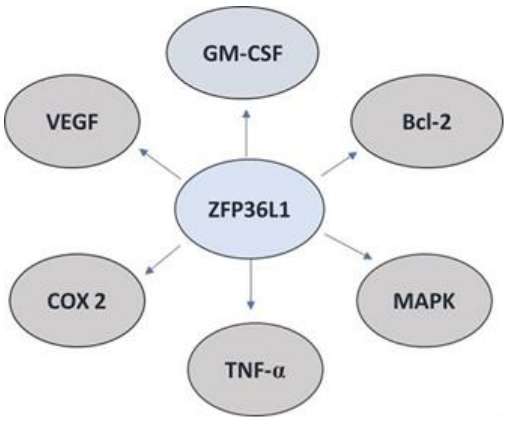

Figure 4: *ZFP36L1 is involved in several pathways of the cell.* These are some of the pathways where the protein has either a negative or positive regulation. ZFP36L1 is known to have an effect on inflammatory pathways such as Cycloxygenase 2, TNF α and GM-CSF.

Another example of negative regulation suggests that ZFP36L1 negatively regulates erythroid differentiation. ZFP36L1 binds to the untranslated region of the Stat5b region of the mRNA directly. It was clearly seen that by down regulation of the Stat5b pathway, due to ZFP36L1, resulted in decreased levels of erythroid cell formation (Vignudelli et al., 2010).

Cell Cycle Regulation

It has been recently proven that ZFP36L1/2 is regulated in a cell cycle-dependent manner (Suk et al., 2019, Noguchi et al., 2018). The phosphorylation of this protein takes place through its C-terminal region and is an important aspect in its role in cell cycle regulation. It has also been shown that ZFP36L1 plays a role in the organisation of spindle fibres during mitotic cell division (Kondo et al., 2018). There is evidence that overexpression of ZFP36L1/2 led to cell cycle arrest at G1 phase and a drastic inhibition in cell growth (Suk et al., 2019). Evaluation of cell cycle proteins such as cyclin A, cyclin B, cyclin D and p21 exhibited decreased levels in

cells that overexpressed the protein. However, p53 was increased exhibiting the role of ZFP36L1/2 in inhibition of cell proliferation in a p53 independent manner (Suk et al., 2019). Therefore it can be said that ZFP36L1 is pivotal in the entire cell cycle process as it influences various checkpoints, affecting the outcome of the cycle (Noguchi et al., 2018, Suk et al., 2019, Kondo et al., 2018).

ZFP36L1 and Cancer

RNA-binding proteins (RBPs) perform critical functions in gene expression regulation (Oliveira et al., 2017). In all eukaryotes, RBPs play major roles across post-transcription processes, such as splicing regulation, mRNA transport, and mRNA translation and decay modulation.

Different pathways and signalling proteins have been investigated to study the effects of zinc finger proteins such as, zinc finger protein 367 and its effects on the Hippo pathway (Wu et al., 2020), MiR-4282 and the its effect in down regulating zinc finger and BTB domain containing 2 (Zhang et al., 2020), Retinoblastoma Protein-Interacting Zinc-Finger Gene 1 expression in cervical cancer (Yang et al., 2019), STAT3 pathway by the Myc-Associated Zinc Finger Protein (Triner et al., 2018) and PRDI-BF1 and RIZ homology domain zinc finger protein 14 and its role in regulation of cancer stemness (Taniguchi and Imai, 2018). The exact functions and implications of our protein of interest ZFP36L1 needs further research for better understanding.

The research exploring the relation of cancer with ZFP36L1 is limited but is of interest, creating a large scope for finding an effective treatment for cancer. For instance, we know that ZFP36L1 influences the VEGF pathway and there is research that shows that angiogenesis inhibitors have shown clinical benefits in patients with advanced cancer (Planel et al., 2010).

It can be concluded that by mutating, deleting or repressing the expression of *zfp36l1* genes, there is direct effect on cell proliferation and division. A study has

shown that mice lacking in these genes developed T cell acute lymphoblastic leukaemia (Hodson et al., 2010). During the process of thymopoiesis, suppression of *zfp36l1* led to lymphoblastic leukaemia which was dependent on the transcription factor Notch 1 (Hodson et al., 2010). The T cells that are not subjected to transformation (β selection in the thymus) accumulated in large numbers and expressed higher levels of Notch 1. However, these cells did not express ZFP36L1/2. This can conclude ZFP36L1/2 bound to the AU-rich regions and prevented the expression of the mRNA responsible for Notch 1. The results from the overall study showed that ZFP36L1/2 is required for the development of T cells and prevention of tumours. The deletion or suppression of these proteins led to the formation of T-cell lymphoblastic leukaemia (Hodson et al., 2010).

While ZFP36L1/2 plays an integral role in the prevention of tumours, it can also lead to inflammatory responses if not controlled and regulated (Sanduja et al., 2011). The targeted binding, if not regulated, can lead to inflammatory problems for example in the resting state of the cell, the ZFP36L1 destabilises the mRNA of pro inflammatory cytokines. The presence of an external stimuli such as lipopolysaccharides of microbes signals the ZFP36L1 to temporarily stop its mechanism. Therefore there is an increase in the cytokines that help prolong the inflammatory response. (Sanduja et al., 2011, Mahmoud et al., 2019). The control and regulation of this family of genes takes place at several levels including, cell signalling, mRNA turnover number, initiation by phosphorylation, the localization and accumulation of cells and interactions with several other proteins, degradation of the protoplasm of the cell. All the regulations of the TIS11 family members affect the ability of the zinc finger protein to hydrolyse mRNA including functions that are independent of decay (Sanduja et al., 2011).

Lee et al, (2005) demonstrated that ZFP36L1 has a role in the ability of patients to respond to chemotherapeutic treatments. The failure of chemotherapeutic drugs is one of the major downfalls of cancer treatments, alongside the long-term side

effects. Cisplatin is a widely used chemotherapeutic drug, used to specifically treat head and neck squamous cell carcinoma (HNSCC). Resistance to cisplatin is common amongst patients (Lee et al., 2005), preventing the effective treatment of cancer. Cisplatin sensitive and resistant cells derived from HNSCC cell lines were used in the study. According to the results, ZFP36L1 showed high expression levels in cisplatin-sensitive cells and can be used to improve chemotherapy for cancer patients (Lee et al., 2005)

Now we know that this zinc finger protein plays an intrinsic role in several pathways that lead to inflammation and cancer, we can study it further, alongside its interactions, to create a therapeutic effect against these issues.

Characteristics of cell surface receptor CD44

Cluster of differentiation 44 (CD44) is a cell surface adhesion receptor that is highly expressed in many cancers and regulates metastasis via recruitment of CD44 to the cell surface. CD44 is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. Its interaction with appropriate extracellular matrix ligands promotes the migration and invasion processes involved in metastases. This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis.

It has been postulated that the two immune receptors CD74 and CD44 work in synergy and play an important role in tumour formation and development (Liu et al., 2016, Ssadh et al., 2017)

CD44 is a type Ⅰ trans-membrane glycoprotein (Fig 5) expressed in all majorities of cell types. In humans, CD44 plays a major role in adhesion, migration, interaction between cells, between cells and extracellular matrix. The specific ligands that bind to this receptor include hyaluron and osteopontin (Ssadh et al.,

2017). Studies show that the expression of CD44 and CD74 is directly proportional to the adhesion rates in tumour cells; this study was done using a reconstituted membrane matrix 'Matrigel' (Ssadh et al., 2017).

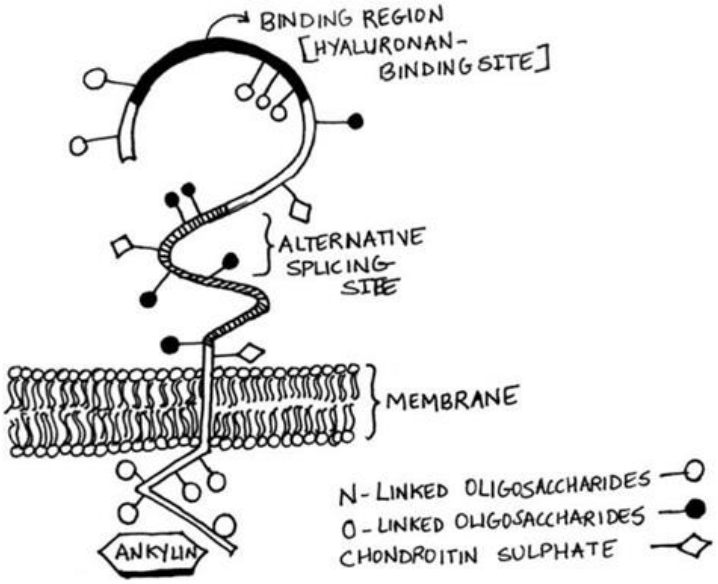

Figure 5: *Basic structure of CD44*. The basic structure includes 3 domains, ligand-binding homologous extracellular domain, trans-membrane domain and an intracellular domain consisting of 20 exons Adapted from (Yasuda et al., 2002).

Some receptors work together in association with each other like CD74 and CD44. This can bring about enhanced tumour development. Is it possible to modulate these interactions? Will it cause an overall effect on the integrity of the cell? Will it affect other signal pathways? These are some of the questions that need to be answered to investigate further possibilities in cancer therapeutics.

Cell signalling works in close association with each other and there is a certain balance. When this balance is disrupted it leads to cancerous or immunological conditions. Recently, it was shown that the collective working of CD44 and CD74 leads to the progression of breast cancer (Ssadh et al., 2017). The levels

of CD44 and CD74 expressions were seen to be different across different types of cells. In some cases, the level of CD44 was higher than CD74. These two cell surface receptors come together in the cytoplasm and assist in the development and metastasis of cancer (Ssadh et al., 2017). By using advanced techniques such as bio imaging and co-immunoprecipitation, the interactions were clearly observed and measured to justify its role in tumour development and metastasis. Since these receptors play such an important role in the development and progression of cancer it can be used as a potential and effective biomarker to diagnose cancer (Ssadh et al., 2017).

Relationship between ZFPs and CD44

Few studies have observed the correlation between zinc finger protein and CD44 in cancer cells. One such study by Perca B et al, 2015 suggested that a specific zinc finger protein; Zinc finger E-box binding homeobox 1 (ZEB1) confirmed that CD44 and ZEB1 expression are reciprocally linked in a positive feedback loop, that is the isoform of CD44 (CD44s) is expressed which further activates more of ZEB1. This suggests that after the initial dependency on external growth factors provided by the microenvironment, cancer cells become autonomous, a process that is very crucial for tumour progression. Of the surviving cells that had undergone Epithelial to mesenchymal transition (EMT), CD44s and ZEB1 expression increased sharply while Epithelial Splicing Regulatory Protein 1 (ESRP1) were reduced. ESRP1 is thought be a regulator of many cancer cells by acting as tumour suppressor (Ishii et al., 2014), hence loss or reduction in ESRP1 levels indicate tumour progression and survival. They also found out that reduced level of ESRP1 was correlated with increased tumour relapse, and the CD44-ZEB1-ESRP1 feedback loop controlling cellular phenotypes and prognosis of cancer patients by determining the CD44 isoform configuration (Preca et al., 2015). The analysis emphasises that targeting

pathways that elevate the expression of ZEB1 and induce a switch that contains isoforms of CD44 is an ultimate goal to increase the survival of patients with tumours. Promoting the expression of ESRP1 will favour the expression of normal CD44, and reduce the isoform subsequently which affects the levels of ZEB1 thereby preventing further tumour relapse (Preca et al., 2015).

TNF α and VEGF A

Tumour necrosis factor alpha is cytokine belonging to the family of TNF/TNFR. TNF α plays a significant role in inflammatory signals and host defence mechanisms. They are known to maintain homeostasis of the cells (Balkwill, 2006). TNF α is produced by macrophages/monocytes in acute inflammatory conditions and gives rise to various signalling pathways that leads to apoptosis or necrosis. Not only does the cytokine support the immune system during inflammation but also takes part in infection resistance and prevention of cancerous conditions (Idriss and Naismith, 2000, Zelova and Hosek, 2013). TNF α binds to cell surface receptors and is involved in three major groups (MAPK) of signalling pathways. These pathways include extracellular-signal-regulated kinases (ERKs), the cJun NH2-terminal kinases (JNKs), and p38 MAP kinases (Sabio and Davis, 2014). As secondary response is instigated which elevated the expression of TNF α. As stated above we have seen that the production of TNF α is regulated by ZFP36L1. Therefore we can say that ZFP36L1 also plays a significant role in inflammation and immune processes, which needs further investigation.

The formation of new blood vessels (angiogenesis) is a highly important process in various processes such as embryonic development, organ development and homeostasis, tissue repair, and disease development. A signalling protein most

extensively researched is VEGF A. Vessel morphogenesis is regulated via VEGF A receptors and is of significance in various roles such as vascular permeability, organ regeneration and network patterning (Matsumoto and Ema, 2014). VEGF A also aids in angiogenesis in cancerous conditions. They help in tumour progression and metastasis, therefore VEGF A inhibitors are vastly studied to prevent the further growth of tumours (Frezzetti et al., 2017). As stated above we have seen that the production of VEGF A is tightly regulated by ZFP36L1. Therefore we can say that ZFP36L1 also plays a significant role in angiogenesis and metastasis, which needs further investigation.

Overall, ZFP36L1 is known to influence several cellular processes that can be studied further to evaluate its significance in potential therapies.

Aims and Objectives

Aims: ZFP36L1 is an RNA binding protein implicated in cancers therefore goal of this project was the analysis of its expression at baseline and upon stimulation in various cell lines. Given the nature of this protein in regulating RNA levels, we hypothesize that its levels are tightly regulated in cell types and several cellular conditions. We also hypothesize that ZFP36L1 regulates expression of effectors and receptors linked to immunity and hence important in controlling cell proliferation and cancer.

Objectives: Our first step is the detection of the protein in cancer cells and quantification of over or under expression of the gene. Our second objective was to investigate its roles in regulating expression of TNF α and VEGF A. The interaction of this protein with immunological surface receptors such as CD44 was also studied.

CHAPTER 2

Materials and Methods

Cell Culture

All cell culture experiments were carried out in the Biosafety cabinet. All chemicals were brought from Sigma Aldrich unless stated otherwise. Materials that were used to carry out all cell culture experiments are as follows. Media: RPMI-1640 with ultra-glutamine (Lonza, cat. no. BE12-704F/U1); DMEM with 4.5g/L glucose and ultra-glutamine (Lonza, cat. no. 12-604F/U1); Foetal Bovine Serum (FBS) (Hyclone cat. no. SV30160.03); Trypsin; Phosphate Buffer Saline (PBS); Trypan Blue; Phorbol 12-Myristate 13-Acetate (PMA); Ionomycin; Dymethylsulphoxide (DMSO); Micropipettes 10ml and 25ml (Sarstedt) Aspiration tips; 25ml Tissue culture flasks, Cell culture dishes (100x20mm and 150x20mm); sterile incubator trays; centrifuge tubes 15ml and 50ml; Haemocytometer; cryovials; 2% Chemgene.

Cell Lines

Cell growth is an important aspect in in-vitro studies. It is important to observe the characteristics of cell growth to assess the effects of any form of treatment. An array of cell lines was cultured and frozen down periodically to prepare stocks. The growth media and cell type of each cell line was cultured according to the information provided by American Type Culture Collection (ATCC).

Cell Line	*Cell Type*	*Disease*	*Growth Type*	*Media*	*Host Species*
MCF 7	Epithelial	Adenocarcinoma	Adehrent	DMEM	Human
B16	Epidermal	Melanoma	Adherent	DMEM	Mouse
EL4	T lymphocytes	Lymphoma	Suspension	DMEM	Mouse
Jurkat	T lymphocytes	T cell leukemia	Suspension	RPMI 1640	Human
T47D	Epithelial	Ductal carcinoma	Adherent	RPMI 1640	Human
MC38/WT	Epithelial	Colon carcinoma	Adherent	DMEM	Mouse
MC38/KO	Epithelial	Colon carcinoma	Adherent	DMEM	Mouse
THP 1	Monocytes	Acute Monocytic Leukaemia	Suspension	RPMI 1640	Human

Table 1: *Summary of cell lines cultured in vitro.*

Guide RNA design and cloning.

The sgRNA oligonucleotides (controls: GCGAGGTATTCGGCTCCGCG, GCTTTCACGGAGGTTCGACG and ZFP36L1: CACC G TCATCGTGGGCGTTCGCACG, TCATCGTGGGCGTTCGCACG), were designed using the Broad CRISPR algorithm (Doench et al., 2016). sgRNAs were cloned into our sgRNA vector using a BsmBI restriction digest. Vector also expresses Vex marker. Cloning was confirmed by sequencing. Frozen aliquots of the knocked down cell lines were used for the following experiments. The results of generation of knocked down cell lines are discussed in the results section below.

Cell Revival

Cryovials retrieved from -80ºC freezers or from liquid nitrogen tanks were allowed to defrost rapidly to room temperature in the biosafety cabinet. Once defrosted, the entire contents of the vial were poured into the culture plate respectively.

Suspension Cells - 25ml TC flasks (THP1, Jurkat, EL4)

Adherent Cells – Culture Dishes (B16, T47D, MCF-7, ZFP36L1^{MC38WT}, ZFP36L1^{MC38KO})

The media was prepared by the addition of 10% FBS to the 500ml bottle of media respectively. This setup was allowed to rest overnight in an incubator with conditions 5% CO2, 37ºC. The following day, cells were checked for adherence and the media was changed to remove the freezing mixture.

Cell passaging/splitting

Cells were checked every two days to check for healthy growth. Once the plate/flask has reached around 80% confluency, cells were split. Splitting ratio: 1:5- 1/5th of the total media, i.e. 1/5th of 10ml. (2ml cell suspension with 8ml media)

Adherent lines: Media was aspirated from the plates. The plates were then washed with 5ml PBS to remove any excess media from the plates. The PBS was then aspirated. The cells were the trypsinised to detach them from the bottom of the plates. (1X) 1ml of trypsin was added to the plates and kept in the warming cupboard for 2min.

Once the cells detach from the bottom of the plate, 9ml media was added (serum inhibits trypsin). The cells were mixed thoroughly to prevent any clumping. 2ml of cell suspension were transferred to newly labelled plate. 8ml of fresh media was added and the cells were allowed to grow for a period of two days.

Suspension lines: Required amount of cell suspension was pipetted out and transferred to a new flask containing fresh media.

Cryopreservation

Cryopreservation is important to preserve cell lines for long periods of times (Jang et al., 2017). It is also required to prepare stocks for future experiments. Cooling to sub-zero temperatures reduces the risk of microbial contamination, cross contamination with other cell lines, genetic drift and morphological changes (Pegg, 2007) The most common freezing mixture used is 90% FBS and 10% DMSO; DMSO helps in preventing the formation of crystals within the cells (Jang et al., 2017). The freezing mixture was cooled before use. The cells were frozen when plates reached around 80% and were 1.5-2 × 10^6 cells/ml. They were frozen at passage number 3.

The cells were trypsinised and centrifuged for 5min at 1200rpm. Supernatant was discarded and the pellet was re-suspended with 10ml of freezing mixture. 1ml aliquots were dispensed into 10 cryovials. The vials were labelled appropriately and transferred to an isopropanol chamber and placed in -80ºC overnight. They were later transferred to liquid nitrogen tanks.

Cell Counting and Viability

It is required to count cells and measure their viability before the start of any in vitro cell culture experiments. The haemocytometer was primarily used to count blood cells and several adaptations have been used since to improve accuracy and increase time efficiency (Cadena-Herrera et al., 2015). Haemocytometer was used for cell counting and trypan blue exclusion assay to calculate the viability of the cell line. The protocol that we used to count cells and viability before every experiment is as follows (Adapted from Abcam website).

The haemocytometer was cleaned and the coverslip was placed on top of the grid. The cell suspension was mixed thoroughly using a pipette for even cell distribution. From the suspension 100ul was pipetted into an eppendorf tube. To this equal

volume (100ul) of trypan blue solution (0.4%) was mixed. Approximately 10ul of this mixture was taken and carefully pipetted at the edge of the coverslip (capillary action). The grid was focused under the microscope and the cells were counted using a tally counter.

Principle: Live cells and healthy cells have a fully functional cell membrane that would not have allowed the entry of any stain such as trypan blue, eosin or propidium (Strober, 2015). Dead cells would be stained due to a degraded cell membrane. Visually, the cytoplasm of dead cells would be stained blue, whereas the live cells would exclude it.

To calculate the number of live cells in 10ml of cell suspension, live cells were counted from 4 large corner squares and recorded. Average was calculated and multiplied by 10^4. Volume of 1 corner square is 10^{-4} cm^3. Value was then multiplied with 2 taking into account the dilution factor of trypan blue (1:2). Final volume was then multiplied was found to be the number of live cells/ml in cell suspension. Viability was counted as,

No. of live cells/ Total no. of cells ×100 = % viability

Western Blot

All chemicals were brought from Sigma Aldrich unless stated otherwise. Resolving gel and stacking gel was prepared according to Sambrook et al. (1989)

12% Resolving Gel	Quantities for 2 gels (15ml)	5% Stacking Gel	Quantities for 2 gels (3ml)
Distilled water	4.9ml	Distilled water	2.1ml
30% Acrylamide	6.0ml	30% Acrylamide	500ul
1.5M Tris (pH 8.8)	3.8ml	1M Tris (pH 8.8)	380ul
10% SDS	150ul	10% SDS	30ul
10% APS	150ul	10% APS	30ul
TEMED	6ul	TEMED	3ul

Table 2: *Reagents for the preparation of resolving and stacking gel.*

Other reagents that were prepared for the blotting procedure included, running buffer (pH 8.3), Loading buffer (2X), Transfer buffer (Made fresh every run), Wash buffer (pH 7.6), Blocking buffer. Finer materials are stated in the protocol which was adapted from Tahrin Mahmood et al. (2012)

Principle: Western blotting is a technique mainly used for the separation of molecules and proteins based on molecular weight and size. Researchers can identify and confirm the presence of the protein of interest from a complex mixture of proteins (Ghosh et al., 2014, Mahmood and Yang, 2012)

The number of cells were counted and recorded as 1.7×10^6 cells/ml; 87% viable. Cells were collected by trypsinisation and centrifuged at 1000rpm for 5min. 60ul of cell lysis buffer was added to the pellet and incubated for 30min on ice. The lysate was then centrifuged at 16,000rpm for 20min at 4ºC. The supernatant was

collected and stored at -20ºC. Bradford assay was carried out and the protein concentration was found to be 15mg/ml.

Sample preparation: From the prepared lysate, 10ul was pipetted into a fresh tube and was mixed with 15ul of loading buffer (SDS reducing buffer). This was heated at 95ºC for 5min. On loading the samples along with a pre stained protein marker (Page ruler plus Pre-stained Protein ladder, cat #26619, Thermo Scientific) the gels were run in an electrophoretic tank at respective times: Stacking gel: 50V for 10-15min. Resolving gel: 120 V for 45-60min until the dye front reaches the bottom of the gel. Blotting: Transfer sandwich was prepared and run.

ANTIBODY	HOST	ISO TYPE	CLASS	TYPE	CONJUGATE	TARGET	COMPANY
BRF1/BRF2 Polyclonal Antibody	Rabbit	IgG	Polyclonal	Primary	Purified	ZFP36L1/2	PA5-17364 (Invitrogen)
HRP Donkey anti-rabbit IgG (minimal x-reactivity) Antibody	Donkey	IgG	Polyclonal	Secondary	HRP conjugated	BRF primary antibody	406401 (Bio legend)
Anti-Tubulin antibody [B-5-1-2]	Mouse	IgG_1	Monoclonal	Primary	Unconjugated (Protein A-Purified)	Tubulin (Control)	
HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody	Goat	IgG	Polyclonal	Secondary	HRP conjugated	Tubulin primary antibody	405306 (Biolegend)
PD-L1/CD274 Antibody	Rabbit	IgG	Polyclonal	Primary (Irrelevant Antibody)	Unconjugated	CD274	17952-1-AP (Proteintech)

Table 3: *Table summarizing the antibodies used for western blot.*

All air bubbles were carefully removed. Transfer was done in a tank containing ice. Blotting was carried out at 150mA at a high voltage of 500V for 90min. On

completion of the transfer the membrane was washed 3 times with wash buffer. Membrane was blocked with 5% blocking buffer for one hour. Membrane was washed 3 times, 5min each wash. Primary antibody (BRF1/2 Polyclonal Antibody) was added at the dilution 1:1000 and was left overnight at 4ºC on a shaker. The membrane was then washed 3 times. The membrane was then stained with secondary antibody (HRP Donkey anti-rabbit antibody) with a dilution of 1:10,000 for 1 hr. This was followed by a washing step 3 times. The membrane was then subjected to primary antibodies (anti- tubulin antibody) as a control and was incubated for 1hr at a dilution of 1:1000 and then with secondary antibody (HRP Goat anti- mouse antibody) at a dilution of 1:10,000 for 1hr. After the following washing steps the membrane was subjected to Enhanced chemiluminescence solution (ECL). [ECL solution was prepared by mixing 5ml of solution A and solution B as per manufacturer]. The membrane was allowed to stand for 90sec. the membrane was then read using the chemiluminescence reader (Fusion).

Pre-clearing cell lysates: 3.5uL of off-target and irrelevant antibody belonging to the same species and isotype as the immunoprecipitation antibody (PD-L1/CD274 Antibody- Rabbit IgG Polyclonal Primary antibody) to 60ul of lysate. The tubes were incubated for 60 min on ice. 6uL of bead slurry was added to the lysate. This was incubated for 30 min at 4°C. The tubes were spun in a micro centrifuge at 12,000 rpm at 4°C for 20 min. Pellet was discarded.

The entire experiment was repeated twice.

Cell stimulation

The cells were stimulated using PMA/ionomycin at a concentration of 5ng/ml. Cells were cultured in 4 dishes and were stimulated with PMA/ionomycin at intervals of 1hr, 2 hr, 4 hr and 6 hr respectively. The cells were then counted and the viability was measured at each time interval.

Enzyme Linked Immunosorbant Assay (ELISA)

Two primary ELISA experiments were carried out to study the positive and negative regulation expressed by ZFP36L1. As previously discussed, we looked into the secretion of two signal proteins TNF α and VEGF A in stimulated ZFP36L1WT^{MC38} and ZFP36L1KO^{MC38} cell lines. Both the experiments were carried out using two ELISA kits as follows, Human TNF α Elisa Ready-Set-Go! (eBioscience, ref: 88-7346-88) and Human VEGF A Elisa Kit (Invitrogen, ref: BM2772). Both the kits measure proteins present in 'human' cells lines whereas, the MC38 cell lines are of murine origin. Although according to the description received on the kits, it is sensitive to measure proteins from both 'human and murine' origin. The kits have been specifically tested on human cell lines, but they are sensitive to TNF α and VEGF A present in mouse cell lines as well.

The experiments were carried out using cell suspensions containing 2.6×10^6 cells/ml. The vials were spun down at 1200rpm for 5min and the supernatant was collected. This supernatant that consisted of TNF α and VEGF A was measured.

TNF α measurement: The 96 well plate was coated with 100ul of capture antibody diluted in coating buffer and incubated overnight at 4ºC. The wells were then aspirated and washed 4 times using 250ul of wash buffer. The wells were blocked with 200ul of ELISA/ELISPOT diluent and incubated for 60 min at room temperature. 100ul of standard concentration (500pg/ml) was added to the appropriate wells. A two-fold serial dilution was carried out for a total of 7 wells. Two wells were used as a blank and to remaining wells 100ul of the samples were added. The plate was incubated overnight at 4ºC. The wells were washed 3 times. 100ul of detection antibody was added to each well and was kept for incubation at room temperature for 60 min. 100ul/well of Avidin-HRP was added and incubated for 30 min at room temperature. Plate was washed 7 times. TMB solution was

added to each well (100ul/well) and left at room temperature for 15 min. After 15 min 50ul of stop solution was added to all the wells and a colour change was observed (blue to yellow). The plates were read at 450nm and analysed using Omega software in the Flurostar plate reader. The experiment was repeated twice to evaluate significance of results.

	1	2	3	4	5	6	7	8
A	Standard (500ng/ml)	Standard (500ng/ml)	ZFP36L1WT Cells only	ZFP36L1WT Cells only	ZFP36L1WT Cells only	ZFP36L1KO 4 hours	ZFP36L1KO 4 hours	ZFP36L1KO 4 hours
B	Standard (425ng/ml)	Standard (425ng/ml)	ZFP36L1WT 1 hour	ZFP36L1WT 1 hour	ZFP36L1WT 1 hour	ZFP36L1KO 6 hours	ZFP36L1KO 6 hours	ZFP36L1KO 6 hours
C	Standard (350ng/ml)	Standard (350ng/ml)	ZFP36L1WT 2 hour	ZFP36L1WT 2 hours	ZFP36L1WT 2 hours			
D	Standard (275ng/ml)	Standard (275ng/ml)	ZFP36L1WT 4 hours	ZFP36L1WT 4 hours	ZFP36L1WT 4 hours			
E	Standard (200ng/m)	Standard (200ng/m)	ZFP36L1WT 6 hours	ZFP36L1WT 6 hours	ZFP36L1WT 6 hours			
F	Standard (125ng/ml)	Standard (125ng/ml)	ZFP36L1KO Cells only	ZFP36L1KO Cells only	ZFP36L1KO Cells only			
G	Standard (50ng/ml)	Standard (50ng/ml)	ZFP36L1KO 1 hour	ZFP36L1KO 1 hour	ZFP36L1KO 1 hour			
H	Blank	Blank	ZFP36L1KO 2 hours	ZFP36L1KO 2 hours	ZFP36L1KO 2 hours			

Table 4: *96 well plate layout to measure the TNF α secretion in MC38 cell line.*

VEGF A measurement: The 96 well plate was washed with 400ul/well with wash buffer. 100ul of standard concentration (1000pg/ml) was added to the appropriate wells containing 100ul of sample diluent. A two-fold serial dilution was carried out for a total of 8 wells. Two wells were used as a blank and to remaining wells 50ul of the sample diluent was added. To the sample wells 50ul of sample was added. The plate was then incubated at room temperature for 120 min. Plate was washed

7 times. 100ul of Biotin-Conjugate was added to all the wells and was allowed to incubate at room temperature for 60 min. Plate was washed 7 times. Streptavidin-HRP was added to all the wells (100ul/well) and was incubated at room temperature for 60 min. Plate was washed 7 times. TMB solution was added to each well (100ul/well) and left at room temperature for 30 min. After 30 min 100ul of stop solution was added to all the wells and a colour change was observed (blue to yellow). The plates were read at 450nm and analysed using Omega software in the Flurostar plate reader. The experiment was repeated twice to evaluate significance of results.

	1	2	3	4	5	6	7	8
A	Standard (1000 pg/ml)	Standard (1000 pg/ml)	ZFP36L1WT Cells only	ZFP36L1WT Cells only	ZFP36L1WT Cells only	ZFP36L1KO 4 hours	ZFP36L1KO 4 hours	ZFP36L1KO 4 hours
B	Standard (500 pg/ml)	Standard (500 pg/ml)	ZFP36L1WT 1 hour	ZFP36L1WT 1 hour	ZFP36L1WT 1 hour	ZFP36L1KO 6 hours	ZFP36L1KO 6 hours	ZFP36L1KO 6 hours
C	Standard (250 pg/ml)	Standard (250 pg/ml)	ZFP36L1WT 2 hour	ZFP36L1WT 2 hour	ZFP36L1WT 2 hour			
D	Standard (125 pg/ml)	Standard (125 pg/ml)	ZFP36L1WT 4 hours	ZFP36L1WT 4 hours	ZFP36L1WT 4 hours			
E	Standard (62.5 pg/ml)	Standard (62.5 pg/ml)	ZFP36L1WT 6 hours	ZFP36L1WT 6 hours	ZFP36L1WT 6 hours			
F	Standard (31.3 pg/ml)	Standard (31.3 pg/ml)	ZFP36L1KO Cells only	ZFP36L1KO Cells only	ZFP36L1KO Cells only			
G	Standard (15.6 pg/ml)	Standard (15.6 pg/ml)	ZFP36L1KO 1 hour	ZFP36L1KO 1 hour	ZFP36L1KO 1 hour			
H	Blank	Blank	ZFP36L1KO 2 hours	ZFP36L1KO 2 hours	ZFP36L1KO 2 hours			

Table 5: *96 well plate layout to measure the VEGF-A secretion in MC38 cell lines.*

Bio Imaging

This visualisation technique has been used by several researchers to record biological processes in real time. The use of fluorescence technology has come a long way in the detection of cell signalling pathways and co-localization. We have used this technique to help us observe the presence of interaction and co-localization between our protein ZFP36L1 and CD44 (cell surface receptor).

A flask with 80% confluency was chosen. The cells were counted and recorded to be 2.3×10^6 cells/ml with 87% viability. A 6 well plate was used to culture cells with a single coverslip in each well. The plate was labelled as follows,

Well 1: ZFP36L1WT^{MC38} cells only unstimulated. Well 2: ZFP36L1WT^{MC38} unstimulated, stained with ZFP36L1 and CD44 antibodies. Well 3: ZFP36L1WT^{MC38} was stimulated with PMA for 4hrs along with ZFP36L1 and CD44 antibodies. Well 4: ZFP36L1KO^{MC38} cells only, unstimulated. Well 5: ZFP36L1KO^{MC38} unstimulated, stained with ZFP36L1 and CD44 antibodies. Well 6: ZFP36L1KO^{MC38} was stimulated with PMA for 4hrs along with ZFP36L1 and CD44 antibodies.

1 **MC38 WT** Cells only	**2** **MC38 WT** BRF + CD44	**3** **MC38 WT** (PMA) +BRF + CD44
4 **MC38 KO** Cells only	**5** **MC38 KO** BRF + CD44	**6** **MC38 KO** (PMA) + BRF + CD44

Table 6: *The 6 well plate layouts.*

1 ml of cells was loaded in each well. It was allowed to grow for 48hrs till the coverslips were completely confluent. Media was changed carefully after 24 hrs. Once confluent, the cells were stimulated with PMA (5ng/ml) for 4 hrs. Media was discarded. Each well was washed with 1ml PBS twice.

CD44 Primary Antibody was added to the specific wells on the cover slip at a dilution of 1:100. The plate was incubated for 60 min incubation at 4ºC. Plate was washed twice with PBS. CD44 secondary Antibody (green) was then added at 1:100 dilutions. The plate was then incubated for 60min at 4ºC. The cells were then fixed with 50ul of 4% Paraformaldehyde (PFA) which was incubated for 20min on an ice bath. The cells were then made permeable by treatment with 200ul of Triton X-100 for 10min. 1ml of blocking buffer was added to each well. (2gm/100ml Bovine Serum Albumin (BSA)). Supernatant was removed. The wells washed once with PBS to remove any excess triton x. BRF primary antibody was added in 1:100 dilutions and was allowed to incubate for 1hr at 4^{o}C. Followed by washing steps, BRF secondary antibody (red) was added in 1:100 dilutions and was incubated for 60min in the fridge. The cells were washed with PBS and the coverslips were removed with the use of a syringe needle. They were allowed to air dry. To the coverslips 10ul of 4′, 6-diamidino-2-phenylindole (DAPI cat. no. H1500, Vectasheild Hard set Mounting medium) stain was pipetted and were inverted over the slides. They were allowed to air dry for 30 min. The slides were then visualised under the confocal microscope. The complete experiment was performed thrice to achieve good quality images.

ANTIBODY	HOST	ISO TYPE	CLASS	TYPE	CONJUGATE	TARGET	COMPANY
BRF1/BRF2 Polyclonal Antibody	Rabbit	IgG	Polyclonal	Primary	Unconjugated	ZFP36L1	PA5-17364 Invitrogen
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Donkey	IgG	Polyclonal	Secondary	Alexa fluor 555 conjugate (Red)	BRF primary Antibody	A-31572 Life technologies
Anti-CD44 antibody [F10-44-2]	Mouse	IgG2a	Monoclonal	Primary	Unconjugated	CD44 receptors	ab6124 Abcam
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Goat	IgG	Polyclonal	Secondary	Alexa fluor 488 conjugate (green)	CD44 Primary Antibody	A-11029 Invitrogen

Table 7: *Summary of the antibodies used for the bio imaging protocol.*

CHAPTER 3

Results

Generation of ZFP36L1KO^{MC38} stable cells lines

MC38 cell lines expressing Cas9 were transduced with lenti-virus continuing control or ZFP36L1 guides vectors. Transduced cells were selected by flow cytometry sorting on Vex expression. Vex is fluorescent marker present within the vector sequence. Cells were grown in culture and selected again by sorting, until purity reached >98%. Gene knockout was confirmed by flow cytometry (FACS) and western blot (Fig 6 and Fig 7).

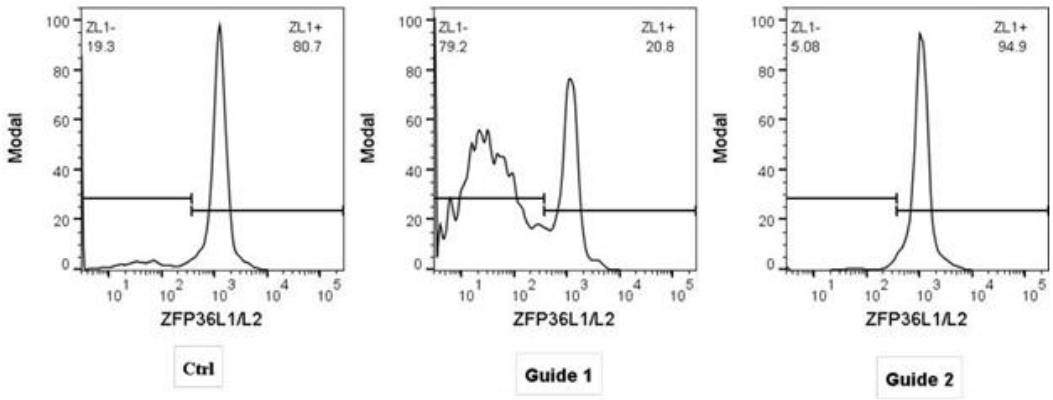

Figure 6: *Crispr-Cas9 knockdown of ZFP36L1 in MC38 cells.* Intracellular staining of ZFP36L1 in control (A), guide 1 KO (B) and guide 2 (C) transduced MC38 cell using primary ZFP36L1 antibody followed by PE-labelled secondary antibody. Prior to staining transduced MC38 cell using primary ZFP36L1 antibody followed by PE-labelled secondary antibody. Prior to staining transduced cells were selected by flow cytometry sorting.

Validation of ZFP36L1KO^{MC38} and other cell lines

A second experiment was carried out to detect ZFLP36L1 by western blotting. On screening various cancer cells, ZFP36L1 was detected as discreet band around 36 kDa molecular weight. The bands (Fig 7) at 50 kDa represent the loading control β tubulin bands. ZFLP36L1 was visualized at 36 kDa as a single band. Absence of the band in the lane containing knocked out MC38 cell line confirmed the knockout of gene in ZFP36L1KO^{MC38} and its precise location on gel in all other cell lines tested. Interestingly MCF 7 and ZFP36L1WT^{MC38} lines showed a slightly higher intense band of ZFP36L1 compared to all other lines. Here we concluded that ZFP36L1 is widely expressed in various tumour lines and therefore warrants its further examination.

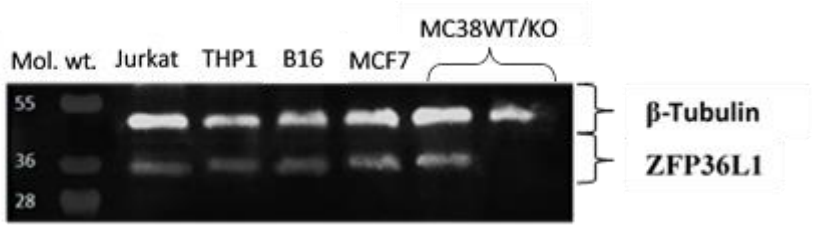

Figure 7: *Visualization and Detection of ZFP36L1.* Nitrocellulose membrane showing β-Tubulin and ZFP36L1 bands in several cell lines. Picturisation of western blot showing bands at 50 kDa (β-tubulin control) and bands at 36 kDa (ZFP36L1). The final lane shows the absence of ZFP36L1 band, confirming its presence in all other lines.

Cell growth and cell test of KO cells

All cell lines were grown for an average period of 10 days and cell densities were plotted. Cells were counted at the end of each day and the values of viable, live cells were recorded. A graph was then plotted to compare growth rates of two cell lines (Fig 8). Wild type MC38 lines showed higher growth rate compared to the knocked out cell lines. The presence of ZFP36L1 could be attributed to the higher growth rate in wild type lines therefore, from the above-mentioned observations, we can speculate that ZFP36L1 has a role to play in the growth, proliferation and development of MC38 cells.

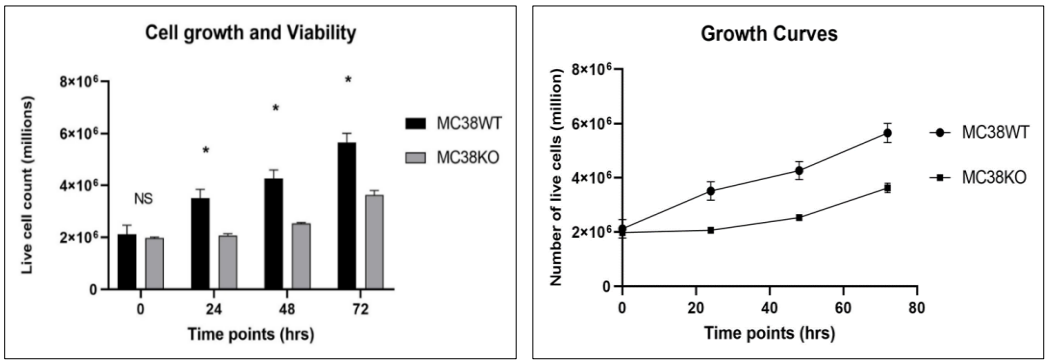

Figure 8: *Comparative growth curves of MC38 cell lines.* It is clearly seen that MC38WT grew at a higher rate than knock out lines rendering them more viable. ”NS” represents non-significant value. “*” represents p value < 0.05, significant values (n=2).

To study roles of ZFP36L1 expression in effector functions of cells we treated cells with PMA. Prior to this, we conducted a plot experiment, to assess the appropriate treatment conditions for chosen cell lines. The stimulation was carried out over a period of 0 to 24 hours. At around 2-5 hours the cells remained stable after which they started to deteriorate. The stimulation of cells over the time periods showed that the cells were the most viable from 2-5 hours with about 70-50% viability. Significant cell death was seen after 4 hours. Therefore, experiments were

conducted following PMA treatment at 4 hours when the cells were at maximum viability to see the clearest effects of stimulant and prevent confounding factors of cell death.

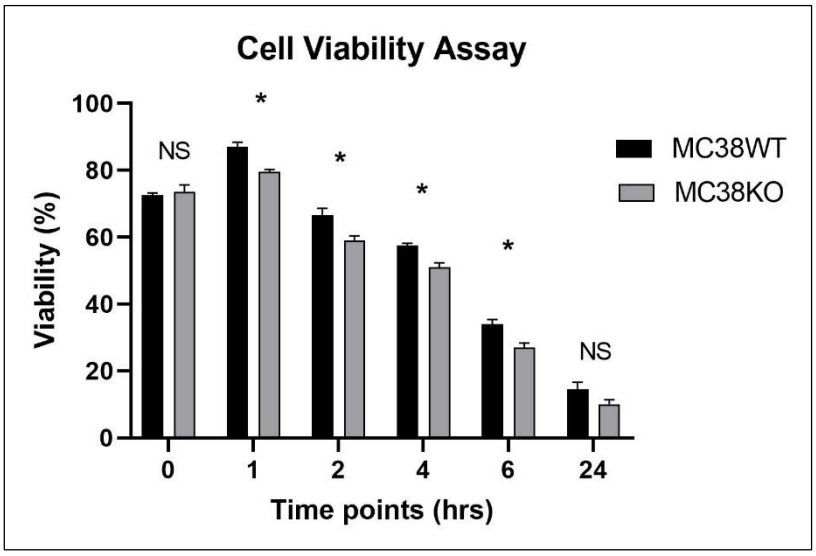

Figure 9: *Cells stimulated with PMA.* THP 1, MC38WT and MC38KO were stimulated over a period of 0 to 24 hours. Cells were the most stable at 4 hours. The significance of the result was calculated from the time points 0hr to 6hr."*" represents the p-value which was calculated as .00041. The result is significant at $p < 0.05$.

ZFP36L1KO^{MC38} cells produce higher amounts of inflammatory cytokine TNF α compared to wild types.

ZFP36L1WT^{MC38} and ZFP36L1KO^{MC38} cells were either left untreated or treated with PMA from 1h to 6h at a concentration of 500ng/mL (Fig. 9). The cells were collected at the time intervals and were subjected to ELISA and the levels of TNF α measured as described in method section. As shown previously (Fig 9), the optimum stimulation time was found to be 4 hours. We observed that 4hrs TNF α secretion was the highest. In addition, we noticed that knockout lines there was a steep increase in TNF α levels whereas in wild type lines there was a gradual

increase (Fig 11). These results clearly showed that secretion of effector molecules was higher in the knocked out cell lines compared to the wild types. The values obtained were of significant difference. We concluded that ZFP36L1 was responsible for the degradation of mRNA that produces TNF α. We also concluded from these results that ZFP36L1 is a negative regulator of TNF α production and stability.

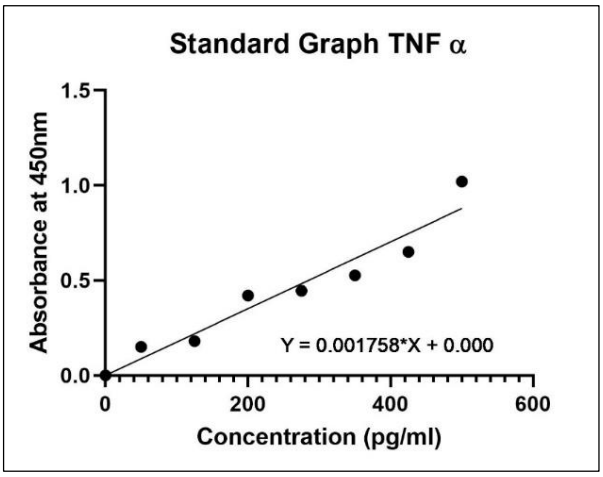

Figure 10: *Linear standardization of TNF α secretion.* A standard graph representing concentrations of TNF α ranging from 0pg/ml to 500pg/ml. Substituting the sample values in the equation of the standard graph, the concentrations of TNF α is calculated for the samples.

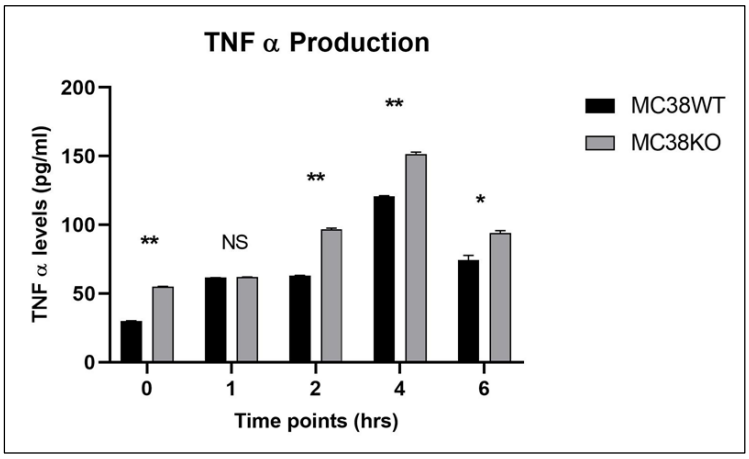

Figure 11: *Secretion of TNF α over 6 hours.* At 4 hours it was clearly seen that the level of TNF α in ZFP36L1WT^{MC38} was lesser than that of ZFP36L1KO^{MC38}. A t test was carried out to obtain the significance of the values. NS- not significant. '*'- the p value is statistically significant ($p<0.05$) with ($n=2$). "**"- the p value is statistically significant ($p<0.001$) with ($n=2$).

ZFP36L1KO^{MC38} cells produce higher amounts of VEGF A compared to wild types.

Our protein of interest, ZFP36L1 regulates the VEGF pathway as mentioned previously. This phenomenon was studied by stimulating ZFP36L1WT^{MC38} and ZFP36L1KO^{MC38} cells with PMA and the VEGF A produced was measured. They were treated with PMA (10ul/ml) over a period of 1, 2, 4 and 6hrs. The cells were collected at the time intervals and were subjected to ELISA and the levels of VEGF A were measured. It was observed that 4hrs was the optimal time for VEGF A secretion. The experiment results showed that in knockout lines there was a rapid increase in VEGF A levels whereas in wild type lines there was a gradual increase. When compared to each other, the levels of VEGF A produced in knock out lines were higher than the wild types (Fig 13). We derived at the conclusion that ZFP36L1 was responsible for the degradation of mRNA that produces VEGF A. This clearly indicates that ZFP36L1 negatively regulates VEGF A production.

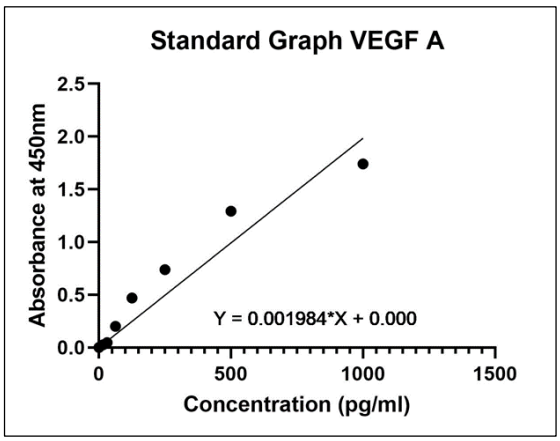

Figure 12: *Linear standardization of VEGF A secretion.* Linear standardization of VEGF A secretion. A standard graph representing concentrations of VEGF A ranging from 0pg/ml to 1000pg/ml. By substituting the sample values in the equation of the standard graph, the concentrations of VEGF A was calculated for the samples.

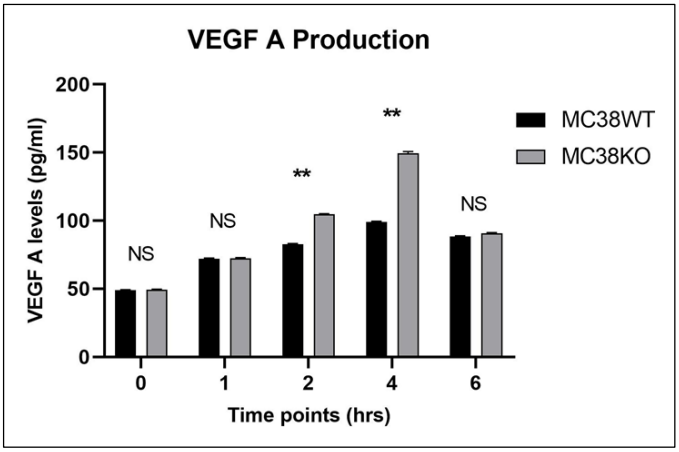

Figure 13: *Secretion of VEGF A over 6 hours.* At 4 hours it was clearly seen that the level of VEGF A in ZFP36L1WT^{MC38} was lesser than that of ZFP36L1KO^{MC38}. A t test was carried out to obtain the significance of the values. NS- not significant. '**'- The p value is statistically significant ($p<0.001$) with ($n=2$).

ZFP36L1 co-localizes with CD44 receptors.

The role of ZFP36L1 in cellular processes such as migration, cell to cell interactions and metastasis were evaluated. CD44 was selected as it is highly expressed in several cancers. This cell surface receptor is known to regulate metastasis and was therefore selected for any possible interactions with ZFP36L1. We would like to see if the protein is not only involved in the regulatory aspect of cell signalling but also metastasis. This study was carried out using bio imaging where the proteins and receptors were stained with specific fluorescence antibodies and were visualised using confocal microscopy. If ZFP36L1 presents any co-localization with CD44 it can be postulated that the zinc finger has a significant role in migration and metastasis (Fig 14). Further studies would be needed to validate the extent of this significance of ZFP36L1 in tumour development as the data presented is merely preliminary results.

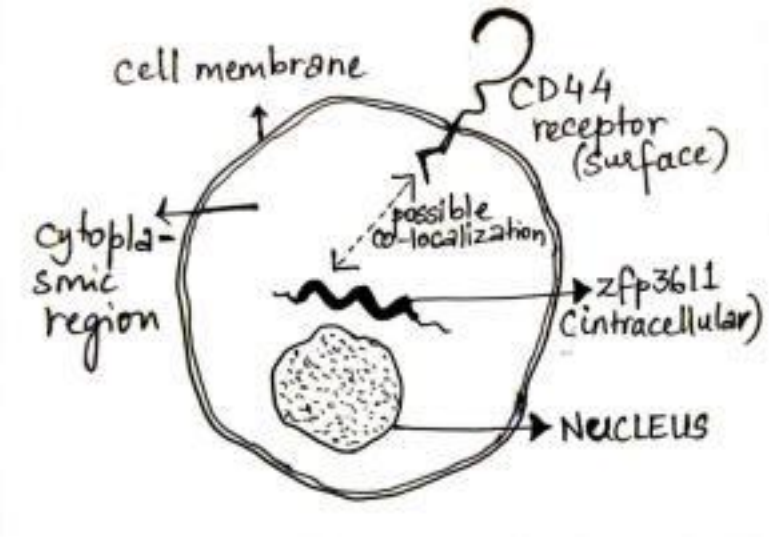

Figure 14: *Possible interactions between ZFP36L1 and surface receptors.*

ZFP36L1KO^{MC38} and ZFP36L1WT^{MC38} cells were stained using two antibodies: Anti-ZFP36L1 (red) and Anti-CD44 (green) in the presence and absence of PMA stimulation. A blue colour was observed as a result of DAPI stain. The cells were permeabilized to allow the red stain to penetrate the cells and stain the intracellular protein. The cells were stained intra-cellularly with anti-ZFP36L1 (red) and the surface were stained with anti-CD44 (green). An overlay of images show yellow visualization if there is any co-localization present. The unstimulated wild type cells show limited interaction as the levels of ZFP36L1 is low. There was a slight co-localization in the unstimulated wild type cells (yellow) (Fig 15 B2). On the other hand, the stimulated wild type cells show a slightly higher interaction between the two proteins (Fig 15 B3). The slight red seen in the knocked down cell lines can be contributed to non-specific staining or background staining (Fig 15 A2 and A3). Interestingly the interactions observed are promising and have not been shown before. The presence of interaction between ZFP36L1 and CD44 can be quantified and studied further.

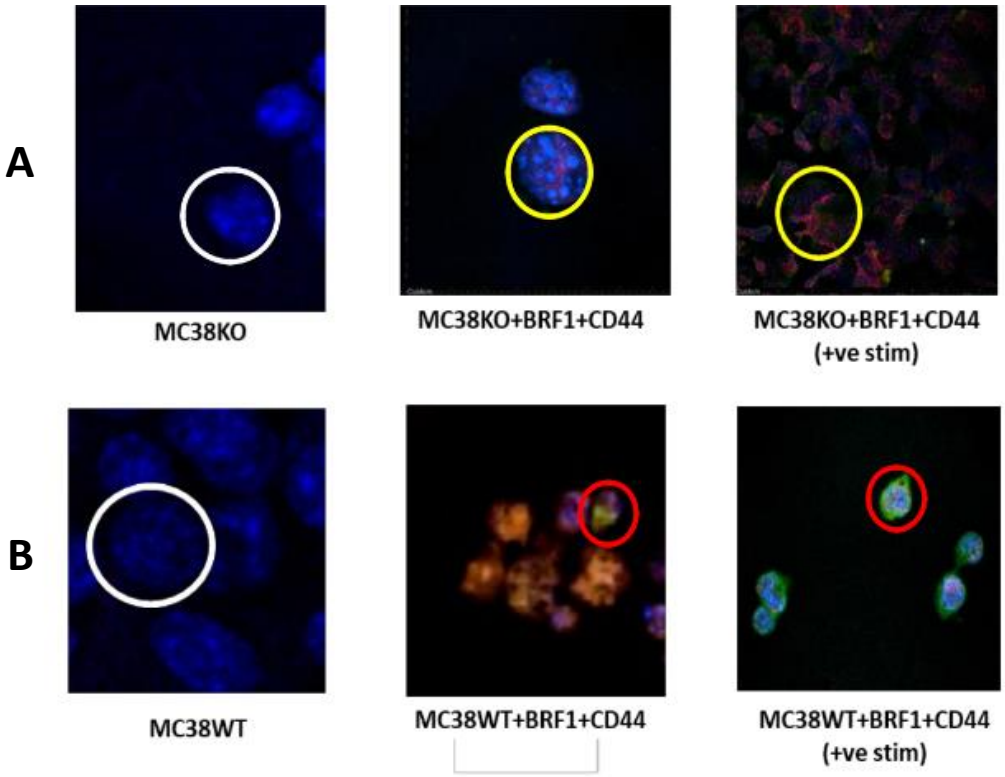

Figure 15: *Confocal microscopic images of intracellular CD44 and ZFP36L1 co-localization.* White circles (A1 and B1) indicate the control showing nuclear staining with DAPI (blue). Yellow circles (A2 and A3) indicate stimulated and unstimulated ZFP36L1KO^{MC38} lines. They presented with no yellow visualisation. Red circles (B2 and B3) indicate stimulated and unstimulated ZFP36L1WT^{MC38}. Yellow colour represents overlay of the two stains (red and green).

Chapter 4

Discussion

It has been postulated that the members of ZFP36 family post-transcriptionally regulate protein expression by binding to the 3' untranslated ARE of mRNA and activating its degradation in various cancer forms (Bye et al., 2018). The approach taken in this study has been to examine a series of cancer derived cell lines including adenocarcinomas, melanomas, lymphomas, colon carcinomas and acute monolytic leukaemia with the aim of establishing differential expression. In this way we narrow down the link between ZFP36L1 and specific forms of cancer.

ZFP36L1 is an RNA binding protein that controls growth, development, differentiation and/or maturation pathways of the cells (Zekavati et al., 2014). Due to the nature and function of this protein in regulating RNA levels by de stabilising mRNA, it is hypothesised that its levels are regulated in different cells under different conditions. An additional aim of this study was to investigate the regulatory effects of ZFP36L1 on effector molecules and the immunological receptors CD44. Firstly, detection of protein expression at basal level and quantification of it's the expression levels were carried out. Secondly, the cell line ZFP36L1KO^{MC38} was generated using the CRISPR-cas9 system to provide as a negative control for all experiments. Thirdly, we examined the role of ZFP36L1 in regulating expression of TNF α and VEGF A. Lastly, the interaction of this protein with immunological surface receptors such as CD44 was observed.

ZFP36L1KO^{MC38} lines were generated using clustered, regularly interspaced short palindromic repeats (CRISPR)/cas9 system (Doench et al., 2016). Two guides (Guide 1 and Guide 2) were incorporated in two MC38 cell lines expressing cas9, an enzyme mediating apoptosis in cells. The cells expressing cas9 were sorted using flow cytometry. These cells were re-cultured until the cell lines showed 100% purity. The peaks present in the graphs indicate that the cells transduced with guide

1 were successfully knocked out, whereas the absence of peaks in graphs indicating guide 2 showed that the knockout was unsuccessful. Therefore, this cell line was selected as the knockout lines. They were confirmed further using western blot.

The observation of cell growth for the presence of ZFP36L1 in ZFP36L1^{MC38WT} and ZFP36L1^{MC38KO} lines has not been shown before, therefore an experiment was conducted to compare the two. The cells examined in this study expressed the zinc protein ZFP36L1. It is therefore reasonable to assume that the growth of these cells may depend on the ZFP36L1 as one of the contributing factors. The THP 1 cells showed the weakest growth rate of all the cells cultured (data not shown). The growth rate of other cell types were observed such as B16, MCF 7 and Jurkat (data not shown) and same cell lines were used for Western blotting. We noticed the presence of ZFP36L1 in adherent cells at a higher intensity compared to suspension cells. Therefore we can say that ZFP36L1 is produced slightly higher in epithelial lines than in monocytic cells. Quantitative analysis needs be conducted to substantiate the statement. Screening of several cancer cell lines for ZFP36L1 has not been done before and is a new and significant experiment.

A steady growing cell line used was the MC38 and further experiments were conducted using the respective line. The reason for selection of MC38 lines is its implications in testing immunotherapy protocols and in studies on the host immune response. MC38 lines are known to be one of the most competent model cell lines for the studies of immunological reactions (Minute et al., 2020, Kuang et al., 2020). Some of the studies with the implication of MC38 cell lines include, targeting immune checkpoints, immune evasion, immune response and used as pre-clinical mouse models. Since these lines are regularly used for immunotherapy and cancer studies they form a reasonably good in vitro model for the investigation of protein expressions (Seyedin et al., 2020).

It was observed that wild types showed better cell growth compared to the knockouts. They quickly reached confluency and required media changes every two days. On the other hand, the knockouts took longer to reach confluency and did not consume media as compared to the wild types. It is known that ZFP36L1 plays a significant regulatory role in cell cycle and proliferation (Suk et al., 2019). Therefore we can assume that the knockouts, due to the absence of this protein did not grow as steady as the wild type which contained the zinc finger protein. There is evidence that ZFP36L1 is involved in growth and cell fate in glioma progenitors and glioma cells (Weng et al., 2019). Therefore expression of ZFP36L1 in this cell line could play a role in tumour genesis, which need further investigation. One of the easiest and most reliable methods to detect the presence of a protein in a cell or tissue sample is to carry out detection by a Western blot (Mahmood and Yang, 2012). Western blot allows to detect the presence of the protein but also to quantify the amount of protein present in the samples. ZFP36L1 has not been confirmed in MC38 colorectal cancer lines before and therefore could be studied further as a possible biomarker. As stated above, the molecular weight of ZFP36L1 is predicted to be at 36kDa, where the bands are present. The higher intensity band above are the control bands tubulin. The bright intensity of the control bands resulted in a higher expression of microtubules in all cells. The results prove the presence of ZFP36L1 in MC38 wild types and this was confirmed by the lack of a band in the knock out lanes. We also noticed that the bands appear fainter in suspension cellular lines such as THP 1 and Jurkat, compared to adherent cell lines such as MCF 7 and B16 although the amount of protein loaded in all the wells were constant.

Regulatory effects of ZFP36L1 was studied upon stimulation of cells with PMA/ionomycin to induce activation pathways within the cell. The over expression or under expression of protein was studied by stimulation of cells. The MC38 wild types and knockouts were stimulated with PMA/ionomycin as per standard

protocol. PMA is known to be a stimulator of differentiation in several lines by inducing protein kinase C pathways (Juneja et al., 2017). According to our previous studies, the activity of ZFP36L1 is known to be initiated by a stimulus such as differentiation and proliferation (Suk et al., 2019, Guo et al., 2019). Therefore the stimulator PMA/ionomycin was used to stimulate the cells. The optimum time at which the PMA/ionomycin stimulated the cells the most was examined. We needed the cells to be the most viable with the highest amount of stimulation.

PMA takes around 4 hours in adherent lines and show increased proliferation (Juneja et al, 2017). The results that we observed were of significant values. The cells were stable up to 4 hours following treatment with PMA/ionomycin after which the cells began to deteriorate at a faster rate. The cells were collected, counted and the viability was calculated. Since we concluded that the cells stimulation is optimum at four hours, we harvested the cells before every experiment performed at the respective time. Stimulation of cells is an integral part of any protocol when investigating protein expression.

The inflammatory cytokine TNF α is a signalling protein responsible for inflammatory responses and the signalling of several other pathways that result in apoptosis (Idriss et al, 2000). This protein is tightly regulated as an over production of this protein can lead to abnormal cell proliferation and an acute inflammatory response (Idriss et al, 2000). Previous studies have shown that ZFP36L1 regulates the production of TNF α in a negative manner (Hyatt et al., 2014, Makita et al., 2020). TNF α expression is controlled by the presence of ZFP36L1 by the degradation of its respective mRNA. If the levels of ZFP36L1 are reduced, the levels of TNF α exponentially increase which might lead to inflammatory and cancerous conditions. Our results showed that ZFP36L1^{MC38KO} expressed higher levels of TNF α compared to that of ZFP36L1^{MC38WT} confirming its regulatory effect.

Previous studies show us that ZFP36L1 regulates the production of VEGF A in a negative manner (Ciais et al., 2004, Planel et al., 2010). VEGF A expression is

controlled by the presence of ZFP36L1, by the degradation of its respective mRNA. If the levels of ZFP36L1 are controlled, the levels of VEGF A exponentially increase leading to inflammatory and cancerous conditions. This regulatory effect of ZFP36L1 was studied experimentally by conducting an ELISA and measuring the levels of VEGF A secreted by the cells. As the results depicted, knock out lines showed lower levels of VEGF A. This confirmed the regulatory effect. The results were promising and the measurements obtained were of significant values.

By studying the regulatory effects of ZFP36L1 further, we can modulate and modify accordingly, to target the proliferation of cells in cancerous conditions. Since the signalling proteins are effected by ZFP36L1, we can assume that the protein can have a significant effect on the downstream signalling pathways associated with the proteins. Which leaves us with the question if it has an effect of one or several signalling pathways and if it leads to any major side effects on other parts of the tissues.

Along with the regulatory effects, zinc finger protein was evaluated with respect to metastasis and invasion. This experiment to study the interactions of ZFP36L1 with a surface immune receptor involved in cell to cell adhesion and metastasis has not been done before. We observed a preliminary level of co-localisation that is promising to make any further progress in the imaging studies. An overlay of the stains used picked up a slight interaction between ZFP36L1 and CD44 allowing us to speculate the presence of any form of interaction between the two. The confirmation of co-localization can be seen by the overlay of red and green giving us a yellow colour.

Further investigations can be carried out to collect more significant data regarding ZFP36L1 and CD74/CD44. The study in this area of research could yield results and open an opportunity to discover a therapeutic avenue to treat certain cancers.

Conclusion

The main aim of this study was to observe potential role on proliferative and effector functions mediated by ZFP36L1. A growth study was carried out to compare the growth curves of three cell lines, THP 1, ZFP36L1WT^{MC38} and ZFP36L1KO^{MC38}. Careful examination of cell growth resulted in the confirmation of relative effects of ZP36L1 in population doubling time of cells over a period of 7 days. The presence of ZFP36L1 was confirmed in several cell lines such as Jurkat, THP 1, B16, MCF 7 and MC38 by the absence of bands in the ZFP36L1KO^{MC38} cell lines. The levels of protein produced by different cell lines were relatively observed by the intensity of the bands on the blot. The confirmation of ZP36L1 in all cell lines provides information of its potential roles in cellular processes. To study its integral role in cellular processes, regulatory effects were examined through ELISA experiments. Results indicated the negative regulatory effect of ZFP36L1 on two signalling proteins TNF α and VEGF A. TNF α is a well-known inflammatory cytokine whereas VEGF A is a known growth factor responsible for proliferation of cells. Negative regulatory effects were one of the key findings and is promising for further experimentation. The effects of ZFP36L1 with immune receptor CD44 was elucidated by performing bio imaging studies. Although the data was relatively unclear there was small but significant co-localisation observed in stimulated ZFP36L1WT^{MC38} cells. In conclusion, ZFP36L1 can be investigated further to study its effect on downstream signalling pathways and its effects on cell proliferation.

References

1. BAHRAMI, S. & DRABLOS, F. 2016. Gene regulation in the immediate-early response process. *Adv Biol Regul,* 62**,** 37-49.

2. BALKWILL, F. 2006. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev,* 25**,** 409-16.

3. BAOU, M., JEWELL, A. & MURPHY, J. J. 2009. TIS11 family proteins and their roles in posttranscriptional gene regulation. *J Biomed Biotechnol,* 2009**,** 634520.

4. BELL, S. E., SANCHEZ, M. J., SPASIC-BOSKOVIC, O., SANTALUCIA, T., GAMBARDELLA, L., BURTON, G. J., MURPHY, J. J., NORTON, J. D., CLARK, A. R. & TURNER, M. 2006. The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression. *Dev Dyn,* 235**,** 3144-55.

5. BYE, A. J. H., PUGAZHENDHI, D., WOODHOUSE, S., BRIEN, P., WATSON, R., TURNER, M. & PELL, J. 2018. The RNA-binding proteins Zfp36l1 and Zfp36l2 act redundantly in myogenesis. *Skelet Muscle,* 8**,** 37.

6. CADENA-HERRERA, D., ESPARZA-DE LARA, J. E., RAMIREZ-IBANEZ, N. D., LOPEZ-MORALES, C. A., PEREZ, N. O., FLORES-ORTIZ, L. F. & MEDINA-RIVERO, E. 2015. Validation of three viable-cell counting methods: Manual, semi-automated, and automated. *Biotechnol Rep (Amst),* 7**,** 9-16.

7. CIAIS, D., CHERRADI, N., BAILLY, S., GRENIER, E., BERRA, E., POUYSSEGUR, J., LAMARRE, J. & FEIGE, J. J. 2004. Destabilization of vascular endothelial growth factor mRNA by the zinc-finger protein TIS11b. *Oncogene,* 23**,** 8673-80.

8. DESROCHES-CASTAN, A., CHERRADI, N., FEIGE, J. J. & CIAIS, D. 2011. A novel function of Tis11b/BRF1 as a regulator of Dll4 mRNA 3'-end processing. *Mol Biol Cell,* 22**,** 3625-33.

9. DOENCH, J. G., FUSI, N., SULLENDER, M., HEGDE, M., VAIMBERG, E. W., DONOVAN, K. F., SMITH, I., TOTHOVA, Z., WILEN, C., ORCHARD, R., VIRGIN, H. W., LISTGARTEN, J. & ROOT, D. E. 2016. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol,* 34**,** 184-191.

10. FREZZETTI, D., GALLO, M., MAIELLO, M. R., D'ALESSIO, A., ESPOSITO, C., CHICCHINELLI, N., NORMANNO, N. & DE LUCA, A. 2017. VEGF as a potential target in lung cancer. *Expert Opin Ther Targets,* 21**,** 959-966.

11. GHOSH, R., GILDA, J. E. & GOMES, A. V. 2014. The necessity of and strategies for improving confidence in the accuracy of western blots. *Expert Rev Proteomics,* 11**,** 549-60.

12. GUO, X., PIAO, H., ZHANG, Y., SUN, P. & YAO, B. 2019. Overexpression of microRNA-129-5p in glioblastoma inhibits cell proliferation, migration, and colony-forming ability by targeting ZFP36L1. *Bosn J Basic Med Sci*.

13. HODSON, D. J., JANAS, M. L., GALLOWAY, A., BELL, S. E., ANDREWS, S., LI, C. M., PANNELL, R., SIEBEL, C. W., MACDONALD, H. R., DE KEERSMAECKER, K., FERRANDO, A. A., GRUTZ, G. & TURNER, M. 2010. Deletion of the RNA-binding proteins ZFP36L1 and ZFP36L2 leads to perturbed thymic development and T lymphoblastic leukemia. *Nat Immunol,* 11**,** 717-24.

14. HYATT, L. D., WASSERMAN, G. A., RAH, Y. J., MATSUURA, K. Y., COLEMAN, F. T., HILLIARD, K. L., PEPPER-CUNNINGHAM, Z. A., IEONG, M., STUMPO, D. J., BLACKSHEAR, P. J., QUINTON, L. J., MIZGERD, J. P. & JONES, M. R. 2014. Myeloid ZFP36L1 does not regulate inflammation or host defense in mouse models of acute bacterial infection. *PLoS One,* 9**,** e109072.

15. IDRISS, H. T. & NAISMITH, J. H. 2000. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech,* 50**,** 184-95.

16. ISHII, H., SAITOH, M., SAKAMOTO, K., KONDO, T., KATOH, R., TANAKA, S., MOTIZUKI, M., MASUYAMA, K. & MIYAZAWA, K. 2014. Epithelial splicing regulatory proteins 1 (ESRP1) and 2 (ESRP2) suppress cancer cell motility via different mechanisms. *J Biol Chem,* 289**,** 27386-99.

17. JANG, T. H., PARK, S. C., YANG, J. H., KIM, J. Y., SEOK, J. H., PARK, U. S., CHOI, C. W., LEE, S. R. & HAN, J. 2017. Cryopreservation and its clinical applications. *Integr Med Res,* 6**,** 12-18.

18. JUNEJA, V. R., MCGUIRE, K. A., MANGUSO, R. T., LAFLEUR, M. W., COLLINS, N., HAINING, W. N., FREEMAN, G. J. & SHARPE, A. H. 2017. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *J Exp Med,* 214**,** 895-904.

19. KONDO, M., NOGUCHI, A., MATSUURA, Y., SHIMADA, M., YOKOTA, N. & KAWAHARA, H. 2018. Novel phosphorelay-dependent control of ZFP36L1 protein during the cell cycle. *Biochem Biophys Res Commun,* 501**,** 387-393.

20. KUANG, Z., JING, H., WU, Z., WANG, J., LI, Y., NI, H., ZHANG, P., WU, W., WU, M., ZHOU, S., QIU, X., WU, D., PRINZ, B., BARUAH, H., CHEN, B., YU, M. & LIU, J. 2020. Development and characterization of a novel anti-OX40 antibody for potent immune activation. *Cancer Immunol Immunother,* 69**,** 939-950.

21. LAI, W. S., KENNINGTON, E. A. & BLACKSHEAR, P. J. 2002. Interactions of CCCH zinc finger proteins with mRNA: non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs. *J Biol Chem,* 277**,** 9606-13.

22. LEE, S. K., KIM, S. B., KIM, J. S., MOON, C. H., HAN, M. S., LEE, B. J., CHUNG, D. K., MIN, Y. J., PARK, J. H., CHOI, D. H., CHO, H. R., PARK, S. K. & PARK, J. W. 2005. Butyrate response factor 1 enhances cisplatin

sensitivity in human head and neck squamous cell carcinoma cell lines. *Int J Cancer,* 117**,** 32-40.

23. LIU, Z., CHU, S., YAO, S., LI, Y., FAN, S., SUN, X., SU, L. & LIU, X. 2016. CD74 interacts with CD44 and enhances tumorigenesis and metastasis via RHOA-mediated cofilin phosphorylation in human breast cancer cells. *Oncotarget,* 7**,** 68303-68313.

24. LYKKE-ANDERSEN, J. & WAGNER, E. 2005. Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev,* 19**,** 351-61.

25. MAHMOOD, T. & YANG, P. C. 2012. Western blot: technique, theory, and trouble shooting. *N Am J Med Sci,* 4**,** 429-34.

26. MAHMOUD, L., MOGHRABI, W., KHABAR, K. S. A. & HITTI, E. G. 2019. Bi-phased regulation of the post-transcriptional inflammatory response by Tristetraprolin levels. *RNA Biol,* 16**,** 309-319.

27. MAKITA, S., TAKATORI, H., IWATA, A., TANAKA, S., FURUTA, S., IKEDA, K., SUTO, A., SUZUKI, K., RAMOS, S. B. V. & NAKAJIMA, H. 2020. RNA-Binding Protein ZFP36L2 Downregulates Helios Expression and Suppresses the Function of Regulatory T Cells. *Front Immunol,* 11**,** 1291.

28. MARTINEZ-CALLE, N., PASCUAL, M., ORDONEZ, R., ENERIZ, E. S. J., KULIS, M., MIRANDA, E., GURUCEAGA, E., SEGURA, V., LARRAYOZ, M. J., BELLOSILLO, B., CALASANZ, M. J., BESSES, C., RIFON, J., MARTIN-SUBERO, J. I., AGIRRE, X. & PROSPER, F. 2019. Epigenomic profiling of myelofibrosis reveals widespread DNA methylation changes in enhancer elements and ZFP36L1 as a potential tumor suppressor gene that is epigenetically regulated. *Haematologica,* 104**,** 1572-1579.

29. MATSUMOTO, K. & EMA, M. 2014. Roles of VEGF-A signalling in development, regeneration, and tumours. *J Biochem,* 156**,** 1-10.

30. MINUTE, L., TEIJEIRA, A., SANCHEZ-PAULETE, A. R., OCHOA, M. C., ALVAREZ, M., OTANO, I., ETXEBERRRIA, I., BOLANOS, E., AZPILIKUETA, A., GARASA, S., CASARES, N., LUIS PEREZ GRACIA, J., RODRIGUEZ-RUIZ, M. E., BERRAONDO, P. & MELERO, I. 2020. Cellular cytotoxicity is a form of immunogenic cell death. *J Immunother Cancer,* 8.

31. NOGUCHI, A., ADACHI, S., YOKOTA, N., HATTA, T., NATSUME, T. & KAWAHARA, H. 2018. ZFP36L2 is a cell cycle-regulated CCCH protein necessary for DNA lesion-induced S-phase arrest. *Biol Open,* 7.

32. OLIVEIRA, C., FAORO, H., ALVES, L. R. & GOLDENBERG, S. 2017. RNA-binding proteins and their role in the regulation of gene expression in Trypanosoma cruzi and Saccharomyces cerevisiae. *Genet Mol Biol,* 40**,** 22-30.

33. PEGG, D. E. 2007. Principles of cryopreservation. *Methods Mol Biol,* 368**,** 39-57.

34. PLANEL, S., SALOMON, A., JALINOT, P., FEIGE, J. J. & CHERRADI, N. 2010. A novel concept in antiangiogenic and antitumoral therapy: multitarget destabilization of short-lived mRNAs by the zinc finger protein ZFP36L1. *Oncogene,* 29**,** 5989-6003.

35. PRECA, B. T., BAJDAK, K., MOCK, K., SUNDARARAJAN, V., PFANNSTIEL, J., MAURER, J., WELLNER, U., HOPT, U. T., BRUMMER, T., BRABLETZ, S., BRABLETZ, T. & STEMMLER, M. P. 2015. A self-enforcing CD44s/ZEB1 feedback loop maintains EMT and stemness properties in cancer cells. *Int J Cancer,* 137**,** 2566-77.

36. REN, J., WEN, L., GAO, X., JIN, C., XUE, Y. & YAO, X. 2009. DOG 1.0: illustrator of protein domain structures. *Cell Res,* 19**,** 271-3.

37. SABIO, G. & DAVIS, R. J. 2014. TNF and MAP kinase signalling pathways. *Semin Immunol,* 26**,** 237-45.

38. SANDUJA, S., BLANCO, F. F. & DIXON, D. A. 2011. The roles of TTP and BRF proteins in regulated mRNA decay. *Wiley Interdiscip Rev RNA,* 2**,** 42-57.

39. SCHMIDLIN, M., LU, M., LEUENBERGER, S. A., STOECKLIN, G., MALLAUN, M., GROSS, B., GHERZI, R., HESS, D., HEMMINGS, B. A. & MORONI, C. 2004. The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B. *EMBO J,* 23**,** 4760-9.

40. SEYEDIN, S. N., HASIBUZZAMAN, M. M., PHAM, V., PETRONEK, M. S., CALLAGHAN, C., KALEN, A. L., MAPUSKAR, K. A., MOTT, S. L., SPITZ, D. R., ALLEN, B. G. & CASTER, J. M. 2020. Combination Therapy with Radiation and PARP Inhibition Enhances Responsiveness to Anti-PD-1 Therapy in Colorectal Tumor Models. *Int J Radiat Oncol Biol Phys*.

41. SSADH, H. A., SPENCER, P. S., ALABDULMENAIM, W., ALGHAMDI, R., MADAR, I. H., MIRANDA-SAYAGO, J. M. & FERNANDEZ, N. 2017. Measurements of heterotypic associations between cluster of differentiation CD74 and CD44 in human breast cancer-derived cells. *Oncotarget,* 8**,** 92143-92156.

42. STOECKLIN, G., COLOMBI, M., RAINERI, I., LEUENBERGER, S., MALLAUN, M., SCHMIDLIN, M., GROSS, B., LU, M., KITAMURA, T. & MORONI, C. 2002. Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. *EMBO J,* 21**,** 4709-18.

43. STROBER, W. 2015. Trypan Blue Exclusion Test of Cell Viability. *Curr Protoc Immunol,* 111**,** A3 B 1-A3 B 3.

44. STUMPO, D. J., LAI, W. S. & BLACKSHEAR, P. J. 2010. Inflammation: cytokines and RNA-based regulation. *Wiley Interdiscip Rev RNA,* 1**,** 60-80.

45. SUK, F. M., CHANG, C. C., LIN, R. J., LIN, S. Y., LIU, S. C., JAU, C. F. & LIANG, Y. C. 2019. Author Correction: ZFP36L1 and ZFP36L2 inhibit cell proliferation in a cyclin D-dependent and p53-independent manner. *Sci Rep,* 9**,** 17457.

46. TANIGUCHI, H. & IMAI, K. 2018. PRDM14, a Zinc Finger Protein, Regulates Cancer Stemness. *Methods Mol Biol,* 1867**,** 3-13.

47. TRINER, D., CASTILLO, C., HAKIM, J. B., XUE, X., GREENSON, J. K., NUNEZ, G., CHEN, G. Y., COLACINO, J. A. & SHAH, Y. M. 2018. Myc-Associated Zinc Finger Protein Regulates the Proinflammatory Response in Colitis and Colon Cancer via STAT3 Signaling. *Mol Cell Biol,* 38.

48. TSENG, K. Y., CHEN, Y. H. & LIN, S. 2017. Zinc finger protein ZFP36L1 promotes osteoblastic differentiation but represses adipogenic differentiation of mouse multipotent cells. *Oncotarget,* 8**,** 20588-20601.

49. VIGNUDELLI, T., SELMI, T., MARTELLO, A., PARENTI, S., GRANDE, A., GEMELLI, C., ZANOCCO-MARANI, T. & FERRARI, S. 2010. ZFP36L1 negatively regulates erythroid differentiation of CD34+ hematopoietic stem cells by interfering with the Stat5b pathway. *Mol Biol Cell,* 21**,** 3340-51.

50. WENG, Q., WANG, J., WANG, J., HE, D., CHENG, Z., ZHANG, F., VERMA, R., XU, L., DONG, X., LIAO, Y., HE, X., POTTER, A., ZHANG, L., ZHAO, C., XIN, M., ZHOU, Q., ARONOW, B. J., BLACKSHEAR, P. J., RICH, J. N., HE, Q., ZHOU, W., SUVA, M. L., WACLAW, R. R., POTTER, S. S., YU, G. & LU, Q. R. 2019. Single-Cell Transcriptomics Uncovers Glial Progenitor Diversity and Cell Fate Determinants during Development and Gliomagenesis. *Cell Stem Cell,* 24**,** 707-723 e8.

51. WU, X., ZHANG, X., YU, L., ZHANG, C., YE, L., REN, D., LI, Y., SUN, X., YU, L., OUYANG, Y., CHEN, X., SONG, L., LIU, P. & LIN, X. 2020. Zinc finger protein 367 promotes metastasis by inhibiting the Hippo pathway in breast cancer. *Oncogene,* 39**,** 2568-2582.

52. YANG, S., LIU, T., CHENG, H., WANG, Z., FENG, Y., YAN, J., LIU, S. & ZHANG, Y. 2019. Decreased Expression of Retinoblastoma Protein-Interacting Zinc-Finger Gene 1 Is Correlated With Poor Survival and Aggressiveness of Cervical Cancer Patients. *Front Oncol,* 9**,** 1396.

53. YASUDA, M., NAKANO, K., YASUMOTO, K. & TANAKA, Y. 2002. CD44: functional relevance to inflammation and malignancy. *Histol Histopathol,* 17**,** 945-50.

54. YE, Q., LIU, J. & XIE, K. 2019. Zinc finger proteins and regulation of the hallmarks of cancer. *Histol Histopathol,* 34**,** 1097-1109.

55. ZEKAVATI, A., NASIR, A., ALCARAZ, A., ALDROVANDI, M., MARSH, P., NORTON, J. D. & MURPHY, J. J. 2014. Post-transcriptional regulation of BCL2 mRNA by the RNA-binding protein ZFP36L1 in malignant B cells. *PLoS One,* 9**,** e102625.

56. ZELOVA, H. & HOSEK, J. 2013. TNF-alpha signalling and inflammation: interactions between old acquaintances. *Inflamm Res,* 62**,** 641-51.
57. ZHANG, Y., ZHANG, Z., HUANG, W. & ZENG, J. 2020. MiR-4282 inhibits tumor progression through down-regulation of ZBTB2 by targeting LIN28B in oral squamous cell carcinoma. *J Cell Physiol*.