Study of the structure and functionality of the zinc finger protein ZFP36L1 and its role in gene regulation

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Abstract

Zinc finger proteins are one of the most structurally diverse protein domains and this includes the Zinc finger protein like 1 (ZFP36L1) also known as Butyrate response factor 1 (BRF1). It has been postulated that ZFP36L1 is involved in both proliferation and angiogenesis. ZFP36L1 has both a negative and positive regulation of cellular protein processes (such as transcription) depending on external and internal stimulations. ZFP36L1 mechanism functions by binding on the Adenylate-Uridylate rich elements (AREs) regions found within the 3' untranslated regions (3'UTRs) within the mRNA, attenuating expression for mRNA degradation. Degradation can remove essential cell regulators such as cytokines, transcriptional factors as well as protein-signalling cells. ZFP36L1 is still considered a novel protein in the field, its structure as well as its mRNA targets is yet to be fully realised. It is postulated that ZFP36L1 may function as a tumour suppressor gene, by interacting with cells during cell replication and arresting cells in the G_0 phase, keeping the cells in a state of Quiescence. A key question is to unravel the role of ZFP36L1 in the immune system. ZFP36L1 seems to interact and control the development of T and B lymphocytes. ZFP36L1 may act to ensure that B lymphocytes progress through the cell cycle, to mature B cells and possible plasma cells engaged in antibody synthesis. Mice deficient in ZFP36L1 and its paralogue, ZFP36L2 had B lymphocyte development blocked. ZFP36L1 and ZFP36L2 are seen to create a thymic β-selection checkpoint, which stopped damaged thymocytes undergoing thymopoeisis. Upregulation of ZFP36L1 and ZFP36L2 regulated the DNA damage response and halted cell cycle progression. Thymocytes were found to be reduced or arrested in the G_0 phase. Taken together, ZFP36L1 might be an important regulator step in various processes including the immune system and cancer cell proliferation.

Abbreviations

ZFP36L1	Zinc Finger Protein 36 C3H Like 1
ZFP36L2	Zinc Finger Protein 36 C3H Like 2
VEGF	Vascular Endothelial Growth Factor
mRNA	Messenger RNAs
AREs	Adenylate-Uridylate Rich Elements
UTR	3’ Untranslated Regions
CD	Clusters of Differentiation
VDJ	Variable Diversity Joining
pre-BCR	Precursor B Cell Receptor
RBP	RNA Binding Protein
MIF	Migration Inhibitory Factor
VPF	Vascular Permeability Factor
MPK-1	Mitogen Activated Protein Kinase (MAPK) Phosphatase 1
TTP	Tristetraprolin
IEG	Immediate Early Gene
AGN2a	Neuroblastoma Cell Line Neuro-2a(N2a) Designated
ZEB1	Zinc Finger E0-box Binding Homeobox 1
EMT	Epithelial to Mesenchymal Transition
ESRP1	Epithelial Splicing Regulatory Protein 1
IL	Interleukin
shRNA	Short Hairpin RNAs
PTX	Paclitaxel

SB203580	(4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole)
PKB	Protein Kinase B
SCs	Skeletal muscle satellite cells
GFPZFP36L1	Green Fluorescent Protein Zinc Finger Protein 36 C3H Like 1
EGFR	Epidermal Growth Factor Receptor
SOS	Sons Of Sevenless

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Chapter 1

Zinc Finger Proteins

1.0.0 Introduction

Within the human body there are multiple factors that appear to contribute to cancer processes. One less understood factor is mRNA regulation. ZFP36L1 as well as related family members TTP and ZFP36L2 are well known mRNA regulators. However, the knowledge around the topic of ZFP36L1 and its roles in cancer and angiogenesis is limited by the current information available. This implies that an accurate theoretical formulation of the protein and its effect on cells is confined to hypothesis for testing.

The purpose of this thesis is to provide an essential background knowledge needed to understand the possible role of ZFP36L1 in malignancies. The structure and the functionality of ZFP36L1 is considered.

1.1.1 Zinc

ZFP36L1 has a zinc group attached to its structure. Thus, it is pertinent to discuss the role of this element and its biology. Zinc is a chemical element with the atomic number of 30 and is denoted on the periodic table with the symbol Zn, found at the top of the group 12 elements (Transition metals) and is the 24^{th} most abundant element found on earth and in humans it is found that around 2-4 grams are within the human body at any given time (Rink, L. et al 2000).

Zinc is found in abundance, in different concentrations within the human body. The importance of zinc is shown by the fact that over 3000 proteins including enzymes and transcription factors are coupled to Zinc (Andreini, C et al., 2007). Zinc is also involved in cell signalling, cell replication as well as DNA repair (Yan, M., et al, 2014). High concentrations of Zinc are found in both the prostate (Song, Y and Ho, E., 2009) and the liver, with the latter being involved in the metabolism of Zinc.

It has been postulated that Zinc is necessary for a healthy immune system (Elmafda, I., Meyer, A.L., 2019). This importance can be seen as far back as 40 years ago with the discovery of thymulin, a zinc dependent nonapeptide that is used for the development of T-lymphocytes found in the thymic gland (Bach, J. et al, 1977).

Zinc has a direct effect on the immune cells. Zinc is easily distinguishable when compared to other trace metals such as iron or copper because Zinc itself is stable under redox reactions. The ZFP36L1 protein has a Zinc ion attached, which come in contact and bind to both the cysteine and histidine residues, in the inhibited state, zinc fingers will have both the cysteine and histidine residues in a state of oxidation, thereby releasing the Zn^{2+} , altering the protein shape and inactivating it. However, when the oxidative state changes, the Zinc will readily bind to the cysteine and histidine residues, allowing for the binding with the Zinc ion. This is the general way that most enzymatic activity that uses zinc is regulated by, by regulating the redox reaction, the functionality of the enzyme can go from activated to inhibited. In some cases, the Zn^{2+} can detach from the protein and can influence other proteins in such a way that it is called a redox transducer (Maret, W. et al 2006).

With the amount of important and vital roles zinc ion plays in biological function, specifically in the field of signal transduction as well as cell proliferation, it is unsurprising that that the trace element itself plays a rather vital role in maintaining immune regulation.

When examining T cells within the adaptive immune system, these cells are found to be sensitive to a deficiency in Zinc, especially when it comes down to maturation and the continuous maintenance of a balance between the different T cell subsets (Ibs., K.G. & Rink, L., 2003) with a decreased function being seen after zinc depletion. This is seen in monocytes, where all function is impaired (Ibs., K.G. & Rink, Lothar., 2003), natural killer cells have decreased cytotoxicity, (Ibs., K.G. & Rink, Lothar., 2003) and in neutrophil granulocytes, where phagocytosis is reduced (Ibs., K.G. & Rink, Lothar., 2003). With a

deficiency in zinc, it becomes evident that there is a decline of type 1 T helper (Th1). Th1 cells are a from a lineage of $CD4^+$ effector T cells that produce interferon gamma, interleukin-2 as well as tumour necrosis factor-beta. Th1 promotes cell mediated immune responses that are required to defend against both intracellular bacterial and virulent pathogens. Subsequently, the decline in Th1 leads to the promotion of inflammatory reactions and leads to an increase in the promotion of interleukin (IL) 1β secretion. Zinc is also found to be important to cells in the innate immune system, especially when it comes to macrophages and monocytes. It's important as it has a regulatory effect on the production of cytokines as well as the release of ROS during respiratory burst (Bonaventura, P. et al 2005). Chelation of Zn^{2+} appears to inhibit the function of neutrophil granulocytes for example, phagocytosis, secretion of cytokines, oxidative burst, phagocytosis as well as chemotaxis (Hasan, R. et al 2016).

With the negatives of chelation becoming apparent, it is only fair to realise that there are also positives to the lack of Zn within the human body. One such positive to chelation of Zn seemed to have increased phagocytosis and respiratory burst activity when combating pathogens, especially the likes of both *Streptococcus pneuomoniae* and *Streptococcus aureus* as well as *Escheria coli* when looked at in human monocytes (Haase, H. et al. 2014; Bonaventura, P. et al. 2015). The proinflammatory IL-6 and TNF-α was found to be lower in cells depleted of Zn. Speculation of the depleted Zn cells and their functionality could be contributed to the immune response has reprogrammed itself in such a way that it has adapted a strategy to instead of relying on cytokine secretion from monocytes has in turn strengthened its innate defence (Mayer, L.S. et al 2014).

Zinc is a critical nutrient, this is made especially critical in diets of poor nutrition based around foods such as legumes, unrefined cereals (cereals that haven't been fortified) as well as other foods rich in phylates. These foods are usually consumed in developing countries as

well as both vegetarian and vegan diets, in which case supplementation of Zinc via other means would become a necessity if consumption of the phylate rich foods makes up a large proportion of the diet (Gibson, R.S. et al. 2018).

1.1.2 Zinc Finger Introduction

Zinc finger proteins were first identified in the African claw toad (*Xenopus Laevis*) by Sir Aaron Klug (Klug., A. 2010) in 1985, a British Chemist and Biophysicist who in 1982 won a Nobel peace prize for chemistry for the development of crystallographic electron microscopy and his work on structural elucidation of the important nucleic acid protein complexes (Nobel Foundation, 1982).

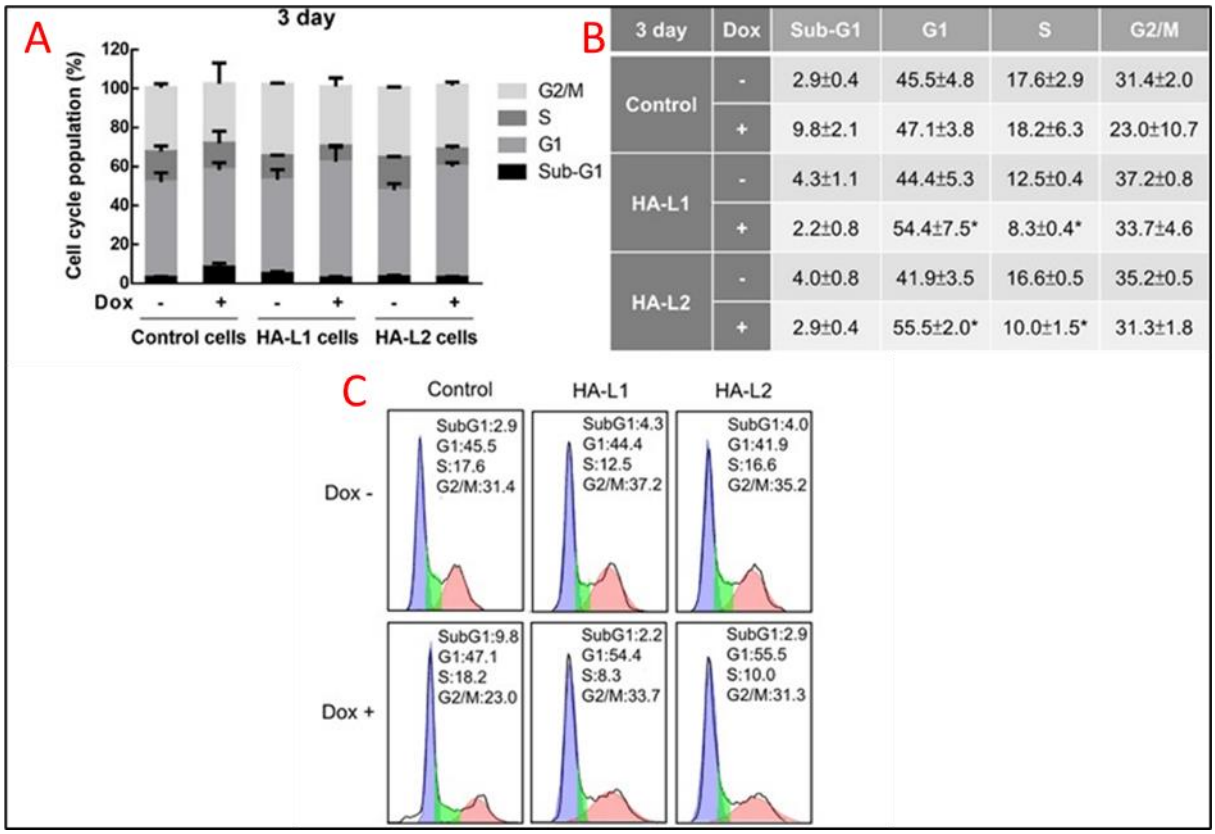
As mentioned above, Zinc finger proteins are one of the most abundant proteins found within eukaryotic genomes. Zinc finger proteins have a diverse range of functions such as: Packaging of RNA, recognising DNA, transcriptional activation (being one of the largest families of transcriptional factors within the human genome), apoptotic regulation, controlling the assembly and folding of proteins as well as lipid binding. ZFP36L1 has the function of playing the putative transcription factor as well as an mRNA binding protein.

1.1.3 ZFP36L1

In this study the focus has been to elucidate the role of ZFP36L1. The fully matured ZFP36L1 protein has two beta strands that have an alpha helix end that folds over to bind to a zinc ion, in ZFP36L1, it consists of two tandemly repeated zinc finger motifs, known as a repeating cys-his motif, that specifically recognise AU-rich elements, but also mediate mRNA decay (Loh X. et al, 2017). This structure is important as it plays a pivotal role in DNA binding proteins. ZFP36L1 is a gene that is a member of the TIS11 family which is made up of immediate early genes (IEGs). These genes are activated as a response to several

cellular stimuli such as phorbol ester TPA and EGF, a polypeptide mitogen, this response takes place before any protein expression takes place. Of the IEG family, there are currently around 40 known early response genes that have been characterised. Generally, IEGs are transcription factors of DNA binding proteins however ZFP36L1 is different as it is a putative transcription factor that is also an mRNA binding protein (Baou, et al, 2009). It has been hypothesised that ZFP36L1 plays a role in degrading down oncogenic mRNA transcripts, which in turn will lead to tumorigenesis. It was found that when ZFP36L1 was heavily mutated, it was found to be downregulated in several different cancers (Loh X. et al, 2017). In the paper written by Loh X. et al, it is suggested that ZFP36L1 might act as an unappreciated tumour suppressor. This Hypothesis gains factual evidence as in the studies conducted, patients with lower expression levels of ZPF36L1 had a reduced survival rate, whereas, forced expression of ZFP36L1 had a massive impact on the cell proliferation, whereby it was seemingly reduced, the same was said about the invasiveness as well as the migration of these cells with upregulated ZFP36L1 were also reduced. Loh X et al report that overexpression of ZFP36L1 led to a reduction of Cyclin D1 protein expression. They propose that ZFP36L1 plays a critical role as a tumour suppressor in breast cancer as well as bladder cancer, it does this by negatively regulating the mRNA. Loh X et al further expanded on this hypothesis where forced expression of ZFP36L1 reduced cell proliferation whilst silencing ZFP36L1 increased tumour growth. However, they in this case find the downstream targets of ZFP36L1 and find that it has a network of 1,410 genes as potential targets for when it comes to ZFP36L1. The findings reveal that ZFP36L1 has an indispensable role as a posttranscriptional safeguard against abnormal cell cycle progression (Lox X et al, 2020). It would also appear that ZFP36L1s antiproliferative abilities are not found just in breast cancer cells as well, a study conducted by Suk F.M. et al found that ZFP36L1 and ZFP36L2 (A close family member) were found to be under expressed in Trex-293 cells. When the ZFP36L1 and

ZFP36L2 were found overexpressed in Trex-293 cells, cell proliferation was found to be drastically limited as it was inhibited. The cell cycle also seemed to have been found arrested in the G1 phase of the cell cycle (the intermediary phase between the end of cell division and the beginning of S phase, S phase is where DNA replication occurs.) (Suk F.M. et al, 2018).



3 day	Dox	Sub-G1	G1	S	G2/M
Control	-	2.9±0.4	45.5±4.8	17.6±2.9	31.4±2.0
	+	9.8±2.1	47.1±3.8	18.2±6.3	23.0±10.7
HA-L1	-	4.3±1.1	44.4±5.3	12.5±0.4	37.2±0.8
	+	2.2±0.8	54.4±7.5*	8.3±0.4*	33.7±4.6
HA-L2	-	4.0±0.8	41.9±3.5	16.6±0.5	35.2±0.5
	+	2.9±0.4	55.5±2.0*	10.0±1.5*	31.3±1.8

Figure 1. **Over expression of both ZFP36L1 and ZFP36L2 arrested the cell cycle in the G1 phase.** Control cells used are T-Rex-293/pcDNA5-TO. HA-L1 cells used are TREx-293/HA-ZFP36L1 and HA-L2 are TREx-293/HA-ZFP36L2. **A.** cells arrested in G_1 phase express ZFP36L1 and ZFP36L2 and did not cause cell death. **B.** Cells were treated with (Doxycylcine) Dox to induce production of either ZFP36L1 or ZFP36L2 over 3 days in different cell states of the cell cycle. **C.** Cell cycles were processed using flow cytometry. Each Haemaglutinin (HA) is a glycoprotein that causes blood cells to clump together (Agglutinate). Original image from (Suk F.M. et al, 2018).

As can be seen in figure 2, the first image confirms that the cell cycles were arrested in the G_1 phase of the cell cycle and that the overexpression of ZFP36L1 and ZFP36L2 did not cause cell death, this can be seen in day 3 of dox treatment, it also has the effect of blocking cells from entering the S phase of the cell cycle. The induced expression of ZFP36L1/ZFP36L2 had an increase population from 44.4.% to 54.4% and 41.9% to 55.5% respectively, in the G_1 phase. (Suk F.M. et al. 2018) it can be seen that dox induced overexpression of both ZFP36L1 and ZFP36L2 lead to an overall decrease of the S phase population from 12.5% to 8.3% and 16.6% to 10% respectively. However, we can see that sub-G_1 phase population did not increase in both HA-L1 and HA-L2 cells when treated with Dox

It was shown that increasing the expression levels of ZFP36L1 and ZFP36L2 in these Trex-293 cells limited Cyclin B, D, A and P21, but in turn, increased the expression levels of p53 in overexpressed Trex-293 cells. The decreased cell proliferation from over expression is not just limited to these few cell lines, it also has the same effect in colorectal cancer cell lines. It was tested in 3 colorectal cell lines, where one had p53 knocked out, one had p53 expressed and the other cell line had a mutated version of p53. These cell lines all were found to have decreased 5cell proliferation; however, it was found that the colorectal cancer cell line that had normal p53 expression, had in fact increased the expression levels of p53 and p21. This experiment shows that both ZFP36L1 and ZFP36L2 can work in a p53 independent manner as both the knocked out p53 cell line as well as the mutated p53 cell line both showed anti cell proliferation. (Suk F.M. et al, 2018).

1.1.4 Zinc finger protein structure

Zinc Finger proteins are created by arranging two cysteines, found to be located on the two antiparallel beta sheets and two histidine's, found to be located on the alpha helix of the zinc finger which are then aligned close together in a chain. This protein can then take on one or more zinc ions into its structure which then leads to tight folding and stability of the protein, thus allowing for conformation into a finger like structure. Zinc finger proteins are classified based upon their 3D structure, leading to their being different and diverse families of Zinc finger proteins, all with specific mechanisms.

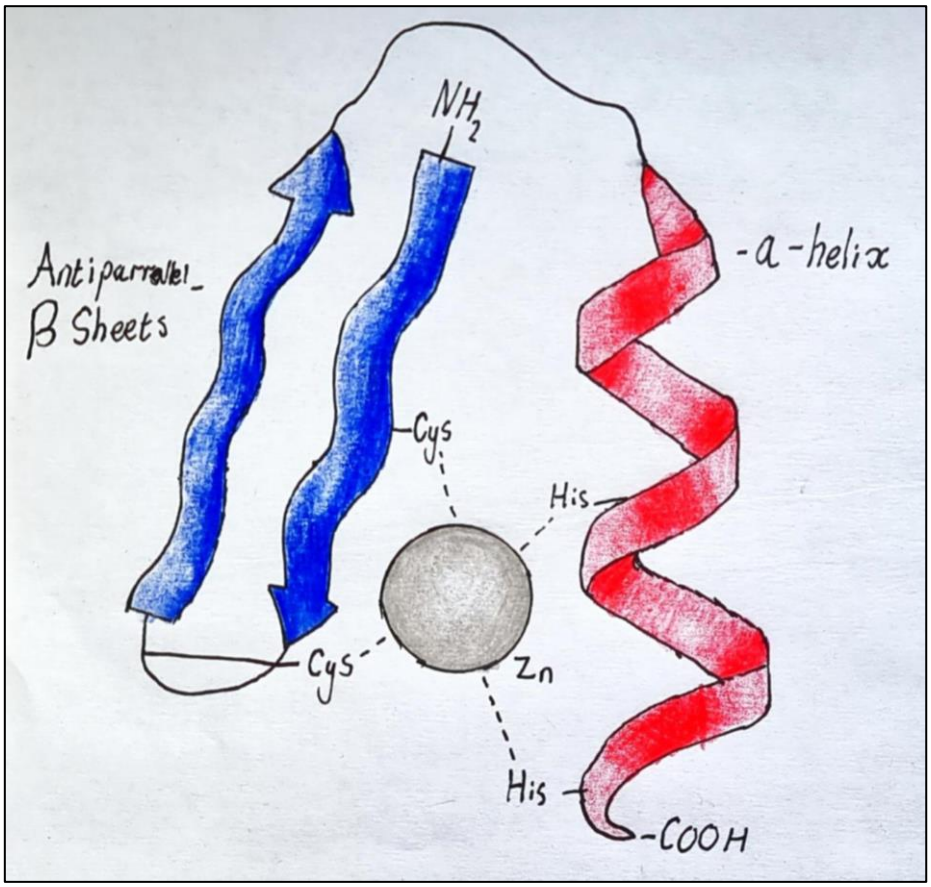


Figure 2. **Cartoon drawing of a Zinc Finger Proteins C_2H_2 motif**

original image adapted from www.slideshare.net/ZeeshanAwan10/zinc-finger-nuclease. In this image the Zinc ion has been attached via the Cysteine and Histidine motives, with the Cysteine motives being located on the antiparallel Beta sheets and the Histidine residues located on the alpha helix. This binding to the zinc in turn allows for the co-ordination of the ion which allows for the Zinc Finger protein to conform to its functional shape. The non-functional shape would be lacking a zinc ion.

In humans, ZFP36L1 has 338 amino acids and has the expected molecular weight of 36.3 kDa (Stumpo et al., 2010). ZFP36L1 was found to be located on the 14th chromosome (14q24.1) within humans and was found to be around 6000 base pairs in length. The ZFP36L1 gene has two type II restriction endonucleases. These endonucleases are both HINDIII and BamHIII.

These are important restriction endonucleases as they are responsible for allowing ZFP36L1 to recognise AU rich regions and induce cleavage of these regions. What is interesting to note is that there is no recorded Pseudogene that has been found so far. The tandem zinc finger domain is conserved and will directly bind to the class II rich elements found located within the 3' untranslated regions.

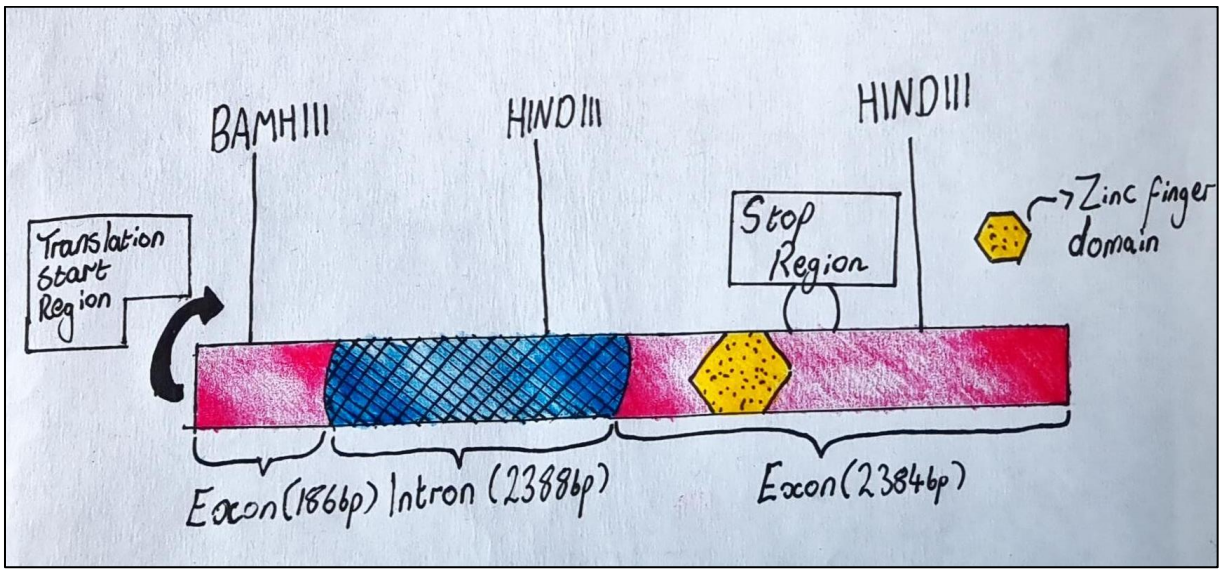


Figure 3. **The human ZFP36L1 gene.**

(Original image from Stumpo et al, 2007) ZFP36L1 consists of two exon regions on either side of the intron. Both exons are 186bp and 2384bp respectively for a combined 5411 total base pairs. The exon of 186bp is found at the 5' end whilst the exon of 2384bp is found at the 3' end. The two exon regions are split in-between by an intron of 2388bp in length. Within the larger exon, is the location of the zinc finger domain. The START and STOP regions are a representation of where translation starts and stops. Both BAMIII and HINDIII type II restriction endonucleases.

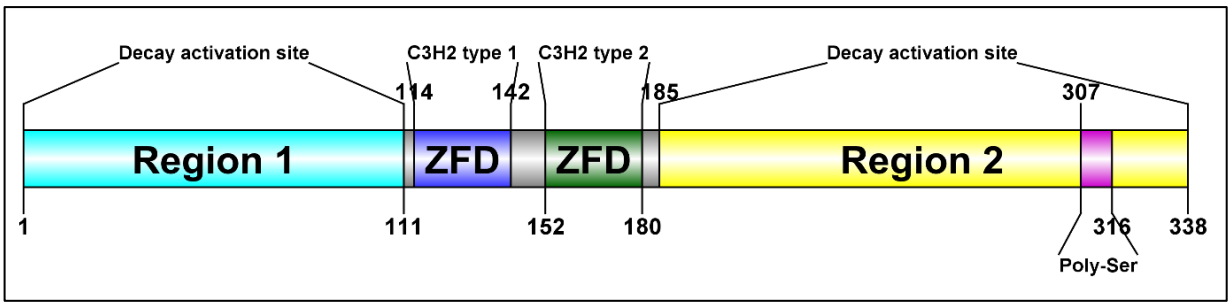


Figure 4. **The five domains of the human ZFP36L1.**

Original image from (Jian Ren et al,2009) created using GPS software. Both region one and region are two are sites for mRNA decay to be propogated. ZFD is indicative of the Zinc Finger Domains. These domains are the location of the repeated Cys_3-His_2 finger motives occur as a tandem repeat of two, this is where coordination of the zinc ion occurs. The fifth domain is the poly-serine 92 domain, where phosphorylation of ZFP36L1 occurs. Phosphorylation of ZFP36L1 leads to mRNA stability.

With ZFP36L1, it is important to note that the structure of this zinc finger protein consists of five domains, which all have a distinct role when it comes to the activity of RNA binding. Two of these domains consist of mRNA decay activation sites which will act upon being bound to an RNA-binding protein which will cause a knockdown affect where propagation of mRNA will occur. These Proteins include exonucleases and de-capping enzymes. (Fig 5.) yet the overexpression of ZFP36L1 will lead to the inhibition of AU rich elements and the decay related to them. ZFP36L1, characterised with having a C_3H_2 (3 Cysteine, 2 Histidine co-ordinating the Zn ion) residues are the only type of zinc finger which have been found to interact and edit the 3' untranslated region of mRNA where the AU rich elements can be found. ZFP36L1 contains 2 C_3H_2 zinc finger domains that are found to be around 20-50 amino acids in length according to PROSITE – ProRule annotations. Within the zinc finger protein there is a significant domain that is fairly small, which is found to be made up of a repeated homologous Serine residues which are found to be 9 amino acids in length, these Serine residues are residues 92 and 203. With ZFP36L1 and its study still in a novel phase, there is a significant lack of knowledge on the proteins structure. Crystallisation of the protein would be essential for accurate understanding of the proteins full structure.

In its inactive form, ZFP36L1 is in a phosphorylated state, regulated via 14-3-3 proteins. Phosphorylation occurs by inducing both serine 92 and 203 within the poly serine region by either Protein kinase B or MAPK phosphatase. In its de-phosphorylated state, ZFP36L1 induces the recruitment of proteins such as de-capping enzymes, exosomes, and exonucleases. This removes/leads to deadenylation of the poly(A)tail which allows for the function of reducing protein synthesis (Stoecklin et al., 2011). The last and final step is the destabilization of mRNA, which is done via hydrolysis of the mRNA, thus degrading the mRNA.

1.1.5 ZFP36L1 Mechanism

ZFP36L1 is a zinc finger protein that regulates a myriad of different cellular processes, such as cell apoptosis. It signals for cell apoptosis by binding to the adenine uridine rich elements which are found in the 3' untranslated regions which targets mRNA so that they can promote degradation (Schmidlin et al., 2004). The importance of ZFP36L1 is quickly made apparent when realised that the protein itself acts as a regulator for a multitude of pathways such as TNF- α, VEGF and MAPK.

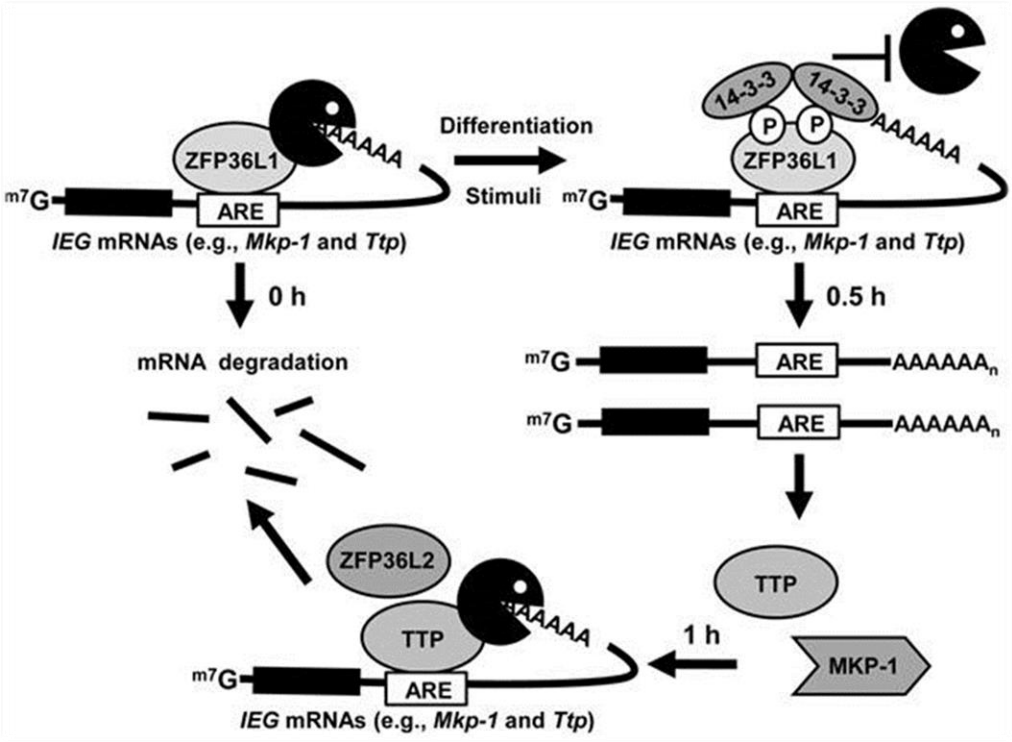


Figure 5. **schematic of ZFP36L1 and ZFP36L2 regulation**.

ZFP36L1/L2 post-transcriptionally and sequentially regulate the expression of AU rich element containing IEGs during differentiation of pre-adipocytes. Pre-adipocytes bind to ZFP36L1 to limit IEG mRNA basal levels. After differentiation, ZFP36L1 is phosphorylated via association with 14-3-3 proteins leading to inhibition of mRNA destabilisation, thus leading to stable IEG mRNAs. Newly produced TTP rapidly degrades induced IEG mRNAs. ZFP36L2 may function similarly, under different conditions. Image from (Lin, N.Y. et al, 2012).

Figure 6 shows how ZFP36L1 interacts with ARE regions. In the top left diagram in figure 6, ZFP36L1 attached to the 3' untranslated region of mRNA, in turn, it creates a complex with the mRNA strand and thus begins navigating towards the AU rich elements, these AU rich elements are found within mRNA which are found to be encoding for genes associated with inflammation as well as cancer associated genes (Lin, N.Y. et al, 2012).

1.1.6 ZFP36L1 expression

The expression of ZFP36L1 has been documented to be expressed in all cell types (Protein Atlas), indicating the importance of the protein, yet the protein itself is considered to have low specificity in the myriad of cells that it can be found in.

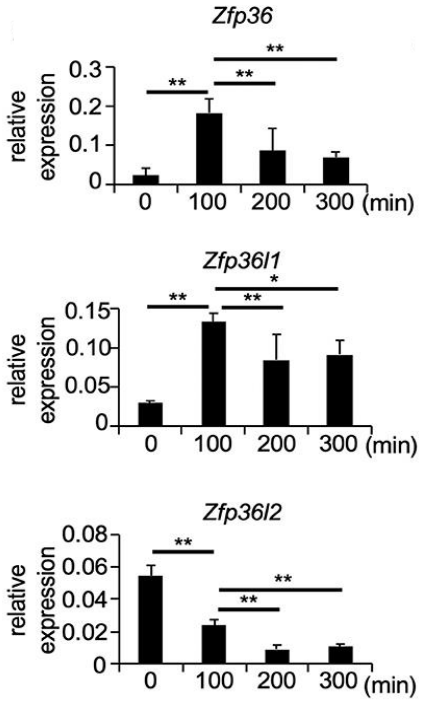


Figure 6. **TCR Mediated stimulation upregulates ZFP36/ZFP36L1 expression and downregulated ZFP36L2 Expression.**Naïve $CD4^+$ T cells were isolated from C57BL/6 Wild type (Spleen) and were stimulated with anti-CD3/anti CD8 mAb under neutral conditions at 100-minute intervals. RNA was prepared from these cells to analyse the mRNA levels of ZFP36/ZFP36L1 and ZFP36L2 and analysed using quantitative PCR. Image from (Makita, S et al, 2000)

it appears that ZFP36L1 is induced in different Cell types, this can be seen in $CD4^+$ cells where the use of TCR-mediated stimulation induced ZFP36 and ZFP36L1 to a 3-fold increase after 100 minutes and gradually stabilising after the 200th and 300th minute, this is in direct contrast with ZFP36L2, where TCR induction significantly decreased the expression levels of ZFP36L2 instead.

This data would give to the idea that ZFP36L1 appears to have an induced expression along with ZFP36, however ZFP36L2 appears to be more so constitutively expressed.

1.2.0 The role of Biomarkers

Many bodily functions have biomarkers that indicate the success of the process that is going on. Take for example in pregnancy, histocompatibility antigen, class I, G, (HLA-G) is a biomarker that has been used to indicate fertilisation and pregnancy success according to (Jabeen A. et al, 2013). However, biomarkers are not only found solely as an indication of fertility success. In fact, many different diseases also have biomarkers. Cancer has biomarkers. According to the National Cancer Institute, a biomarker is a biological molecule that can be found in blood, tissues, or other bodily fluids. They appear when there is a sign of normal or abnormal processes. These can either be a substance or a process that is indicative of the cancer it came from. These biomarkers could also be secreted by a tumour or in a response that is specific to the body when in the presence of the cancer. Biomarkers have a huge potential when it comes to the applications it has for oncology, such things like Risk assessments, different diagnosis, screenings, and others to name a few. Biomarkers are involved in all stages of the disease (Henry N.L. 2012). Biomarkers however are not just limited to proteins, but it seems that there is an extremely large variety of biomarkers ranging from antibodies to peptides. Biomarkers in tumours are special because specific biomarkers are only secreted by specific tumours. This brings the question though, what if a tumour

produces the same biomarker as another, completely different tumour? In which case we would need to associate more than just one biomarker to each type of tumour to increase the accuracy of the medical diagnostics because a singular biomarker will not ever be 100 per cent accurate. Proper prognosis of cancer will lead to effective and accurate treatment.

1.2.1 Possible association of ZFP36L1 with Clusters of Differentiation receptors

It is possible that the ZFP has connection with some CD receptors know to control important functions. These include the CD74 and CD44 receptors (N. Fernández, personal communications). Thus, it is of importance to discuss these receptors.

1.2.2 CD74

CD74 is a nonpolymorphic type II integral membrane glycoprotein that is expressed in antigen presenting cells (APCs) (Becker H.S. et al, 2005). It has been found that improper interactions can occur in the endoplasmic reticulum (ER) when CD74 forms a homotrimeric structure with MHC class II. This bonding prevents the MHC class II molecule from being able to bond to endogenous peptides (Liu Z. et al, 2016). It has been seen that CD74 has a low expression level in normal epithelial cells, but when looking at the expression levels of CD74 in tumour cells, it is highly expressed. In clear cell-renal cell carcinoma, the grade of the tumour was related to that of the expression level of the CD74. Down regulation of CD74 appears to have induced cell cycle arrest and apoptosis, whilst also supressing cell proliferation as well as invasion (Ji S.Q. et al, 2014). Triple negative breast cancer is found to be difficult to treat because there is a lack of rationale-based therapies. The Biomarkers for triple negative breast cancer are not established, which means treating it cannot happen as there are no targets for the therapy. However, it should be noted that triple negative breast cancer can be segregated into two phenotypes. One such phenotype has high expression

levels of Stat1, Mx1 as well as CD74. It was found that overexpression of CD74 in triple negative breast cancer, increased cell adhesion. This was accompanied with a marked increase in the expression of a variety of proteins involved in cell adhesion and cancer metastasis (Greenwood C. et al, 2012). CD74 tends to work simultaneously with CD44. CD74 and CD44 has been identified and witnessed to work together in multiple types of cancer and would appear to play a role in the development of tumours (Stein R. et al, 2007).

1.2.3 CD44

CD44 is a type I transmembrane glycoprotein that is expressed in most cell types, being a receptor for hyaluron and osteopontin (Jaggupilli A. et al, 2012). CD44 appears to be involved in cell adhesion and migration, as well as extra cellular-matrix interactions. CD44 is a topic of interest as it may have a potential role in breast cancer. CD44 appears to be able to stop signalling of oncogenes in a response to an extracellular response (Louderbough J.V. and Schroeder J.A., 2011). CD44 expression appears to be upregulated in premalignant lesions and has been associated with a variety of cancers. These cancers include melanoma, prostate cancer, and breast cancer to name a few. CD44 gene undergoes extensive amounts of DNA splicing across a variety of different exons which have been positioned in a cassette that's placed in the middle of the gene (Screaton G.R. et al, 1992). CD44 is found amongst most cell types, and the standard CD44 state being comprised of exons 1-5 and 16-20. There is a variant to CD44 known as CD44v isoforms, which have variances in their splicing, which in turn leads to exons of varying differences. one theory suggests that the alternative CD44 exons might in fact be associated with the progression of tumours. CD44 is not so cut and dry as being a negative in tumour progression however, because in human breast cancer, it is found to have both a favourable and unfavourable effect when it comes to clinical outcomes (Gotte M., Yip G.W., 2006).

1.2.4 CD74 and CD44 Co-Expression

Previous studies by Hussain A.S. et al, 2017 suggest the roles of CD74 and CD44 and their likely functions in breast cancer cells (Hussain A.S. et al, 2017). The current data has suggested that the co-expression of both CD74 and CD44 has played a role in the pathogenesis of solid tumours. However, it leads to a gap in knowledge as there has not been any direct quantification of the association of CD44 and CD74. (migration inhibitory factor) MIF induces ERK1 and ERK2 kinase phosphorylation but requires CD74 along with its co-expressor the full length of CD44 to do so (Shi X.R., et al, 2006). MIF also appears to interact with p53 tumour suppressor gene, it does this by inhibiting the p53-responsive gene activating and its apoptotic state (Fingerle R.G. et al, 2003). MIF facilitates signalling by binding to the extracellular domain of macrophage and B cell CD74 (Verjans E. et al, 2009). However, it should be noted that it requires CD44 for the MIF signal transduction to occur, this is because CD74 has a lack of direct signalling on its cell surface domain. When CD74 and CD44 become a complex, the CD74 undergoes a structural change, as it's modified by the inclusion of chondroitin sulphate. This is essential for the MIF-induced signalling cascade (Gil Y.N. et al, 2014). It has been hypothesised that the complex made up of the CD74, CD44 and MIF (CD74-MIF-CD44 complex) is actually required to start the pro survival signal that leads to increased cell proliferation and reduced apoptosis (Gore Y. et al, 2008).

CD74 has been considered a potential target for therapeutics against cancer as its seen that it involved with MIF-mediated signalling, as well as CD44. This leads to the proposition that it is relevant to quantify the association of CD74 and CD44 in cancer cells (Hussain A.S. 2017). a paper by (Meyer S.K.L. et al. 2004). has shown that CD44 has been seen to bind to p35 in bladder cancer cells, this suggests that it is an isoform that is involved in antigen presentation.

(Meyer S.K.L. et al. 2004) also found that in prostate cancer, the CD44 expression is lost, and instead it over-expresses the variant forms of CD44, this happens due to the alternating splicing of exons 7-10. Therefore, the new CD74-MIF-CD44 complex would instead be CD74-MIF-CD44v complex and shows that there is a potential involvement of this new complex in prostate cancer. It should be noted that not all cancers do not express CD74 on their cell surface, for example, one such cancer is the LNCaP prostate cancer cells, as well as Benign hyperplasia epithelial cells (BPH-1). From the fact that these two lines of cells do not have CD74 receptors on their cell surfaces, we can conclude that both these cell lines do not actually interact with CD44. LNCaP does interact with MIF, whilst BPH-1 does not (Meyer S.K.L. et al. 2004).

It appears that the expression levels of CD44 appears to be significantly higher compared to that of CD74. This could mean that higher levels of CD74 expression could render tumours less immunologic. It does this by stopping and barring the MHC class II peptide-binding cleft. This prevents the antigenic peptides from presenting themselves to CD4+ lymphocytes (Zheng Y.X. et al, 2012). Many studies have shown that there is a direct correlation between the expression of CD74 and it being proportionate to the grade of a tumour. It is seen that cell surface CD74 is only seen as a prerequisite for when it comes to internalising HLA-DR complexes. These complexes are targeted by endosomes. Yet, with alteration to CD74 and how its expressed on the surface of the cell, it doesn't have to show a correlation when looking at the level of the internalisation activity. It is also seen that CD44 expression can be associated to a high rate of cell proliferation and cell division (Sneath R.J.S. 1998).

The main agreement within this subject is that ZFP36L1 and its related family members play a pivotal role in the defence against cancerous cells. biomarkers are needed, especially within the triple negative breast cancer cells, where there aren't enough clear biomarkers. CD74 and CD44 also have a huge impact in the control of cell proliferation of cancer cell lines, but

whilst they are both effective together, one slight change to the CD44 to make it a CD44v can jeopardise the CD74-MIF-CD44 complex and make it ineffective at dealing with the problems.

1.2.5 Interactions with Zinc finger proteins with CD74/CD44

Tumours become prone to metastasis as well as increase in mortality rates the more solid the tumour becomes; an example of a solid tumour would be Neuroblastomas. These types of cancers are known to be associated with pro-inflammatory as well as high inflammation mediators. This is proven by the fact that cancers such as neuroblastomas produce relatively high amounts of a cytokine known as MIF. MIF as well as other cytokines are essential when it comes to innate immunity. With homeostatic regulation, MIF can coordinate and initiate both cellular and humoral responses, which can lead to the destruction of cancer. (Calandra T and Roger T., 2003). MIF was discovered in the late 1960s from a product found from activated T cells. MIF has been recently seen to be able to carry out functions such as mediation for the innate immune system. MIF is released when it is exposed to a response to stress, this MIF then induces pro-inflammatory responses which will become like regulators for immune response within animal models. (Calandra T. and Roger T., 2003). However, a paper by (Qiang, Z. et al. 2013) have found that when neuroblastomas produce high amounts of MIF, the MIF in turn will assist to promote tumour genesis, although this has only been recorded in pre-clinical studies. The reasoning behind MIF and how it plays an important role in tumour progression is because the increased levels of MIF actively suppress and inhibits T cell proliferation in vitro, further proving that MIF promotes tumorigenesis., due to in part, by supressing anti-tumour immunity. A paper by (Penticuff, et al, 2019) further proves the idea of tumour genesis via MIF expression, via actively inhibiting MIF in cancer cell lines, which in turn lead to a reduced rate of cancer, with evidence suggesting that inhibition of MIF

reduces cell invasion, cell proliferation, angiogenesis, the aggressive nature of tumours as well as the down regulation of system pathways. Whilst information is available for MIF, the most part comes from these pre-clinical studies, with such a novel cytokine being such an important part of tumour progression, more studies and investigations would be needed to further understand the potential of MIF fully. However, the understanding of MIF in genitourinary cancers appears to be vastly slower. (Penticuff et al, 2019)

There are relatively few studies that have observed the correlation between ZFP6L1 and CD44 in cancer cells. in a study created by (Perca, B. et al, 2015), it has been suggested that a family member of ZFP36L1, known as zinc finger E-box binding homeobox (ZEB1) has been confirmed to have a link to CD44 reciprocally, found in a novel positive feedback loop, in which CD44 isoforms expressions will in turn further activate more ZEB1. This fact links to the idea that although there is initial dependency on external growth factors that are found to be provided by the microenvironment, such as the use of the cytokine TGF-β, the cancerous cells will inevitably become autonomous – a necessary requirement for tumour progression. When looking at cells that have survived the administration of Gemzar that had undergone Epithelial to mesenchymal transition (EMT), the expressions of both CD44 as well as ZFP36L1 had both drastically increased, whilst the Epithelial splicing regulatory protein 1 (ESRP1) being found with having reduced expression. ESRP1 is theorised to be a regulator of a multitude of cancer. ESRP1 does this by acting as a tumour suppressor (Ishii et al, 2014). This loss of ESRP1 levels indicates an increase in tumour progression and also its survival. Ishii et al, 2014 continue on about ESRP1 and indicate that the reduced levels of ESRP1 had a direct correlation to an overall increase in tumour relapse and that the CD44-ZEB1-ESRP1 feedback loops that control the cellular phenotypes as well as the prognosis of cancer patients can be determined via the CD44 isoforms configurations (Perca B et al, 2015). This analysis concludes that there should be an emphasis on targeting pathways that

will increase the overall expression of ZEB1 that will in turn increase the amount of CD44 isoforms which would be an overall goal as it would increase the survivability of cancer patients. Increasing the overall expression levels of ESRP1 will in turn, increase the expression of normal CD44, which would then lead to an overall decrease of CD44 isoforms in turn affecting the levels of ZEB1 and returning the body to the point that further tumour relapses wouldn't occur (Perca B et al, 2015)

There, as of yet is very little if at all any significant data to show a correlation between CD78 and Zinc finger proteins in cancer, yet there is a link between CD74 and CD44 in breast cancer, as well as other types of cancers. Both MIF and CD74 contribute to cancerogensis, but the contribution affects the disease in various ways purely based upon the stage the disease is currently in, this could be due in part to the pathways the complex activates. The CD44-MIF-CD74 complex activates, ERK1, as mentioned earlier, as well as both AKT and PI3K. all three of these pathways attribute themselves to the upregulation of cell migration, a decrease in apoptosis as well as an increase in cell proliferation (Leng et al, 2003).

A paper by (miller et al, 2008) indicate a correlation between MIF and CD44 inducing the activation of the AMPK related pathway – which leads to a decrease in cell proliferation and cell viability in certain cancers (Park et al, 2012) whilst the inactivation of AMPK is linked to hepatocellular carcinomas according to Miyoshi et al, 2009. A study produced by (Lui Z. et al, 2016) concluded that CD74 was found to be highly expressed in breast cancer tissues, with clinical analysis indicating that there is indeed a correlation between the metastasis of lymph nodes and CD74 expression. The paper also further proves that there is a connection between CD74 and CD44 expression in breast cancers stem cells. Both CD74 and CD44 are found to be highly expressed in breast cancer which was proven in a paper by (Shi x et al 2003) in COS-7/M6 cell lines which were transduced to have MIF induced signalling. With this information coming into play, it can be theorized that CD74 may play a crucial role in the

maintenance of cancer stem cells, done by regulating the expression of CD44, thus it can be assumed that both CDs play pivotal roles in zinc finger protein as CD44 has a direct correlation to ZEB1.

1.2.6 ZFP36L1 as a Biomarker

ZFP36L1 is found to be apparent across a whole host of different types of cells, such as tissue, brain, single cell, the immune system as well as in both subcellular and cell lines (Protein Atlas), thus showing the importance of this protein as a post-transcriptional safeguard all across the body, as ZFP36L1 works by keeping the cell in a state of quiescence when being induced (Conne B et al, 2005), yet. there are very few clear indicators as to what binding proteins ZFP36L1 binds to. This leads to the idea that ZFP36L1 could be selected as a biomarker for early signs of cancer, due to the role ZFP36L1 fills as an IEG. As an IEG, ZFP36L1 expression would be induced in early signs of cancer, as ZFP36L1 arrests the cells in the G_0, phase, which would only occur in damaged cells (Vogel, K.U., et al. 2016). The issues arise with ZFP36L1 being a novel protein within the field, as there would be a need for further research to understand the binding proteins that physically interact with ZFP36L1.

Chapter 2

Aspects of Cancer

General aspects of cancer processes

2.1.1 Ageing of cells

Age is one of the most studied risk factors of cancer. Cancer is considered an age-related disease as the incidence of many cancers increases with age (US cancer statistics 1999-2009). With many cancers, the disease is caused by an accumulation of damage to DNA. All somatic cells carry chromosomes; these chromosomes are capped on their chromosome ends. These ends are called telomeres and consist of a repeat of 6 base pairs (TTAGGG) (Moyzis et al, 1988). The reason telomeric ends are important is because during chromosome replication, the DNA polymerase does not duplicate the full chromosome, and in doing so, shortens the chromosome on each successful replication (Chow, T.T. et al, 2012). Telomeres hence act like a buffer and are removed gradually during each replication. Telomere ends are non-coding and as such are deemed permissible to be lost. Telomeric ends are protected by an enzyme called telomerase that slow down the degradation of the ends. (Jafri, M.A., et al. 2016). In older cells (cells that have duplicated often and have likely obtained errors during replication process), the production of telomerase is significantly less and as such the telomeres are inevitably lost or are shortened considerably. In aged cells, the telomere ends are around 4kbps whilst in younger cells it is around 11kbps (Okuda, K. et al, 2002). The shortening of these telomeres thus signals for cell apoptosis. This is a natural process and reduces cell proliferation. However, in the case of cancerous cells, these cells will produce an abundance of telomerase, which will consequently protect the telomere ends, and in doing, allow for the cancer cells to constantly proliferate (Jafri, M.A., et al. 2016). This leads to the eventual formation of immortal tumours. One of the reasons that cancer occurs is because of the accumulation of damage to genes (Aunan, et al. 2017). The damage caused to these genes

is not repaired and in turn leads to uncontrollable cell growth, allowing for the formation of a tumour if not dealt with correctly (Bernstein et al, 2013).

When cancerous cells accumulate into a single tissue, they become a tumour, and like any other tissue in the body, tumours require oxygen and nutrients to be able to survive (Yadav, L., et al. 2015). Tumours are particularly dangerous as they have the ability to divert a normal blood supply that's carrying oxygenated blood to healthy tissues in the body to instead, take it towards itself. They do this by sending out angiogenic factors. The affect that this has on the body is that it starves normal, healthy tissues. The lack of blood flow causes the tissues to die off or work incorrectly. Tumours that produce these angiogenic factors can then instruct the body to create a direct blood supply towards itself and allows it to feed (Nagy J.A. et al, 2009). It has been found that when a solid tumour cannot induce its own blood supply via angiogenic factors, the tumour itself cannot grow any larger than 2-3mm in diameter (Folkman J., 1995).

Tumours tend to have an invasive feature in which they can become malignant, migrating from one infected area, and spread into the surrounding tissue, as well as distant tissues, known as tumour metastasis. Tumour metastasis requires blood vessels that can carry the tumour to distant sites. These satellite tumours can in turn, implant and begin growing as secondary tumours (Park S.A. et al, 2018). The effect this can have is catastrophic, completely debilitating a person's defences and spreading tumour cells to different parts of the body. The process of angiogenesis and tumour metastasis is not well understood but is thought to be controlled by chemical signals in the body. One of these chemical signals is called Vascular Endothelial Growth Factor (VEGF).

Intracellular Processes leading to cancer

2.2.1 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), often referred to as Vascular permeability factor (VPF). VEGF is a highly conserved, disulphide-bonded dimeric glycoprotein found to be between 34-45 kDa. It is produced by different cell types such as neutrophils, endothelial cells as well as peripheral mononuclear cells, such as T lymphocytes and macrophages (Neshad, M.A. 2014) VEGF is found to bind to the VEGF receptors located on the surface of cells. this binding leads to the activation of intracellular tyrosine kinase, leading to the initiation of a series of signalling cascades that are found to be involved in both vasculogensis and angiogenesis. VEGF is a potent angiogenic factor by both indirect and direct mechanisms. VEGF was initially described as a cell specific mitogen, which is a small protein that allows for cell division to occur. In an experiment conducted by (Duffy A.M. et al, 2013) showed that VEGF appears to be upregulated in a myriad of different tumours and has been noted to be a well-known contributor towards tumour angiogenesis. It's been seen that anti-VEGF therapies that stimulate tumour cells by VEGF appear anti-apoptotic and increases the resistances against both chemotherapy and radiotherapy. These two therapies appear to decrease the amount of VEGF that the tumour cells are expressing.

It is seen in malignant animal and human tumours, as well as a multitude of transformed cell lines. VEGF is overexpressed in many carcinomas arising in the stomach, kidney, breast, bladder, and colon. However, it does appear that some carcinoma expression of VEGF is not actually overexpressed at all, but instead was seen to have produced little to no signal of VEGF which was seen using *in situ* hybridization. This lack of expression of VEGF was seen in the following carcinomas: lobular carcinomas of the breast and papillary kidney tumours which were shown to be overexpressed at not just the protein level, but also the mRNA level.

It's suggested that VEGF is a powerful angiogenic factor, however there are other angiogenic factors other than VEGF being utilised in other cancers (Lee, A.H., et al. 1998).

These VEGF receptors also appear to be expressed in non-endothelial cells such as tumour cells (Duffy A.M. et al, 2013). VEGF is produced by many cell types such as platelets, keratinocytes, and macrophages, (Duffy A.M et al, 2013). VEGF plays a crucial role in the maintenance of healthy cell growth, from bone formation to wound healing and formation. VEGF plays a pivotal role in certain cancers and their survival.

(Dvorak. H.F., et al, 1995) has considered VEGF's effect on endothelial cells. They have shown that it leads to induction of transient accumulation of cytoplasmic calcium as well as leading to cell division and migration. The review article also states that VEGF alters the pattern of the endothelial cell gene expression and leads to induction of angiogenesis *in vivo.*

The possibility exists that ZFP36L1 plays a role in angiogenesis. In an experiment on mouse embryos homozygous for a targeted mutation with ZFP36L1 locus it was observed that mice died mid-gestation; upon close examination, it was found that the mice exhibited vascular abnormalities including a defective heart. This was due to the overproduction of VEGF due to the embryonic fibroblast and possibly the influence of ZFP36L1, indicating that ZFP36L1 is a negative regulator of VEGF during development, and this is further proven by their being AU rich regions found within the 3' untranslated regions within VEGF, which have been reported to interact with ZFP36L1 when in vitro (Bell, S.E., et al. 2006)

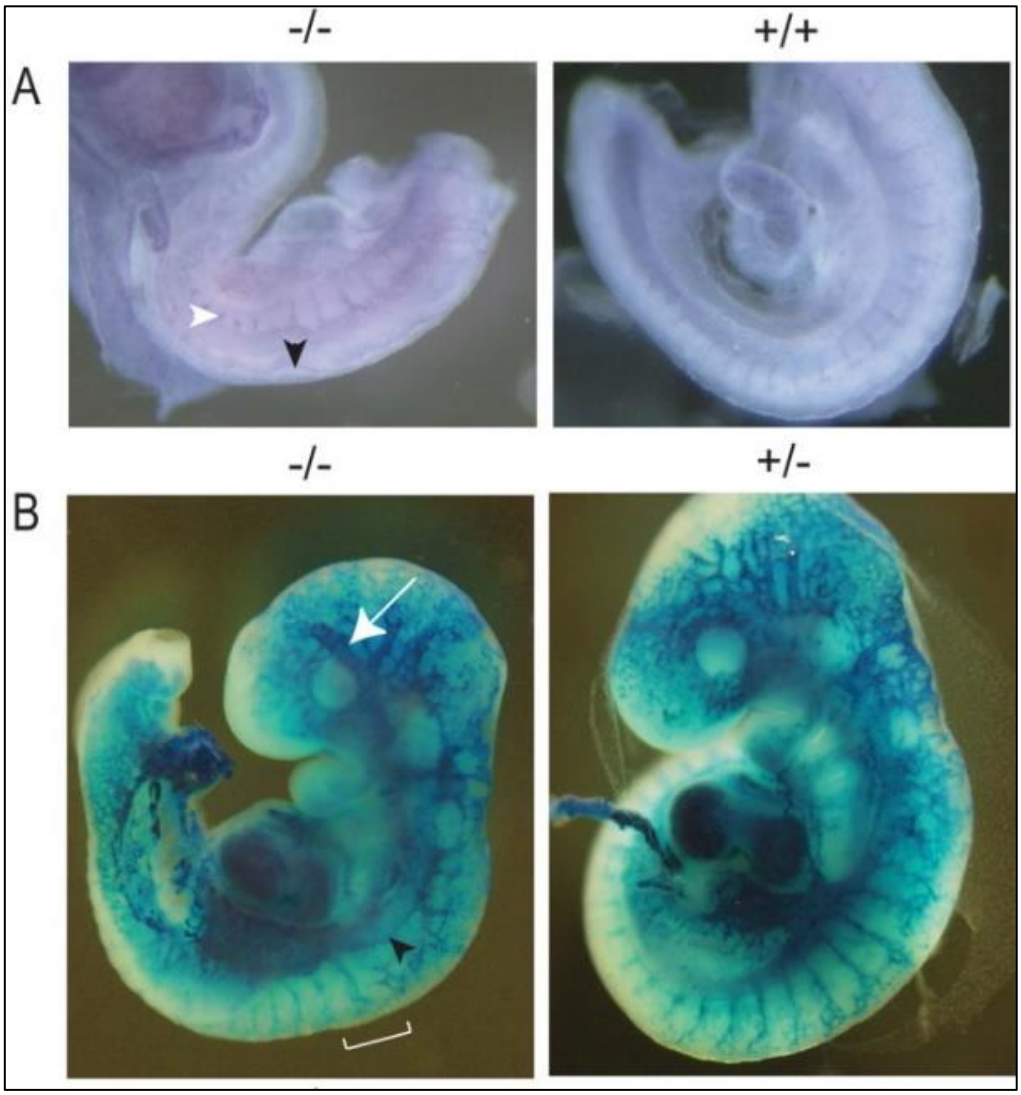


Figure 6. **Vascular abnormalities in mouse embryos.**

A. Embryos stained with an antibody to PECAM-1. Mutant embryo (-/-) and control embryo (+/+). 25x magnification. Black arrowhead indicates regions of neural tube distortions. White arrowhead indicates irregular condensation of somites. **B.** X-Gal staining of SCL *lacZ* transgenic mutant (-/-) and control embryo (+/-). Black and white arrowhead indicates enlarged dorsal aorta. Disorganised intersomitic vessels are seen in the mutant and are indicated by a bracket. Original image from (Bell, S.E. et al, 2006).

2.2.2 The role of Tumour Necrosis Factor Alpha

Tumour necrosis factor alpha (TNFα) is a small cytokine used by the immune system for cell signalling. TNF α is used as a regulator for inflammatory response, with the expression of TNFα indicative to that of an immune response. TNFα is known to maintain homeostasis (Balkwill, F. 2006). The production of TNFα comes from both macrophages and monocytes when in an acute inflammatory state. The TNFα signals for a myriad of pathways such as necrosis or apoptosis. TNF α signals for three major MAPK signalling pathways, these being the ERKs, p38 MKP1 and JNKs (Sabio, G & Davis, R.J., 2014). The production of TNFα might be regulated by ZFP36L1. TNFα playing a key role in the immune system and inflammation processes. The interactions between ZFP36L1 and TNF α are still to be determined. Additional studies are needed to fully realise the implications of ZFP36L1 and its role in the secretion of TNA alpha. The interaction between ZFP36L1 and monocyte/macrophage, the cells that secrete TNF alpha, have not been systematically studied yet.

2.2.3 mRNA in the immune system

The importance in controlling the messenger RNAs (mRNA) stability is an essential process as it allows for cells to limit and adjust the expression of regulatory factors that when overexpressed, can be detrimental to the host organism. Adenylate-uridylate-rich elements (AU-rich elements) are found in the 3'untranslated regions (UTR) of mRNAs. These mRNA are special as they can code for cytokines such as interferons, which are released in the presence of several viruses, as well as interleukins, which are synthesised by helper CD4 lymphocytes (Palanisamy, V. et al. 2012; Murphy G.M. et al. 1995). The CD4 lymphocytes promote the development of T and B lymphocytes as well as hematopoietic cells.

2.2.4 AU-Rich Elements and Their Role in Maintaining Cell Quiescence

AU-rich elements are a region encoded in the RNA with a frequent abundance of adenine and uridine bases. These AU-rich elements when looked at in mammalian cells are found to target the host mRNA for a fast and rapid degradation (Barreau C. et al, 2006). Generally, the AU-rich elements target mRNA encoded proteins that seem to be able to regulate cell growth. These genes require precision when it comes to their transcriptional control. This is done by regulating the translation and how stable the mRNA is. It is found that in unstimulated or resting cells, the AU-rich elements inhibit the expression levels within these cells (Audic Y., Hartley R.S., 2005). These cells that are in an unstimulated state are said to be in a state of quiescence. Cell quiescence is the state of a cell in which it does not divide but retains the ability to re-enter cell proliferation (Galloway et al., 2017). A recent study has shown indication that ZFP36L1 and ZFP36L2 may have a functional role in promoting cell quiescence (Galloway et al., 2017).

The progression of lymphocyte development requires accuracy and coordination within the cell cycle. This coordination allows for genomic integrity whilst allowing for the cells to somatically rearrange their antigen receptor genes. This process is called Variable diversity joining (VDJ) recombination. When this rearrangement becomes successful, it expands the now bigger pool of progenitor lymphocytes. Galloway A. et al states that in B lymphocytes, both the RBP, ZFP36L1 and ZFP36L2 play a vital role in the maintenance of cell quiescence. This is done before precursor B cell receptor (pre-BCR) expression, but also for stabilising quiescence after the pre-BCR expansion. The role of these RBPs is made evident in that they suppress the conserved posttranscriptional regulon which consists of mRNA, the proteins of which promote the transition to the S phase of the cell cycle. This mechanism was found to promote VDJ recombination and effectively select only cells expressing immunoglobulin- μ at the pre-BCR checkpoint. Why exactly do we want a low expression rate for these

unstimulated cells? The reason is that pathological states, such as cancerous tissues and auto immune diseases (Conne B et al, 2005) have been associated with the downregulation of the AU-rich elements that are contained within mRNA.

What exactly regulates these AU-rich elements? There are certain proteins known as RNA-binding proteins (RBP) which bind to either the single or double stranded RNA found within cells, creating ribonucleoprotein complexes. So, in the case of AU-rich elements, the RBP will bind to that region of the RNA, creating this ribonucleoprotein complex that protects this area from enzymes such as RNase. RBPs contain structural motifs such as zinc finger proteins.

Chapter 3

ZFP36L1 and Related Family

ZFP36L1 and Related Family

ZFP36L1 appears to work in conjunction with a variety of different IEG proteins. When looking at different types of these IEGs, we can see how ZFP36L1 affects IEGs and how the upregulation/ downregulation of ZFP36L1 can alter the effectiveness of these proteins. The First IEG looked at is MAPK

3.1.1 MAPK/MKP-1

Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) is an enzyme encoded by the MAPK1 gene. MAPK is specific to the acid's threonine and serine. MAPK regulates cell functions such as apoptosis, proliferation, differentiation, gene expression and mitosis (Pearson, G. et al, 2001). Like ZFP36L1, MAPK can be found in eukaryotes, and are found to be diverse in all eukaryotic kingdoms, as well as unicellular eukaryotes. MAPK are close relatives to Cyclin-dependent kinases (CDK's), belonging to the CMGC kinase group (made up of CDK/MAPK/GSK3/CLK).

The first MAPK discovered was ERK1 (MAPK3) found in mammals. ERK1 as well as MAPK1 were found to be involved in growth factor signalling, they deemed the family term to be 'Mitogen activated' however the term mitogen activated shouldn't be used, as with the discovery of other members of the family, it has become increasingly clear that the majority of MAPKs are involved in a response to stress stimuli, DNA damage and infection. ERK1/2 do not have a generic function, but rather a highly specialised function.

MAPK are always in an inactive state when found in the base form. To become active, there is a necessity for the MAPKs to become phosphorylated within their activation loops. Generally, the conformational change for MAPKs to go from inactive to active is induced via long range allostery. In the case of ERK2s activation loop, the loop contains a threonine-x-

tyrosine (TEY) motif that needs to be phosphorylated in on both threonine and also the tyrosine residues so that the kinase domain can be locked into a catalytically competent conformation (activated state).

Signal Cascade

The MAPK/ERK pathway is a chain of proteins found within a cell that will confirm and communicate a signal sent from a receptor on the cells surface and bring it to the DNA found in the nucleus of the cell. Receptor linked tyrosine kinases such as epidermal growth factor receptor (EGFR), are found to be activated by extracellular ligands such as EGF. When EGF binds to EGFR, the tyrosine kinase activity of the cytoplasmic domain of the receptor. EGFR becomes phosphorylated on the tyrosine residues. Docking proteins bind to the phosphotyrosine residues located on the activated receptor (Schulze, W.X., et al. 2005). The SH2 domain on the docking protein binds to the Sons of Sevenless (SOS) (Zarich, N., et al. 2006). residues found on the phosphorylated EGFR. This creates a complex with the docking protein, activating SOS. Activated SOS then promotes the removal of GDP from a Ras family member, which then allows the Ras protein to bind to GTP, thereby becoming active. Raf kinase phosphorylates and activates MKK1/MKK2. MKK1/2 activate and activate MAPK.

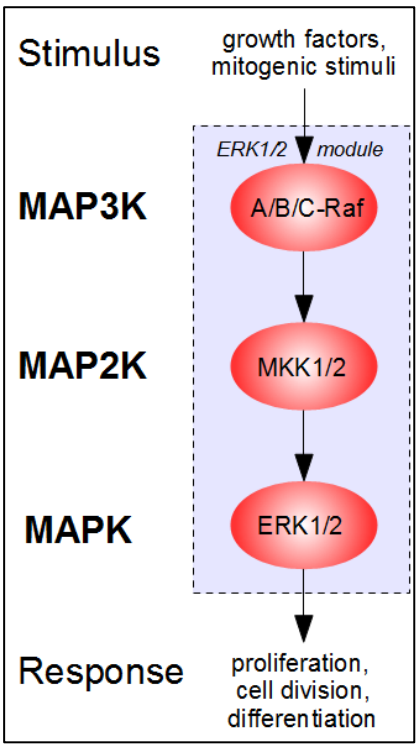


Figure 7. **simplified overview of MAPK cascade for MAPK1 (ERK2)**

binding of tyrosine kinase receptors to extracellular ligands enables phosphorylation via association with Raf proteins dropping GDP and binding to GTP, hence becoming active. Activated Ras proteins activate MKK1/2 by phosphorylating the kinase enzymes. Phosphorylated MKK1/MKK2 activate MAPK. MAPK signals for responses such as proliferation, Cell division and differentiation.

ZFP36L1 and ZFP36L2 modulation of MAPK

In a paper by Wang., et al, 2015, they hypothesised and experimented on the idea that both ZFP36L1 and ZFP36L2 may have some form of control over the expression of MAPK within rodent cells. When observing control macrophages, in resting conditions, there was regular expression of ZFP36L1 and ZFP36L2. Wang., et al, 2015 knocked out both ZFP36L1 as well as ZFP36L2 using lentivirus-carrying short hairpin RNAs in RAW267.4 cells. Western blot was utilised to confirm that the knockdown of ZFP36L1 and ZFP36L2 had been produced. what was found was that the knockdown cell lines had an increase expression of mkp-1 mRNA, with the half-life increasing from 19 minutes to over 5 times with a near 100-minute half-life when found within the knockdown cells. when looking at the knockdown cell lines for other expression levels, it was found that Ttp, Ccl -2, Tnfa and Icam-1 mRNA levels had all decreased or increased, but the increase and decrease was not significant. Wang., et al, 2015 findings suggest that Mpk-1 mRNA stability in macrophages is downregulated by both ZFP36L1 as well as ZFP36L2, however, the expression of both Tnf-a and Ccl-2 are both enhanced.

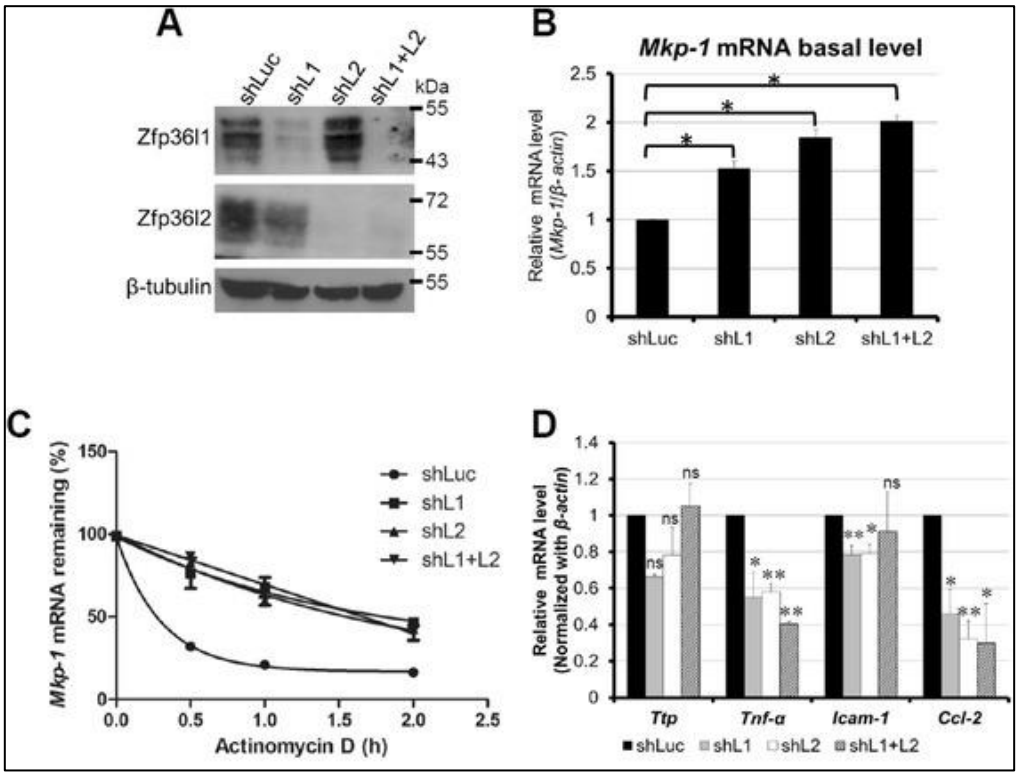


Figure 8. **ZFP36L1 and related family's effect on MAPK**

a. shLuc, shL1, shL2, shL1+shL2 represent the different luciferase cell lines and their knockdown cells. **b**. basal levels of Mkp-1 were detected using quantitative PCR within the different knockdown cell lines. **c.** mRNA half-life of Mkp-1 of the different knockdown cells. **d.** the levels of Ttp, Tnf-a, Icam1 and Ccl-2 were observed using quantitative PCR when looking at the different knockdown cell lines. Image reproduced from Wang, et al 2015

Paclitaxel (PTX) is a form of chemotherapy medication that is used to treat various types of solid cancers such as breast cancer, ovarian cancer, and lung cancer to name a few. Delivery is by injection into the vein. Haiquan., Lu et al, 2018 have shown that the treatment of paclitaxel induces phosphorylation of ZFP36L1 thus leading to inactivation. Co-treatment with the p38 MAPK inhibitor SB203580, blocked the phosphorylation of ZFP36L1 which allowed for mRNA degradation to occur. Both the p38 MAPK pathway and ZFP36L1 are found to work in conjunction with each other, with ZFP36L1 have a direct regulatory role for the p38 MAPK pathway.

3.1.2 Tristetraprolin

Tristetraprolin, also known as Zinc finger protein 36 homolog (ZFP36) is a protein encoded by the ZFP36 gene and found on the 19th chromosome of humans. TTP binds to AREs found in the 3' UTRs of the mRNA region and allows for the promotion of degradation of said regions, in a sense, working very similar to the way ZFP36L1 does. TTP is found to be a negative feedback loop that interferes with TNF-alpha production, it does this by destabilising the mRNA. Mice that were deficient in TTP were found to have had a myriad of various complex syndromes and inflammatory diseases.

mRNA turnover is a tightly regulated process, due to in part, playing a critical role in gene expression of mammals. The importance of this regulation is found evidently when the loss of regulation contributes to an overexpression of certain factors such as genes encoding for protooncogenes, inflammatory mediators and growth factors. A well conserved mRNA sequence element found in a plethora of these cancer associated genes are the AU rich elements. AU rich elements, which are found to be located within the 3' untranslated regions of the mRNA mediate the actions of the transcript via interactions with a trans-acting RNA-binding protein. AU rich elements are deemed to be significant as post-transcriptional

regulatory elements as it has been found that around 8-10% of the human transcriptome includes AU rich element transcripts. (Bakheet et al., 2006). Tristetraprolin, just like ZFP36L1, is an AU rich element binding protein, and one of the best characterized. The importance in regulation of AU rich elements becomes rather apparent when in fact 8-10% of the human transcriptome contains these AU rich element transcripts.

3.1.3 TTP in Breast Cancer

TTP and related families are quintessential for targeting and regulating the AU-rich elements within the 3'-UTRs, via the destabilisation the target mRNAs found within. It's been confirmed that the lack of TTP expression leads to a predictable disease-associated survival. A paper by Milke., et al, 2014. Provided an answer to the effect of knockdown TTP expression in T47D breast cancer cells. what was found to be the case that both in vivo and in vitro growth of TD74 had enhanced growth in the Knockdown lines. downregulation of TTP also lead to increased infiltration of Monocytes in vitro in the 3D tumour spheroid, and also had increased infiltration of macrophages found in vivo in tumour xenografts. This movement of both monocytes and macrophages was found to be due to IL-16 promoting this monocyte and macrophage migration, indicating that TTP is in fact a critical chemotactic regulator of IL-16. (Milke., et al, 2014).

The overexpression of AREs containing genes encodes for factors such as cell proliferation, inflammation, tumour invasion as well as angiogenesis – all factors that allow for the initiation and progression of tumorigenesis (Benjamin & Moroni., 2007; Lopez et al., 2007) this overexpression comes from a lack of regulation within ARE-mediated mRNA decay, due to a mutation which would have incurred alterations to regulatory factors. With TTP being a regulator of mRNA, there has been a lot of extensive work around the interactions TTP has on cancer, with many papers proving that the loss of TTP expression will consistently lead to

various malignancies. Expression of TTP leads to specific cell-type inhibitory effects, giving TTP a strong inclination to being a proposed tumour suppressor gene. TTP also has the function of being able to control the expression of anti-inflammatory processes, and as such, can be considered to have positive cancer regulation due to its affect as a regulator for pro-inflammatory mediators.

3.1.4 14-3-3 Proteins

14-3-3 proteins are a family of regulatory proteins that are found to be conserved and expressed in all eukaryotic cell. Regarding the signalling cascade of both ERK2 and TTP, the 14-3-3 protein would be in this case be the Raf family of proteins. RAF proto-oncogene serine/threonine-protein kinase, also known as proto-oncogene c-RAF or simply c-Raf or even Raf-1, is an enzyme that in humans is encoded by the RAF1 gene. c-Raf protein is found to be a part of the ERK1/2 pathway and a MAP3K as seen in figure 4, which functions downstream of the Ras family of GTPases.

There is evidence to suggest that Protein kinase B (PKB) may act as a stabiliser for ZFP36L1 by phosphorylating within the serine domain at serine 92 (Benjamin, D. et al, 2006), with this phosphorylation occurring, ZFP36L1 suffers impaired mRNA promotion of decay. AU-rich element binding still occurred with ZFP36L1, however the binding of PKB allowed for a complex to form between ZFP36L1 and 14-3-3 proteins, which in turn, increased mRNA stability of the transcript.

Chapter 4

Discussion

4.0.0 DISCUSSION

The zinc gene product known as ZFP36L1 is an RNA binding protein that has the potential to regulate and control both homeostatic as well as cancerous cells. The aim of this study is to explore a plethora of studies and information found on the protein and provide a baseline of knowledge to help in aiding towards deciphering the potential role that ZFP36L1 has as a tumour suppressor gene, as well as play a vital role in cell proliferation, acting as a safeguard against uncontrollable cell growth.

It has been hypothesised over the past two decades that the members of the ZFP36 family post transcriptionally regulates expression of proteins via the actions of binding to these conserved AU-rich regions found withing the 3' untranslated regions of mRNA and recruiting factors that incur the act of degradation when looked at in a variety of different cancer models (Mahtani, M.R., et al. 2000). It is evident that the ZFP36 family is an important protein regulator when looking at how many cancerous cell lines inhibit the effect of ZFP36 and its family members on cell cycle regulation, the down regulation of these zinc finger proteins is becoming an important issue in tumorigenesis (Park., et al., 2018).

ZFP36L1 is an RNA binding protein that has a direct regulatory impact on cell growth, development, differentiation as well as the maturation of the pathway of the cells. (Zekavati et al., 2014). ZFP36L1 is theorised to be regulated extensively at a variety of different levels within different types of cells. ZFP36L1 has a lot less research on it unlike its related family members of Tristetraprolin which is already a very well-known tumour suppressor as well as ZFP36L2.

In a study carried out by (Adachi et al. 2014) their findings and experimental data suggested that both ZFP36L1 and ZFP36L2 have an active role in regulation of the dysfunctional low density lipoprotein receptor (LDLR) mRNA, it does this via the same mechanism of binding

to the 3’ untranslated region of mRNA, but also actively taking up a deadenylase complex, in this case it would be (Carbon Catabolite Repression-Negative On TATA-less) CCR4-nOT, a multiprotein complex that regulates transcription in the cytoplasm, where it will associate with translating ribosomes as well as RNA processing bodies(Miller, J.E. and Reese, J.C., 2012). and thus, leading to the destabilization of the mRNA transcript, (Adachi et al, 2014) also take into account that the deadenylase complex is only temporary to ZFP36L1, and can be reversed via the phosphorylation of p90 ribosomal S6 kinase, allowing for the disassociation with CCR4-nOT to occur. There is multiple studies showing that the dissociation of the deadenylase complex as a regulatory factor of ZFP36L1 as found in observations made by (Wang, et al. 2015) found with substantial evidence that hyper-phosphorylation with ZFP36L1 with a 14-3-3 protein complex lead to decreased interactions with deadenylase (in this case, deadenylase Caf1a), this can be further expanded upon and it could be suggested that this lead to mRNA stability of the transcript. (Wang, et al. 2015) suggest via the results of the study, the complex between the 14-3-3 protein with a phosphorylated ZFP36L1 may have led to MKP-1 expression levels to stabilise thereby having a direct control on the p38 MAPK activity, as found during lipopolysaccharide stimulation.

ZFP36L1 is a potent regulator of VEGF, however its regulatory effect of VEGF is not only experienced in response to cancerous stimulus, but its regulation may also be seen in wound healing as observed by (Hacker, et al. 2010) In which testing on HaCaT keratinocytes with ZFP36L1 knockdown via the use of SiRNA. It was observed that the cell lines had prolonged expression of VEGF upon epidermal growth factor stimulation of the HaCaT cells. This experiment leads to the idea that inhibition of ZFP36L1 allows for prolonged cell proliferation to occur, inciting the idea that ZFP36L1 may possibly provide a key regulatory effect on wound healing when found in vitro (Hacker, et al. 2010). In in vitro studies

conducted by (Loh, et al. 2020) as well as In silico analysis of data across a myriad of cancers revealed that ZFP36L1 as well as related family members ZFP36L2 and ZFP36 were found to be mutated, epigenetically silenced and downregulated in a large variety of cancers, with a large percentage of truncating mutations (frameshifts, stop-gains and indel deletions) of ZFP36L1 and ZFP36L2 being found in bladder and breast cancers (Loh, et al. 2020). These truncating mutation effectively silencing ZFP36L1 and ZFP36L2. One of the most prominent mutated genes found in bladder and breast cancer is ZFP36L1 as found when analysed using MutSig CV. The analysis of ZFP36L1 Q values of false discovery rate was found to be comparable to that of the tumour suppressor gene p53. (Loh et al, 2020). In vitro testing was done on bladder cancer cell lines (HT1376, T24, J82 and UMUC3) as well as breast cancer cell lines (HS578T and BT474), with forced expression of ZFP36L1. It was found that cell proliferation was reduced, with silencing of ZFP36L1 being a tumour enhancer. (Loh, et al. 2020) further the work and identify 1410 genes being potential direct targets to ZFP36L1, with targets including key oncogenic factors such as HIF1A, CCND1 and E2F1, with wildtype ZFP36L1 being able to fully bind and destabilise the HIF1A 3'UTR region via the AU-rich elements, causing mRNA degradation, whilst the truncating mutant variant not being able to bind to the HIF1A 3'UTR region. Several studies support the claim that ZFP36L1 acts as a tumour suppressor (Martinez-Calle et al. 2019., Suk et al. 2018) where (Martinez-Calle-et al., 2019) tested on a form of blood cancer known as myelofibrosis. It was found that in the hypermethylation of myelofibrosis, 27 genes were found to be in the cohort, with a focus on ZFP36L1, (Martinez-Calle., et al. 2019) found that ZFP36L1 was downregulated and hypermethylated in myelofibrosis. Hypermethylation inhibited ZFP36L1s role of inhibiting cell proliferation and induced apoptosis in SET-2 cells – yet when ZFP36L1 was rescued in vitro, the expression had a significant impact on cell proliferation and apoptosis. As pointed earlier, ZFP36L1 can work just as well in a cyclin D dependent and p53 independent manner

as show by (Suk., et al. 2018). With their studies showing that ZFP36L1 loses its antiproliferative ability when mutated. The idea of ZFP36L1 acting as a tumour suppressor can be seen as far back as 2003. (Stoecklein et al., 2003) stated that in mast cell tumours overexpressing IL-3, ZFP36L1 can be witnessed to be acting along the lines of a tumour suppressor. It does this through the ectopic expression, which initiated mRNA degradation of IL-3, and as such, tumour progression was found to be decreased as a direct correlation.

However, with that being said, ZFP36L1 can also be the cause of cell proliferation as well, when functioning improperly. In a study conducted by (Guo, et al. 2020) they conducted analysis of glioblastoma multiforme, and found that the expression levels of ZFP36L1 were upregulated nearly 2-6.5 fold in the glioblastoma cell lines in comparison to normal brain cell (glioblastoma cell line n=162, regular brain tissue n=5). What can be hypothesised from this is that ZFP36L1 expression has increased due to its anti-prolific nature, however ZFP36L1 intended affect – of indicting tumourgenesis, is instead having an adverse effect, instead increasing tumour growth, this is elaborated on by (Guo, et al. 2020) that indicate that miR-139-5p instead acts as the tumour suppressor, via binding to ZFP36L1. (Guo, et al. 2020) results indicate that miR-129-5p negatively regulates ZFP36L1 expression levels in glioblastoma malformed.

Glioblastoma is not the only area that the miR family suppress ZFP36L1, with an increase in miR-29b expression inducing the suppression of ZFP36L1 in renal cancer cells (Sinha et al., 2009). When looking at mice deficient in both ZFP36L1 as well as ZFP36, the mice have acquired T-cell acute lymphoblastic Leukaemia (Hodson et al. 2010).

When looking at ZFP36L1 as a safeguard for cell quiescence, we can see that there is various papers and literature supporting this hypothesis. A paper by (Galloway, A., et al, 2017) state that both ZFP36L1 and ZFP36L2 are necessary to maintain Cell quiescence before precursor BCR (pre-BCR) as well as re-establishing quiescence after pre-BCR induced expansion.

Looking through the meta-analysis of their data, we can see that in vitro testing on double knockout mice of both ZFP36L1 and ZFP36L2, there was a reduced cellularity from pre-b stage onward, at around a 98% reduction in the amount of mature B cells present (Galloway, A., et al, 2017). The cells cycle then went under analysis and it was found that the Knockout lines had higher amounts of pro-B cell in the S phase of the cell cycle. Cyclin dependent kinase inhibitor p27 is a well-known regulator of the cell cycle during pre-B cell development (Boehm, M. et al, 2002), with high expression levels of p27 equating as a marker for cellular quiescence (S, Pathak., et al, 2010). (Galloway, A. et al, 2017) state that in the knockout pro-B cells, the proportion was highly reduced, whilst in the knockout early and late pre-B cells, the expression was moderately reduced. These findings suggest that both ZFP36L1 and ZFP36L2 can keep the cells in the state of cell quiescence and arrest cells before the S phase of the pre-BCR before expression. (Galloway, A. et al, 2017) further support the hypothesis by demonstrating that transcription factors that had been induced by the pre-BCR that are promoting cell quiescence in late pre-B cells were found to be expressed in the knockout mice and p27 mRNA was also being induced. There were also factors that mediate VDJ recombination being expressed as well as the IgK locus being transcriptionally active. These show that the Knockout mice late pre-B cells are transcriptionally ready to enter cell quiescence as well as undergo VDJ recombination; however, without the mediation of ZFP36L1 and ZFP36L2, the posttranscriptional regulation does not occur in these knockout mice. As such, the full activation process does not occur (Galloway, A. et al, 2017). As B-lymphocytes develop, they rearrange their own antigen receptor gene and profusely proliferate, this proliferation may lead to adverse changes to the B lymphocytes genomes. (Galloway, A et al. 2017) found that ZFP36L1 were helping the B-lymphocytes through their entry and exit of the cell cycle, this process allowed for the B-lymphocytes to keep their genomic integrity. This was proven when mice found deficient in both ZFP36L1 and

ZFP36L2 had a block in B cell development. It was found that ZFP36L1 and ZFP36L2 suppressed certain mRNAs that allowed for the B lymphocytes to progress through their cell cycle, this ensured that the B lymphocytes success in cell quiescence as well as keeping genomic integrity.

The posttranscriptional safeguard that ZFP36L1 presents is not only limited B lymphocytes but is also seen in skeletal muscle satellite cells (SCs) (Hausberg, M.A. et al, 2015) as well. SCs are maintained as a population of stem cells in a state of quiescence. SCs will activate and will enter S phase to generate proliferating myoblasts upon the injury of muscle. The change between going from the quiescent state of SCs to the activated state of SCs requires a large transcriptional change in the mRNA as well as changes in miRNA (Farina, N.H. et al, 2012). During the activation of SCs. It was seen that TTP as well as its family members, ZFP36L1 and ZFP36L2 were all found to be decreased upon activation of the SCs (Hausberg, M.A. et al, 2015).

ZFP36L1 and ZFP36L2 have also been witnessed being able to create a thymic β-selection checkpoint by limiting damaged DNA response signals and halting cell cycle progression during thymopoeisis (Vogel, K.U., et al. 2016). Thymopoeisis is the process in which thymocytes turn into mature T cells (Lee, D.K. et al, 2010) depending on negative or positive selection. ZFP36L1 and ZFP36L2 were found to be forcing a β-selection checkpoint during thymopoeisis when seen in primary mouse thymocytes on a genome wide scale (Vogel K.U., et al. 2016). It was also seen that ZFP36L1 and ZFP36L2 regulated the DNA damage response as well as the cell cycle transcripts so that proper β-selection was ensured. (Vogel, K.U., et al. 2016) provide evidence to back this claim. Mice were bred with the endogenous Rosa26 locus which were engineered to express an encoded (Green Fluorescent Protein ZFP36L1) GFPZFP36L1 fusion protein. It was found that in double negative thymocytes, there was a high abundance of GFPZFP36L1 being expressed as well as endogenous

ZFP36L1. This was then assessed via western blotting where both proteins came back at the predicted molecular size. The GFPZFP36L1 had an effect on the thymic development. This is because GFPZFP36L1 the mice had a reduction in the total thymocytes. However, in another double negative cell line, it was found that GFPZFP36L1 had actually increased the percentage and number of the cells. (Vogelm, K.U. et al. 2016). Yet a significant proportion of these cells in S-phase were found to be less in other double negative cell lines. This shows an indication that GFPZFP36L1 acts to inhibit the proliferation of developing thymocytes (Vogelm, K.U. et al. 2016).

4.0.1 Conclusions

The aim of this study was to understand ZFP36L1 and to propose the idea that ZFP36L1 may fill the role as a tumour suppressor gene due to the nature of its anti-proliferation like ways, as well as inducing of tumorigenesis. The presence of 3'UTRs containing AU- rich elements in their transcripts is a key determining factor in the post-transcriptional regulation of many cytokines, as the AU-rich element binding role ZFP36L1 has, can either stabilise or decay the mRNA region. For ZFP36L1 to be considered as a potential tumour suppressor gene, ZFP36L1 would also need to have a way to be regulated. One such regulator of ZFP36L1 is protein kinase B, via the phosphorylation of serine 92 in ZFP36L1. With that being said, ZFP36L1 can also have a negative effect on the body, with their evidence pointing to ZFP36L1 actively promoting cell proliferation as seen in Glioblastoma multiforme.

ZFP36L1 as well as the related family member: ZFP36L2 has been seen to promote cell Quiescence in a variety of cells, and most interestingly, In a variety of immune cells, being seen as a key post-transcriptional safe guard for T cell development by controlling the progression of thymocyte development, as well as helping B cells through the process of the cell cycle. ZFP36L1 and ZFP36L2 would appear to both be good proteins to promote cell quiescence as both their mechanisms work post transcriptionally, and as such, their effects on cells can be more readily reversed, compared to at transcription level. ZFP36L1 and ZFP36L2 are easily phosphorylated and are found to be downstream targets of p38 MAPK and are phosphorylated by MAPKAPK2, leading to reduced mRNA decay. The p38 activity is found to be downstream of pre-BCR, which provides the mechanism ZFP36L1 and ZFP36L2 need to stop mRNA decay.

We can see that ZFP36L1 has exciting areas of where it works, and has a different effect depending on where it is. We have seen that it is found in an abundance of different cancers

and is seen to interact with both T and B lymphocytes, being utilised as a protein to promote Cell Quiescence.

It can be concluded that ZFP36L1 works as a tumour suppressor gene by entering the cells into a state of cell Quiescence, arresting the cell cycle in the G_0 phase. This arrest would therefore not allow the cells to proliferate further. Mutated variants of ZFP36L1 can be seen as a target and can be considered to have similar properties to that of a proto-oncogene.

4.0.2 Future Research

There are minimal studies on ZFP36L1 and given that the AU rich elements are found to be proportionally high in the transcriptome, it would be fair to assume that ZFP36L1 may not be the only protein that is regulating the same mRNA decay sites. We can see the effect that ZFP36L1 has with MAPK, a well-known IEG, so an option for study that could be highlighted is how ZFP36L1 works in conjunction with other IEGs, and whether ZFP36L1 is utilised as a primary response to an inflammatory stimulus, or whether its response is invoked as a safeguard when other IEGs fail. With the cancer rates increasing year by year, the topic for new targets of interest have become to pop up more often, ZFP36 and its family's role in the progression of cancer have become the target area of research in recent years. ZFP36L1 is found to be selective, with a bias towards both bladder and breast cancer where it is mutated mostly. An application of the knowledge from this thesis may prove to be useful for the development of cancer drugs that target specifically ZFP36L1 and its related family members, these drugs could in theory restore ZFP36L1 and its family members to a point of normal expression or preventing the abnormal expression altogether. Moreover, with the high specificity of these proteins, it may be fathomable to utilise them in such a way that they can be used as biomarkers for prognosis in certain cancers.

Another point for further research for ZFP36L1 is to discover other proteins that it readily binds to. These results could be found using various types of techniques such as co-immunoprecipitation as well as proximity ligation assay which both detect physiological protein to protein interactions. These methodological approaches can also be used on already known ZFP36L1 binding proteins to confirm the affinity to ZFP36L1.

Chapter 5

Appendix

5.0.0 Appendix

Due to the disruptions caused by the COVID-19 lockdown, it should be known that there was an attempt at wet lab experiments, and unfortunately, there was no significant data created

These experiments consisted of western blot, where I was looking at whether ZFP36L1 was expressed in a plethora of cell lines. the knockout cell line: MC38KO was provided, this would have been the control.

As I knew the molecular weight of ZFP36L1, I would have separated the proteins by molecular mass, and as such. SDS Page would have been used. The main reason for these experiments was to create images of the protein ladders, to give evidence towards the notion that ZFP36L1 was located in a variety of different cells.

There was also Cell culture work, the idea was to give an abundance of cells for further testing, but to also see how time affected MC38KO. I can confirm that the cell line grew slower compared to MC38 over a period of 3 days, however the idea was to keep a colony going for an extensive period of time as to see whether the MC38KO cell growth would match the cell growth of MC38, as this would have been an indicator that MC38KO would have mutated in such a way that the cell growth would have been working independently of ZFP36L1 from being present.

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