REGULATION OF THE HUMAN RNA NUCLEOTIDYL TRANSFERASE ZCCHC11

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ABSTRACT

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Terminal uridylation of RNA 3' ends has recently been recognised as an important biological regulatory mechanism. The human terminal uridyl transferase ZCCHC11 was shown previously to uridylate and thereby regulate replication-dependent histone mRNAs and several miRNA precursors and mature miRNAs. Studies in cancer cell lines, mouse models and patient samples suggest that ZCCHC11 is a potential oncogene. However, very little is known about regulation of its expression and subcellular localisation. In this study, the regulation of the expression of ZCCHC11 was investigated, especially in relation to the roles played by the untranslated regions of its mRNA. The second aim of this study was to investigate the subcellular localisation of ZCCHC11, both under physiological conditions and under cellular stress. The results presented show that ZCCHC11 expression is regulated negatively through its 5' and 3'UTRs. Upstream open reading frames in the 5'UTR decrease its translation, while regulation through the 3'UTR involves the combined activity of positive and negative factors. In this study it was shown that HuR positively regulates ZCCHC11 expression, whereas TTP contributes to its negative regulation. An homologous enzyme, ZCCHC6, negatively regulates ZCCHC11 expression. Regulation through the 3'UTR of ZCCHC11 is particularly marked following S-phase arrest. ZCCHC11 expression increases under cellular stress and both UTRs take part in this regulation. Regulation under cellular stress also involves an alteration in sub-cellular localisation. ZCCHC11 is predominantly cytoplasmic in unstressed cells, but re-localises to p-bodies and stress granules under cellular stress. Mutating one of three zinc knuckle motifs within ZCCHC11 significantly decreased differential co-localisation with stress granule marker TIA-1 under oxidative stress. Taken together, the data presented provide insights into the post-transcriptional regulation of this post-transcriptional regulatory protein and its subcellular localisation, especially under cellular stress.

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Glossary of Abbreviations

ARE	AU-rich element
AUBP	AU-Rich element binding protein
AUF1	AU-rich element RNA-binding protein 1
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cid	Caffeine induced death suppressor
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
eGFP	Enhanced Green Fluorescent Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HRP	Horseradish peroxidase
HuR	Human antigen R
IgG	Immunoglobulin G
KSRP	KH-type splicing regulatory protein
miRNA	microRNA
mRNA	Messenger RNA
MTPAP	Mitochondrial poly(A) polymerase
Mut	Mutant
ORF	Open reading frame
PABP	Poly(A) binding protein
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride

PPS	Proximal polyadenylation signal
qRT-PCR	Quantitative reverse transcription polymerase chain reaction (RT-PCR)
RACE	Rapid amplification of cDNA ends
RBP	RNA binding protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	Sodium dodecyl sulphate
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
TBS	Tris-buffered saline
TIA-1	T-cell-restricted intracellular antigen-1
TTP	Tristetraprolin
TUTase	Terminal U transferase
uAUG	Upstream AUG
UMP	Uridine monophosphate
uORF	Upstream Open Reading Frame
UTR	Untranslated region
WT	Wild type
ZCCHC11	Zinc finger, CCHC domain containing 11
ZCCHC6	Zinc finger, CCHC domain containing 6

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CHAPTER ONE

INTRODUCTION

Chapter 1: INTRODUCTION

The regulation of gene expression through the regulated stability of RNA molecules has been studied in detail in recent decades. The half-lives of RNA molecules range from minutes to many hours *in vivo* (Chen, Ezzeddine, and Shyu 2008). Eukaryotic cells have the capacity to regulate the half-lives of most mRNAs in order to modify the expression of particular genes under different conditions. RNA turnover mechanisms and their regulatory pathways have been widely conserved during evolution.

In addition to pre-mRNA splicing, modifications on both ends of the nascent transcript are important steps during mRNA maturation. 5' capping and 3' polyadenylation are required for the efficient transport of the mRNA from the nucleus to the cytoplasm, its interaction with the translation machinery, and thus its efficient translation, as well as for the protection of the mRNA against the exonucleases that might attack the transcript from either the 5' or the 3' end. However, when mRNAs complete their lifespan or when internal or external stimuli promote earlier turnover of the mRNA, it is necessary to remove these protective structures in order to allow mRNA degradation. The process of removing the 5' cap (decapping) usually follows a modification at the 3' end of the mRNA. More recently it has been found that, in addition to the well-characterised deadenylation-dependent pathway, 3' uridylation of RNAs also leads to 5' decapping.

1.1. Mechanisms of RNA turnover

1.1.1. Deadenylation-mediated RNA turnover

The poly(A) tail, when present at the 3' end of the transcript, binds to poly(A) binding protein (PABP), which in turn interacts with eIF4G; this interaction circularizes the mRNA, which allows efficient translation as well as preventing exonucleolytic cleavage from either end of the mRNA (Wilusz, Wormington, and Peltz 2001). However, once the mRNA is deadenylated, PABP can no longer bind the transcript and perform this function. Deadenylation is mediated by enzyme complexes with exonucleolytic activity (Parker and Song 2004; Wilusz, Wormington, and Peltz 2001). Once the deadenylated mRNA can no longer bind PABP, it instead recruits the Lsm1-7 complex, which leads in turn to interaction with the decapping enzyme complex (Tharun and Parker 2001). Overall, deadenylation leads to the decapping of the mRNA, and deadenylated, decapped mRNAs are then degraded either by 5'-3' exonucleases or from their 3' ends by the exosome complex (Parker and Song 2004). As discussed later, many *trans*-acting factors that regulate mRNA stability act primarily by governing the rate of mRNA deadenylation.

1.1.2. Uridylation-mediated RNA turnover

Terminal adenylation of RNAs has been the most extensively studied type of template-independent RNA 3' modification. Although studies as early as the 1960s suggested the addition of nucleotide residues other than adenylation, such as uridylation, it took several decades for this phenomenon to attract the attention of the wider scientific community (Rissland and Norbury 2008). The development of more efficient 3' sequencing methods made it possible to study this phenomenon in more detail. The

terminal uridylation of RNAs was then shown to be a mechanism, which has a significant role in the regulation of RNA stability, and is widely conserved in many organisms from *S. pombe* to humans. Terminal uridylation of RNAs at their 3' ends is performed by a family of enzymes called RNA terminal uridyl transferases (TUTases). TUTases add mono-, oligo- or poly- uridyl tails to the 3' ends of RNAs in a template-independent manner (Aphasizhev, Aphasizheva, and Simpson 2003; Schmidt and Norbury 2010). Although the terminal uridylation of U6 snRNA by an enzyme called RBM21 (U6 TUTase) conferred increased stability, many TUTases are characterised by their role in conferring decreased stability on their target RNAs (Schmidt and Norbury 2010).

1.1.2.1. Terminal uridylation in non-human organisms

The characterisation of Cid1, a TUTase in *S. pombe*, has provided a model for understanding the concept, mechanism, and function of uridylation in other systems. Cid1 (*c*affeine-*i*nduced *d*eath suppressor) was first identified in a cell cycle study in an attempt to identify the downstream effectors of the S-M checkpoint activated in response to the DNA replication inhibitor hydroxyurea, in the presence of the checkpoint inhibitor caffeine (Wang et al. 1999). Cid1 is a member of the Pol β superfamily of nucleotidyltransferases. It is a constitutively cytoplasmic protein, which was soon after its identification found to be involved in RNA metabolism (Read et al. 2002; Rissland and Norbury 2008). *In vitro*, Cid1 can incorporate ATP, but has a stronger affinity for UTP as substrate to add to the 3' end of RNAs. *In vivo*, it was found to add mono- and di-uridyl tails to mature polyadenylated mRNA transcripts in a manner that was independent of the poly(A) length. Alpha actin mRNA was found to be uridylated by Cid1 upon S-phase arrest. This study showed for the first time that mRNAs already

modified at their 3' ends by polyadenylation can be further modified by uridylation (Rissland, Mikulasova, and Norbury 2007). It was later found in *S. pombe* that decapped transcripts are often uridylated, suggesting an involvement of uridylation in RNA degradation from the 5' end. Decapping was thought to be induced by deadenylation, as it was firstly described in the budding yeast *Saccharomyces cerevisiae.* However, in *S. pombe*, terminal uridylation of mRNAs was deadenylation-independent, with uridyl residues often added to mRNAs with long poly(A) tails. Decapping was found to follow uridylation, and an increase in stability of *urg1* mRNAs in strains lacking Cid1 further supported the role of uridylation in RNA turnover (Rissland and Norbury 2009). The Lsm1-7 complex, known to be capable of binding oligo(U) tracts *in vitro*, is involved in uridylation-mediated mRNA decay, and is proposed to stimulate decapping following uridylation (Tharun et al. 2000; Song and Kiledjian 2007; Rissland and Norbury 2009). The majority of uridylation in *S. pombe* is Cid1-dependent (Rissland and Norbury 2009), with the remaining uridylation attributable to the related Cid16 protein (Scott, D.D., personal communication). In the budding yeast *S. cerevisiae*, which is a yeast distantly related to the fission yeast S. pombe, no mRNA uridylation appears to take place. Indeed, the *S. cerevisiae* genome encodes for two nucleotidyl transferases, related to Cid1, Trf4 and Trf5, but unlike Cid1 these enzymes are non-canonical poly(A) polymerases, and transfer adenyl residues to their substrates (Schmidt and Norbury 2010). Another fungus, *Aspergillus*, was shown to contain enzymatic activity capable of adding CU residues to polyadenylated mRNAs (Morozov et al. 2010). More recently, a homologue of Cid1 has been identified in *Arabidopsis thaliana*, and a role of uridylation in preventing trimming of oligoadenylated mRNAs has been proposed in this organism. This mechanism was suggested to favour 5' to 3' degradation, and thereby to prevent

the translation of aberrant transcripts partially degraded from their 3' ends (Sement et al. 2013).

In addition to the role of uridylation in the degradation of intact transcripts, it was found that TUTases are also involved in the degradation of miRNA-directed 5' cleavage products. miRNAs are a group of non-coding RNAs (ncRNAs) that bind to complementary sequences in their target mRNAs, and regulate their expression (Bartel 2004). The RNA-induced silencing complex (RISC) is the name for the heterogenous molecular complexes, which function in small RNA guided gene silencing (Pratt and MacRae 2009). In *Arabidopsis*, after endonucleolytic cleavage by the RISC, the 3' products of mRNAs are degraded 5' to 3', while the 5' fragments can also be degraded 5' to 3' after uridylation and decapping instead of being degraded from the 3' end by the exosome (Shen and Goodman 2004). Similar mechanisms for the degradation of 5' cleavage products exist in other organisms and the activity of TUTases in such regulation is not limited to oligouridylation, but also includes oligoadenylation of 3' ends of these fragments (Ibrahim et al. 2006; Scott and Norbury 2013).

Interestingly, another RNA modification, methylation, is performed on 3' ends of small RNAs such as siRNAs and miRNAs in *A. thaliana* by the methyltransferase HEN1, which prevents uridylation of these RNAs and thereby maintains RNA stability. HENN1, a homologue of HEN1 in *C. elegans,* was identified, and similarly its mutation destabilised several small RNAs, while the uridylation rate of 26G siRNA, one of the destabilised RNAs, increased. In *Chlamydomonas reinhardtii*, the enzyme Mut68, which is suggested to perform destabilising RNA uridylation, is also inhibited by the activity of methyltransferases that modify the 3' ends of small RNAs. In summary, the opposed

functions of methylation and uridylation appear to be a conserved feature of RNA metabolism in diverse eukaryotic organisms (Scott and Norbury 2013).

Terminal uridylation also occurs in mitochondria, and this phenomenon has been studied in detail in *Trypanosoma brucei*. RNA Editing TUTases 1 and 2 (RET1 and RET2) perform this function. RNA editing of mitochondrial RNAs, including uridylation and deletion of uridyl residues, occurs in complexes called editosomes, and guide RNAs (gRNAs) involved in this editing are themselves also uridylated (Rissland and Norbury 2008; Aphasizhev, Aphasizheva, and Simpson 2003).

1.1.2.2. Terminal uridylation of human RNAs

Several studies have identified uridylation in human cells. Given the regulatory role of uridylation in other organisms, the capacity of human TUTases to regulate the expression of key genes in human physiology has been investigated. It has been suggested that alterations in terminal uridylation in human cells might be important in the pathogenesis of diseases such as cancer and inflammatory disorders. Somewhat confusingly, several human enzymes have been designated as TUTases by some authors, despite clear indications that they add adenyl rather than uridyl residues to their RNA targets. In this section, these enzymes are also briefly mentioned. Table 1.1 shows a summary of human TUTases.

1.1.2.2.1. U6 TUTase (TUT1)

U6 snRNA is a spliceosomal RNA, which is highly conserved, and has an important role in pre-mRNA splicing (Brow and Guthrie 1988; Trippe et al. 2006). U6 snRNA terminates in a 3' oligouridyl tract, which is the main binding platform for the La protein provided it

ends with 3'-OH (Terns, Lund, and Dahlberg 1992). This binding is thought to be stabilising, and the stabilisation of U6 snRNA was suggested to be maintained in yeast by the redundant functions of the La protein and the Lsm2-Lsm8 complex, which also binds to the 3' ends of U6 snRNAs (Pannone et al. 2001). The U6 snRNA is unique among all snRNAs in that its 3' end is modified by two functionally opposing mechanisms, one of which removes the uridyl residues while the other adds them. Indeed an exonuclease activity removes the terminal uridyl residues (Booth and Pugh 1997), and after a long search for the enzyme responsible, the Mpn1 RNA exonuclease was found to trim 3' oligouridine tails of U6 (Shchepachev et al. 2012). On the other hand, the enzyme responsible for the uridylation of the U6 snRNA was identified in HeLa cells, and as it was suggested to uridylate U6 snRNA exclusively, it was named U6 TUTase. The function of the U6 TUTase was suggested to restore the oligouridyl terminal tail, which requires four Us, and hence the U6 snRNAs ending in three or fewer Us are substrates for this enzyme (Trippe, Sandrock, and Benecke 1998). The biological significance of U6 TUTase is substantial, as shown by knock-down experiments. HeLa cells transfected with siRNA against U6 TUTase displayed a significant decrease in cell survival. The same study also found that the subcellular localisation of U6 TUTase is predominantly nucleolar (Trippe et al. 2006). Contrary to these early studies, which proposed a unique function for U6 TUTase in uridylating U6 snRNA, more recent studies suggested that miRNAs, in addition to U6 snRNA, are substrates for U6 TUTase (Wyman et al. 2011; Knouf, Wyman, and Tewari 2013). Indeed, knocking down U6 TUTase broadly affected miRNA levels negatively. This decrease in miRNA levels was not accompanied by a decrease in primary miRNA transcripts, on the contrary, a slight increase occurred, suggesting an effect on later steps in miRNA maturation. However, the role of this enzyme in miRNA biogenesis

is currently not fully understood. Finally, in addition to its activity as a terminal uridyl transferase, the U6 TUTase was suggested to possess poly(A) polymerase activity (Mellman et al. 2008). Although it is surprising that no lethality was mentioned after the knockdown of the U6 TUTase in this study, it would be quite interesting to understand the multi-functions of this enzyme in the future.

1.1.2.2.2. PAPD4 (TUT2/ GLD2)

PAPD4 was identified more than a decade ago in *Caenorhabditis elegans* as a cytoplasmic enzyme with poly(A) polymerase activity. This activity was shown to positively regulate target mRNAs, and to require the presence of the RNA binding protein GLD3 (Schmidt and Norbury 2010; Wang et al. 2002). While PAPD4 localises to the cytoplasm in *X. laevis*, *C. elegans* and *D. melanogaster* (Burroughs et al. 2010), mice exhibit both nuclear and cytoplasmic localisation of PAPD4, which is ubiquitously expressed in all tissues, with testis and ovary expressing relatively higher amounts. The same study also showed that PAPD4 has an essential role in oocyte maturation, as its absence results in arrest in meiotic metaphase I (Nakanishi et al. 2006). *miR-122*, an abundantly expressed miRNA in liver, is 3' terminally adenylated by PAPD4 in mammalian cells, and is stabilised by this modification. The regulation of *miR-122* also leads to an indirect regulation of its targets, such as BCKDK, Cd320, CyclinG1 and Ndrg3, which were upregulated in the absence of PAPD4. *miR-122* was suggested to be adenylated preferentially in its single-stranded form (Katoh et al. 2009). PAPD4 is also thought to be involved in synaptic translation in mammalian brains, and an interaction between PAPD4 and CPEB (Cytoplasmic Polyadenylation Element Binding Protein) exists (Rouhana et al. 2005). Decreased expression of mRNAs is conferred through their 3'UTRs

by hCPEB1 in the case of p53 and HPV-16 mRNAs, as shown by overexpression studies. On the other hand, PAPD4 has a positive effect on the expression of these genes when it is expressed as a fusion protein hCPEB1-PAPD4. Hence the presence of PAPD4 functions to relieve the negative effect of hCPEB1 (Glahder and Norrild 2011; Glahder et al. 2010).

Such an opposition of function between these proteins was also observed during oocyte maturation. PAPD4, PARN and CPEB all reside in the same complex, where CPEB and PARN function to favour the maintenance of short poly(A) tails while PAPD4 has the capacity to elongate them. Cyclin B1 mRNA has a shortened poly(A) tail during oocyte maturation owing to the higher efficiency of PARN in deadenylating in comparison with the efficiency of PAPD4 in elongating the poly(A) tail. However, when oocyte maturation is induced by progesterone, phosphorylation of CPEB by Aurora A, a serine/threonine-protein kinase, leads to dissociation of PARN from this protein complex, resulting in the predominance of the activity of PAPD4 in maintaining the poly(A) tail (Kim and Richter 2006). The evolutionary advantage of two opposing mechanisms, one of which adenylates while the other deadenylates mRNAs, and the fact that these enzymes reside in the same protein complex, is currently not fully understood and requires further studies.

1.1.2.2.3. MTPAP (PAPD1)

Human mitochondrial PAP (hMTPAP), as the name implies, adenylates mitochondrial transcripts. It is the only identified PAP, which resides and functions in mitochondria, despite being encoded by the nuclear genome. hMTPAP knock-down in HeLa cells led to a decrease in mitochondrial poly(A) tail lengths and a parallel increase in mRNAs with oligo(A) at their 3' ends, suggesting that this protein is required for the long poly(A) tails

in mitochondria (Tomecki et al. 2004). The function of polyadenylation differs between organisms and among organelles. Polyadenylation in bacteria, as well as polyadenylation in plant chloroplasts and mitochondria, is usually degradative, whereas polyadenylation by canonical PAPs in plant nuclei maintains RNA stability (Dreyfus and Regnier 2002; Gagliardi and Leaver 1999; Chang and Tong 2012). In comparison, human mitochondrial polyadenylation is less understood, and may be more complicated in terms of its effects. For some mitochondrial mRNAs, polyadenylation is thought to complete the UAA stop codon (Bobrowicz, Lightowlers, and Chrzanowska-Lightowlers 2008). One group suggested that the phenomenon of degradative polyadenylation in human mitochondria is a vestige of their bacterial origin, showing that in addition to the presence of stable polyadenylated mitochondrial mRNAs, mRNAs that are truncated and polyadenylated coexist (Slomovic et al. 2005). Reducing hMTPAP expression by gene silencing, on the other hand, resulted in decreased stability of several mitochondrial transcripts such as CO1, CO2, CO3 and ATP6 mRNAs but the stability of ND3 mRNA, another mitochondrial transcript, was not affected (Slomovic et al. 2005). Conversely, overexpression of PDE12, a mitochondrial deadenylase, resulted in a parallel increase in the level of three mitochondrial transcripts, ND1, ND2 and CytB, whereas the same situation led to a decrease in CO1 and CO2 transcripts (Rorbach, Nicholls, and Minczuk 2011). To sum up, the effect of mitochondrial polyadenylation in human cannot be generalised as degradative or stabilising. According to current literature the effect of polyadenylation on mitochondrial mRNA is transcript-dependent. Other factors interacting with mitochondrial mRNAs may also have roles in determining whether polyadenylation leads to increased or decreased stability. Though currently little is known about mitochondrial polyadenylation, there is evidence that mutation of hMTPAP is associated with a severe

neurodegenerative disorder, highlighting the importance of the integrity of this mechanism (Crosby et al. 2010). More recently it was shown that hMTPAP mutation resulted in increased radiosensitivity, increased DNA damage and increased apoptosis, possibly owing to a parallel increase in reactive oxygen species (ROS) and disruption of DNA repair (Martin et al. 2014). Surprisingly, hMTPAP was also suggested as one of the TUTases involved in miRNA 3' modification. (Knouf, Wyman, and Tewari 2013; Wyman et al. 2011). Very little is known about this function, however if MTPAP is exclusively mitochondrial, a function in modifying miRNAs seems unlikely, but cannot be excluded as some studies suggest that miRNAs are present in the mitochondria (Kren et al. 2009; Barrey et al. 2011). An earlier study also suggested a role for MTPAP in regulating replication-dependent histone mRNAs (Mullen and Marzluff 2008), which considering its localisation in mitochondria also seemed implausible; a more recent study from our group showed that hMTPAP is not involved in such regulation in human cells (Schmidt, West, and Norbury 2011). Finally, similarly to the opposed PAPD4-PARN/CPEB system, hMTPAP was found to interact with the deadenylating SUV3-PNPase complex, at least transiently, and depending on cellular energy status this interaction seems to play a role in determining mitochondrial mRNA poly(A) tail lengths (Wang et al. 2014).

1.1.2.2.4. PAPD5 and POLS

The identification of the human proteins PAPD5 and POLS is based on the comparison of sequence with the yeast non- canonical PAP Trf4. Trf4 associates with Mtr4, an RNA helicase, and the zinc knuckle proteins Air1/ Air2 to form the TRAMP complex, which is responsible for nuclear polyadenylation (LaCava et al. 2005b; Wyers et al. 2005; Vanacova et al. 2005). This complex functions in the surveillance of nuclear RNAs such as

rRNAs, snRNAs and snoRNAs. It plays a role in targeting these nuclear RNAs for degradation by the exosome (LaCava et al. 2005a; Schmidt and Norbury 2010). Although the function of their homologue Trf4 is relatively well studied, very little is known about the functions of human orthologues PAPD5 and POLS. Polyadenylation of aberrant pre-RNA transcripts generated by the RNA polymerase I was shown to be performed by PAPD5, and is important in the degradation of these transcripts by the exosome complex (Shcherbik et al. 2010). Similar to its yeast homologue Trf4, PAPD5 was also found to localise exclusively to the nucleus in mammalian cells (Rammelt et al. 2011). A role for PAPD5 as the enzyme primarily responsible for the 3' oligoadenylation of H/ACA box snoRNAs was also suggested recently (Berndt et al. 2012). On the other hand, there is currently no identified function of POLS in mammalian cells. However, POLS resides in a genomic region that is amplified and represented with high copy number in a number of cancers (Walowsky et al. 1999), but this amplification was not found to result in increased POLS gene expression in cervical cancer cells (Huang et al. 2006).

1.1.2.2.5. ZCCHC6 and ZCCHC11

ZCCHC6 and ZCCHC11 are two human orthologues of *S. pombe* Cid1; they were identified by BLAST searches, on the basis of their similarity to other members of the Cid1 family. These proteins transfer non-templated nucleotides at the 3' end of RNA molecules, and unlike the known nucleotidyl transferase PAPD4, which adds adenyl residues, these proteins add uridyl residues to the 3' ends of RNA substrates (Kwak and Wickens 2007; Rissland, Mikulasova, and Norbury 2007). In this section these two proteins, which share extensive sequence similarity and partially redundant functions, will be discussed together.

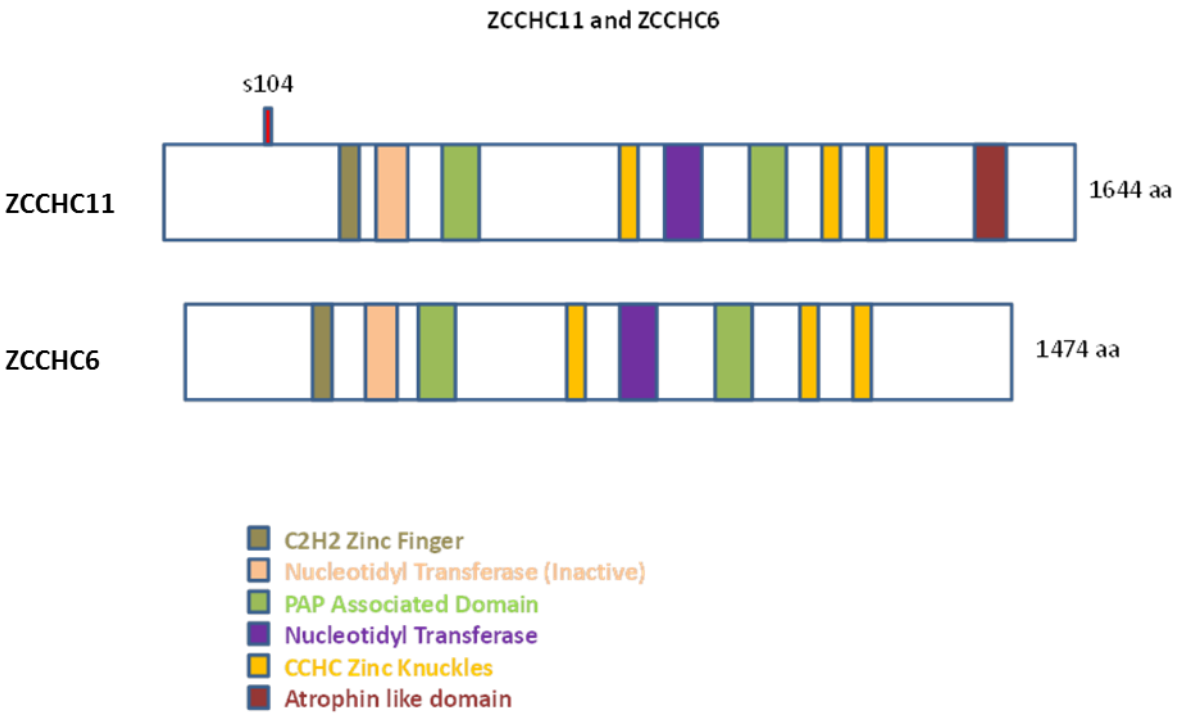


FIGURE 1.1. Schematic representation of the domains and motifs of ZCCHC11 and ZCCHC6. (not to scale)

ZCCHC11 contains three CCHC zinc fingers (zinc knuckles) and one C2H2 zinc finger motif. It contains two PAP-associated domains and other domains, such as an Atrophin like domain, and the catalytically active and inactive nucleotidyl transferase domains (Fig. 1.1.). Soon after its identification, potential roles were identified for ZCCHC11 in the regulation of key elements in human physiology, as well as in pathological conditions such as inflammation and cancer biology. In a study investigating the interacting partners of TIFA (TRAF-interacting protein with a forkhead-associated (FHA) domain), an adaptor protein important in activating NF-kappa-B and AP-1 pathways under IL-1 stimulation, ZCCHC11 was found as one of the proteins that interacts with TIFA in a lipopolysaccharide (LPS)-dependent manner. The study, carried out in RAW 264.7 cells,

suggested that ZCCHC11 is mainly localised to the nucleus under normal circumstances, but re-localised to the cytoplasm after LPS stimulation. This was contrary to several later studies, and results presented in this thesis, where ZCCHC11 was observed predominantly in the cytoplasm. However, none of these later studies were carried out in RAW 264.7 cells, and the difference between these studies in terms of ZCCHC11 localisation may reflect the usage of different cell lines. ZCCHC11, relocalising to the cytoplasm and interacting with TIFA following LPS stimulation, was suggested by the same study to suppress LPS-induced NF-κB activity (Minoda et al. 2006). Knocking down ZCCHC11 resulted in decreased levels of several cytokines, including IL-6, VEGF, TGF-alpha and RANTES, in cells pre-treated with TNF-α. This was not a global effect on cytokines, as IP-10, MCP-1, fractalkine, G-CSF and GM-CSF levels did not change, and IL-8 level was even increased when ZCCHC11 was targeted by siRNA. ZCCHC11 knock-down led to a decrease in IL-6 mRNA poly(A) length and, given the role of ZCCHC11 as a terminal uridyl transferase, a possible indirect effect on IL-6 was investigated. *miR-26b* was shown to be uridylated by ZCCHC11 in its mature form, and this uridylation did not affect its expression levels. Interestingly, the ability of *miR-26b* to suppress IL-6 mRNA decreased when the mature *miR-26b* had 3' non-templated uridyl residues, suggesting the possibility that uridylation of mature *miR-26b* by ZCCHC11 resulted in its diminished function in suppressing IL-6 expression. Terminal modification of *miR-26a* by ZCCHC11 with the addition of one to three non-templated uridines was also suggested in this study (Jones et al. 2009). Later, it was suggested that ZCCHC11 was itself targeted by *miR-26a* through its 3'UTR (Fu et al. 2014). However, the results presented later in this thesis do not support such regulation of ZCCHC11 by *miR-26a*.

Until now, the most extensively studied ZCCHC11 function is in the regulation of *let-7* miRNA biogenesis and degradation. *let-7* is a family of miRNAs known for their tumour suppressor functions. Targets of *let-7* with oncogenic roles include RAS, Hmga2 (High Mobility Group A2) and several cell cycle-associated proteins (Johnson et al. 2005; Mayr, Hemann, and Bartel 2007; Wang et al. 2012). Decreased *let-7* levels were shown to be associated with poor prognosis in human lung cancer (Takamizawa et al. 2004), while ectopic overexpression of *let-7* in non-small cell lung tumour xenografts led to growth reduction and increased cell death (Kumar et al. 2008). An inverse association between *let-7* family member *let-7b* levels and several parameters for the outcome of breast cancer, such as patient survival and breast cancer lymph node metastasis was noted (Ma et al. 2014). Another *let-7* family member, *let-7d*, decreases in head and neck squamous cell carcinoma (HNSCC) tumours, and this decrease is related to poor prognosis (Childs et al. 2009). Finally, many other cancer types including prostate cancer, melanoma, ovarian cancer and lymphoma show deregulation of *let-7*, usually down-regulation, but less frequently and surprisingly in some cases up-regulation (Boyerinas et al. 2010b). Hence it is important to understand the regulation of *let-7* in normal cells as well as cancer cells. Lin28 is an RNA binding protein, which has crucial roles in regulating let-7 expression. It contains a cold shock domain and two zinc finger domains (Moss, Lee, and Ambros 1997). Lin-28 was first identified in *C. elegans* (Ambros and Horvitz 1987), where it plays important roles in the maintenance of pluripotency, and functions as an oncogene, in addition to its requirement in developmental timing. In mammals, there are two homologues of Lin-28: Lin28A and Lin28B. Most of the functions of Lin-28 mentioned here are functions of Lin28A in regulating let-7 (Thornton and Gregory 2012; Scott and Norbury 2013). In fact, in many cancers Lin28A expression increases while let-

7 levels decrease (Boyerinas et al. 2010b). Lin28A suppresses *let-7* biogenesis by interacting with primary and precursor *let-7* miRNAs, and the uridylation of *let-7* precursors is an important mechanism for this regulation (Mayr and Heinemann 2013). The regulation of *let-7* by Lin28 and the importance of uridylation in this regulation were suggested by several groups. These findings will be discussed together as they have considerable overlap. These groups showed that Lin28 associates with pre-*let-7* and regulates it post-transcriptionally. Lin28A was also shown to be regulated by *let-7*, suggesting the existence of a feedback loop (Rybak et al. 2008). Terminal uridylation of pre-*let-7* was found to occur in cells overexpressing Lin28A. When the cells were transfected with a zinc finger domain of Lin28A mutant instead, no uridylated pre-*let-7* was seen, suggesting a requirement for the zinc finger domain in recruiting the enzyme responsible for pre-*let-7* uridylation. In addition, the uridylation conferred by Lin28A was specific to pre-*let-7* as no significant uridylation was observed among several other miRNA families tested. Uridylation activity was shown to occur mainly in the cytoplasm and uridylation of pre-*let-7* inhibited its downstream processing by Dicer and made it less stable (Heo et al. 2008). However, as Lin28A does not possess uridyl transferase activity itself, an additional protein, which bears a ribonucleotidyl transferase activity, and is recruited to pre-*let-7* by Lin28A, was sought. Using mass spectrometry and RNA affinity purification, ZCCHC11 was found associated respectively with Lin28A and pre-*let-7a-1*. Uridylation of pre-*let-7* by ZCCHC11 was observed, and it was shown that it requires a catalytically active enzyme, as a mutation inhibiting the catalytic activity of ZCCHC11 also inhibited uridylation *in vitro*. Knocking down ZCCHC11 in embryonic stem cells increased the abundance of mature forms of *let-7* significantly without affecting pri-*let-7* levels, suggesting a mechanism of regulation at a level downstream of Drosha

processing. It also led to repression of *let-7* target genes (Hagan, Piskounova, and Gregory 2009b). In line with the earlier study (Rissland and Norbury 2009) suggesting that the uridylation occurs mainly in the cytoplasm, ZCCHC11 was found to reside in the cytoplasm, as shown by cell-fractionation and immunocytochemistry, and its binding to pre-*let-7* was also found to occur in the cytoplasm. Both Lin28A and ZCCHC11 are important for the uridylation of pre-*let-7*, as the impact of their double knockdown was greater than individual knockdown of these proteins. Although it was more pronounced in double knockdown of these proteins, the decrease of the pluripotency markers Oct4 and Nanog was observed both in single and double knockdown of Lin28A and ZCCHC11 (Heo et al. 2009; Hagan, Piskounova, and Gregory 2009a). It was subsequently suggested that Lin28A, but not Lin28B, regulates *let-7* maturation through recruiting ZCCHC11 (Piskounova et al. 2011). It was shown that several cancer cell lines express Lin28A or Lin28B in a mutually exclusive manner. Though previous *in vitro* studies had suggested that Lin28B is also capable of directing the uridylation of substrates through ZCCHC11 recruitment, studies in cancer cell lines expressing Lin28B showed firstly that it does not interact with ZCCHC11, secondly that it localises mainly in the nucleus, but not in the cytoplasm where ZCCHC11-dependent uridylation occurs, and thirdly that knock-down of ZCCHC11 in these cell lines led to no significant change in *let-7* levels. These results suggested that the Lin28A, but not Lin28B, enhances ZCCHC11-mediated uridylation in physiologically relevant conditions, though both proteins can perform this function *in vitro*. Lin28B appears to operate independently of ZCCHC11, possibly directly associating with pri-*let-7* in the nucleus and preventing its maturation as suggested by the same study (Piskounova et al. 2011).

In summary, Lin28A, but not Lin28B, recruits ZCCHC11 in order to uridylate pre-*let-7*, which then leads to decreased processing by Dicer endonuclease, and increases its turnover. More recently, a 3' to 5' exonuclease, Dis3L2, was shown to interact preferentially with the uridylated forms of pre-let-7. Dis3L2, ZCCHC11, and ZCCHC6 associated with Lin28A, and the interaction between Dis3L2 and Lin28A was shown to be RNA-dependent. In addition to its interaction with Lin28A and uridylated pre-*let-7*, it was revealed that Dis3L2 degrades uridylated pre-*let-7*, strongly suggesting that it is the exonuclease required for Lin28A-ZCCHC11-mediated turnover of pre-*let-7* (Chang et al. 2013).

Although the involvement of ZCCHC11 in miRNA biology has been its most widely studied role, our group has previously shown that ZCCHC11 is also capable of uridylating replication-dependent histone mRNAs (Schmidt, West, and Norbury 2011). These mRNAs are unique among metazoan mRNAs in that they lack poly(A) tails, but instead have a stem loop structure near their 3' ends (Dominski and Marzluff 1999). A previous study proposed that MTPAP and PAPD5 uridylate histone mRNA 3' ends (Mullen and Marzluff 2008). However, because of their subcellular localisation and usage of adenyl residues as major substrate respectively, it seemed unlikely that these proteins were involved in histone mRNA uridylation. Our group suggested that another human TUTase is responsible for this modification (Schmidt, West, and Norbury 2011). Indeed, ZCCHC11 was shown to associate with replication-dependent histone mRNAs in human cell lines, whereas the other Cid1 orthologue TUTase, ZCCHC6, did not show such an association (Schmidt, West, and Norbury 2011). It was revealed that ZCCHC11 is required for replication-dependent histone mRNAs turnover following DNA replication stress such as

that induced by hydroxyurea treatment. ZCCHC11 levels decreased in a cell cycle-dependent manner, decreasing during S-phase, possibly related to its role in downregulating histone mRNAs and the cellular requirement to maintain histone levels during DNA replication. On exit from S-phase, ZCCHC11 levels returned to their pre-S-phase level (Schmidt, West, and Norbury 2011). This regulation is potentially important in degrading histone mRNA when they are not required, thus preventing their toxic accumulation in cells. Knock-down experiments suggested the involvement of Lsm1-7 in ZCCHC11-dependent histone turnover (Schmidt, West, and Norbury 2011). Though decapping and subsequent 5' to 3' decay of histone mRNAs was demonstrated (Su et al. 2013), and considering the role of Lsm1-7 in mRNA degradation by a decapping mediated mechanism in *S. pombe* (Rissland and Norbury 2009), some aspects of this process are still unclear.

Recently a novel mechanism that involves 3' to 5' directionality for histone mRNAs decay was revealed. Exoribonuclease (Eri1), a 3' exonuclease, was shown to play an important role in replication-dependent histone mRNA decay, as hydroxyurea-induced decay decreased under Eri1 depletion. Eri1 was shown to bind histone mRNAs and Lsm1-7 complex, and to degrade histone mRNAs when they were oligouridylated (Hoefig et al. 2013). However, Lsm1-7 has a role in decapping-mediated mRNA turnover in other organisms (Scott and Norbury 2013). Besides, Eri1 depletion does not cause a cell cycle progression defect and histone mRNAs still undergo decay, albeit more slowly, in the absence of Eri1 (Hoefig et al. 2013). Therefore, further research is still required to clarify how oligouridylation by ZCCHC11 confers decreased stability to histone mRNAs, especially following DNA replication stress.

By decreasing the levels of tumour suppressor miRNA pre-*let-7*, and by promoting the degradation of replication-dependent histone mRNAs, excess accumulation of which are toxic, ZCCHC11 has potential implications in cancer biology as a candidate oncogene as well as a potential target for cancer therapy. The first indications that ZCCHC11 might play an oncogenic role came from a study suggesting that ZCCHC11 knock-down decreased cancer cell numbers, owing to a decreased proliferation because of G1-arrest rather than increased apoptosis or senescence (Blahna et al. 2011). G1-arrest upon ZCCHC11 knockdown was shown to happen through two mechanisms, one of which involved phosphorylation of the retinoblastoma protein (Rb), a modification that inactivates Rb and is a key step in the G1 to S-phase transition. Several cell cycle-associated proteins with roles in Rb phosphorylation, such as CDK4 and cyclin D1, significantly decreased after ZCCHC11 depletion. However, the impact of ZCCHC11 on cell cycle progression was suggested to be independent of its uridyl transferase activity, but required the N-terminal region of the protein. Though this finding is unexpected considering the requirement of ZCCHC11 catalytic activity in regulating histone mRNA and *let-7* amounts, these results supported the previous observation that the N-terminal region of ZCCHC11 is sufficient to confer its suppressive effect on NF-kappa-B (Minoda et al. 2006). ZCCHC11 might have a key role in gene regulation completely independent of its uridyl transferase activity. In order to understand the underlying biochemistry of such a different function, further studies are required.

The potential of ZCCHC11 to behave as an oncogene was supported by a second study. The study, which showed the difference between Lin28A and Lin28B in terms of their requirement for ZCCHC11 in pre-*let-7* regulation, also suggested that ZCCHC11 might be

a potential candidate for cancer therapy in Lin28A-expressing cells but not in those expressing Lin28B (Piskounova et al. 2011). Depletion of ZCCHC11 resulted in decreased invasiveness in Lin28A-expressing cell lines as well as decreased growth of tumours expressing Lin28A in mouse xenografts. Further evidence in favour of a role for ZCCHC11 in cancer came from clinical studies. A prognostic role for ZCCHC11 in a two-gene expression signature with E2F1 in breast cancer patients was identified. In other words, high expression of ZCCHC11 is associated with poor prognosis in patients only when the tumours also express average or high E2F1 levels (Hallett and Hassell 2011). Finally, ZCCHC11 levels were found to be higher in colorectal and liver cancer tissues in comparison with the adjacent non-tumour tissues (Fu et al. 2014). In summary, several lines of evidence suggest a role for ZCCHC11 in various cancers as a protein promoting cell proliferation, invasion and survival, and these features make ZCCHC11 a potential target for cancer therapy.

In addition to its comparatively well-studied role in cancer and inflammation, ZCCHC11 may play roles in the regulation of several other genes. Expression of mature *miR-1* in myotonic dystrophy was suggested to be decreased by a Lin28A-mediated uridylation of pre-*miR-1* by ZCCHC11 (Rau et al. 2011). Similar to its role in regulating mature *miR-26*, where the uridylation activity inhibits the function of mature miRNA by a currently unknown mechanism instead of causing its decay, it was recently suggested that ZCCHC11 knockout mice exhibited decreased length and decreased frequency of terminal uridylation of several miRNAs (Jones et al. 2012). The terminal uridylation of these miRNAs was proposed to inhibit their function in supressing IGF-1, which would explain decreased survival and growth after birth in ZCCHC11 knockout mice.

In comparison with its orthologue ZCCHC11, current understanding of ZCCHC6 function is very limited. Sharing extensive sequence similarity with ZCCHC11, ZCCHC6 has been found to have redundant functions in regulating pre-*let-7* levels (Thornton et al. 2012). Biochemical assays suggested that both ZCCHC6 and ZCCHC11 could uridylate RNA substrates, and that the uridylation activity of these proteins was enhanced by Lin28A. ZCCHC6 and ZCCHC11 were knocked down individually in embryonic stem cells. ZCCHC11 knock-down resulted in increased mature *let-7g* levels, whereas ZCCHC6 depletion did not alter *let-7g* expression. However, interestingly, knocking down ZCCHC6 in addition to ZCCHC11 caused an enhancement in the amount of mature *let-7g* up-regulation, suggesting a partial redundancy of function (Thornton et al. 2012). Finally, another study proposed that pre-*let-7* uridylation is more complicated. Group II pre-miRNAs, which have a one-nucleotide overhang, were found to require terminal monouridylation for further processing by Dicer. This was a Lin28A-independent activity, which involved ZCCHC6, and favoured *let-7* biogenesis in contrast to Lin28A-dependent oligouridylation (Heo et al. 2012). Group I miRNAs on the other hand have a two-nucleotide overhang and therefore do not require terminal monouridylation for Dicer processing. Fig 1.2. summarizes the role of Lin28A and ZCCHC11 in *let*-7 metabolism.

TABLE 1.1. The function, activity and subcellular localisation of human TUTases (Schmidt and Norbury 2010; Scott and Norbury 2013; Wyman et al. 2011; Knouf, Wyman, and Tewari 2013; Heo et al. 2012; Berndt et al. 2012).

TUTase	Activity	Localisation	Function
U6 TUTase	Uridylation Adenylation	Nucleolus	• Terminal uridylation and stabilisation of U6 snRNA • Potential role in miRNA regulation
PAPD4	Adenylation	Nucleus Cytoplasm	• Stabilization of *miR-122* and mRNAs • Translational activation of deadenylated mRNAs
MTPAP	Adenylation	Mitochondria	• Adenylation and hence either stabilization or destabilization of mitochondrial mRNAs • Potential role in miRNA modification
PAPD5	Adenylation	Nucleus	• Adenylation of abberant pre-rRNAs, targeting them for degradation by the exosome • 3' oligoadenylation of H/ACA box snoRNAs
POLS	Not known	Not known	• No known function
ZCCHC6	Uridylation	Cytoplasm	• Oligouridylation of pre-*let-7*, targeting it for degradation in the presence of Lin28a • Monouridylation of pre-*let-7,* promoting its processing by Dicer
ZCCHC11	Uridylation	Cytoplasm	• Oligouridylation of pre-*let-7*, targeting it for degradation in the presence of Lin28a • Monouridylation of pre-*let-7,* promoting its processing by Dicer • Uridylation and destabilization of replication-dependant histone mRNAs • Uridylation of pre-*miR-1,* targeting it for degradation in the presence of Lin28a • Uridylation and inhibition of the function of mature miRNAs

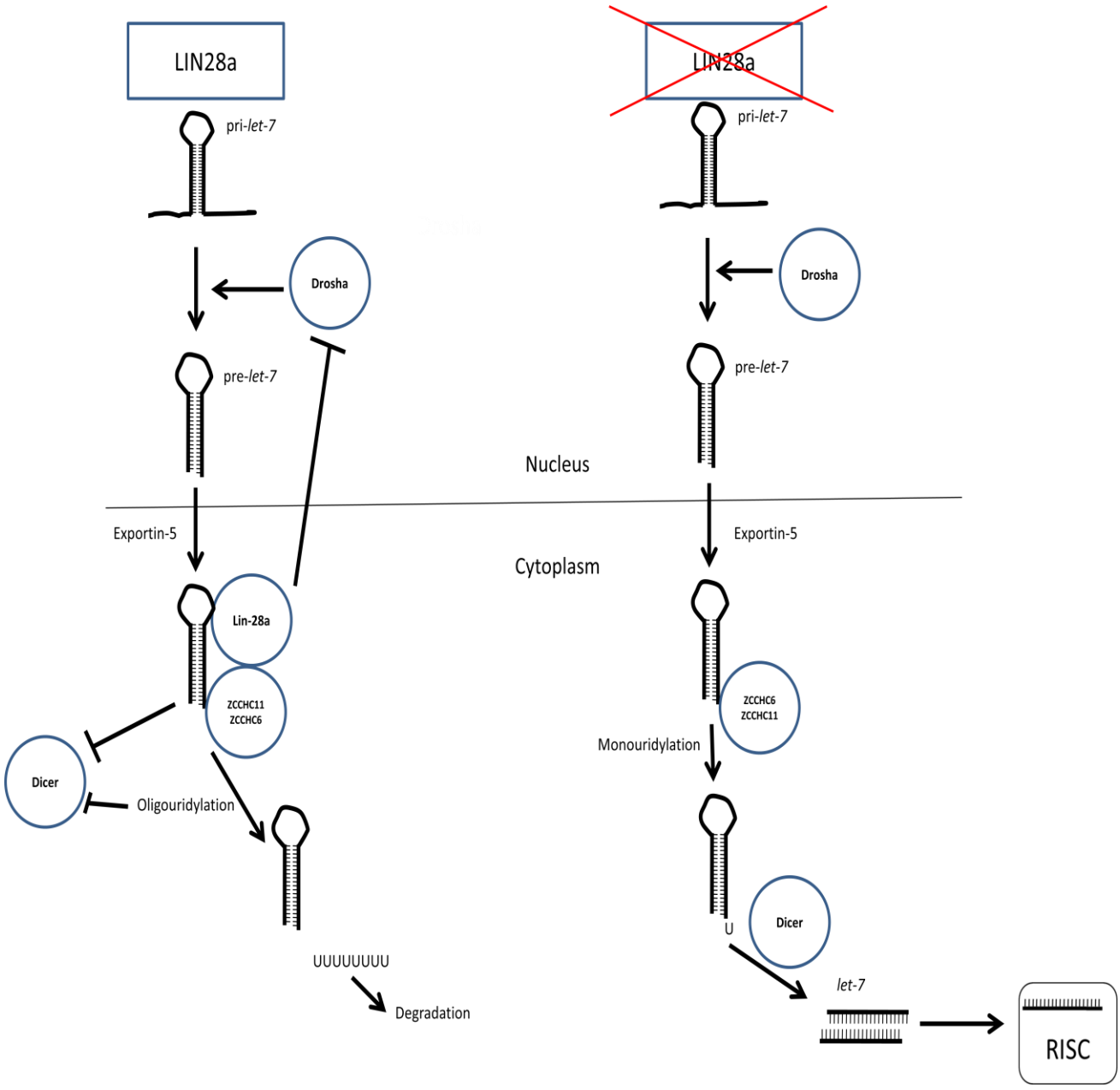


FIGURE 1.2. The role of Lin28A and ZCCHC11 in let-7 metabolism. In the presence of Lin28A, ZCCHC11 and also ZCCHC6 are recruited to oligouridylate pre-*let-7* which results in the inhibition of its further processing by Dicer and leads to its degradation. On the other hand, in the absence of Lin28A, ZCCHC6 and ZCCHC11 monouridylate pre-*let-7* and this is an important step in the maturation of *let-7* miRNA.

1.2. The importance of untranslated regions in the regulation of gene expression

The 5' and 3' untranslated regions (UTRs) of an mRNA may exert regulatory functions either by influencing mRNA stability or by affecting translational efficiency.

1.2.1. The 5'UTR and gene expression

The structure and specific sequence of the 5'UTR is often important in determining the translation efficiency of the mRNA. The 5'UTR may contain upstream open reading frames (uORFs) that are scanned and translated into short polypeptides before the authentic polypeptide product. Reinitiation of the translation machinery at the authentic AUG usually results in decreased translation relative to what would be seen in the absence of uORFs (Somers, Poyry, and Willis 2013; Wethmar, Smink, and Leutz 2010).

uORFs confer a stronger inhibitory effect on translation when present in multiple copies in a given 5'UTR and when further away from the cap (Calvo, Pagliarini, and Mootha 2009). However, the presence of uORFs in the 5'UTR does not necessarily imply a regulatory function, and in some cases they inhibit gene expression only slightly (Horvath et al. 1995; Lammich et al. 2004)). Regulation through uORFs is complex. If the ribosome recognizes and scans the uORF, it can then translate and dissociate, or may start translating and stall, thus blocking translation initiation by other ribosomes. It can also target the mRNA for decay, or continue scanning the transcript and reinitiate at the distal authentic AUG with low efficiency. Cellular stress decreases the translation of most mRNAs, though the translation of several transcripts increases under stress conditions. Due to the limiting availability of the ternary complex under stress conditions, the scanning of some uORFs, which normally interfere with the translation of

authentic peptides, is skipped, and hence the translation of certain mRNAs increases as this is the case for the mRNAs encoding the transcription factor Gcn4 in yeast and the activating transcription factor 4 (*ATF4*) in mammals (Barbosa, Peixeiro, and Romao 2013b). In line with the importance of uORFs as regulatory elements, polymorphisms and mutations in these elements in several genes result in various human phenotypes and diseases (Calvo, Pagliarini, and Mootha 2009; Barbosa, Peixeiro, and Romao 2013b).

Although regulation through uORFs is mainly driven by a decrease in translation efficiency of the authentic ORF, the presence of uORFs may also have a role in determining mRNA stability. Aberrant mRNAs containing premature translation termination codons are degraded by a cellular mechanism termed nonsense-mediated decay (NMD). mRNAs containing uORFs are also targets of NMD (Barbosa, Peixeiro, and Romao 2013a). The knockdown of hUPF1, an essential component of NMD, results in up-regulation of transcripts containing uORFs (Mendell et al. 2004). Therefore, stalling of ribosomes at uORF termination codons can also decrease gene expression by triggering NMD (Gaba, Jacobson, and Sachs 2005).

Finally, the secondary structure of the 5'UTR and its (G+C) content can also be important in determining the level of translation, as both increased hairpin stability and increased GC-richness decrease mRNA translation(Babendure et al. 2006).

1.2.2. 3'UTRs and regulation of gene expression

The mRNA 3'UTR is arguably the most extensively studied part of the transcript with regard to post-transcriptional regulation of gene expression. Most miRNAs exert their function through target sites in 3'UTRs. In addition, there are sites for alternative

polyadenylation in many primary transcripts, and regulation through alternative polyadenylation is of substantial importance in the molecular biology of many diseases including cancer. Finally, 3'UTR regions may contain *cis*-acting AU-Rich Elements (AREs), which have important roles in determining mRNA stability as well as translation efficiency. Indeed, AREs interact with RNA binding proteins (RBPs) that exert these regulatory functions on the transcript.

1.2.2.1. miRNA-mediated post-transcriptional regulation

miRNAs are approximately 22 nucleotides in length and their expression is usually tissue- and developmental stage-specific (Feinbaum and Ambros 1999; Reinhart et al. 2000; Lau et al. 2001). miRNAs are transcribed as longer transcripts and further processed to generate shorter mature forms. When transcribed by RNA polymerase II, primary transcripts are generally polyadenylated and capped. However, it has been reported that some miRNAs are instead transcribed by RNA polymerase III (Borchert, Lanier, and Davidson 2006).The primary transcript (pri-miRNA) is initially cleaved in the nucleus by Drosha, an RNAseIII type endonuclease, to form a 60-70 nucleotide long precursor miRNA (pre-miRNA). In order to perform this function, Drosha requires a cofactor called DGCR8 (or Pasha) and these two constitute the nuclear microprocessor complex (Gregory et al. 2004). Following nuclear processing, pre-miRNA is then exported via exportin-5 to the cytoplasm (Yi et al. 2003), where Dicer, another RNAseIII enzyme, cleaves it forming the 22 nucleotide mature miRNA (Finnegan and Pasquinelli 2013). Mature miRNAs regulate gene expression post-transcriptionally by interacting with the target mRNAs, mainly via their 3'UTRs. In plants, miRNA/mRNA interaction requires nearly perfect complementarity, however most metazoan miRNAs do not require

perfect complementarity (Filipowicz, Bhattacharyya, and Sonenberg 2008). Seed regions are short (~7 nucleotide) sequences in the 5' half of the miRNA, which are perfectly complementary with the target mRNA in metazoan miRNAs (Lewis, Burge, and Bartel 2005; Lewis et al. 2003; Filipowicz, Bhattacharyya, and Sonenberg 2008). Mature miRNAs are components of RNA-induced silencing complexes (RISC) and function as guide RNAs, which direct RISC to repress target mRNA expression (Pratt and MacRae 2009). The catalytic component of RISC is a protein called Argonaute (Ago), which, in plants, is guided by the miRNA, and cleaves the target mRNA endonucleolitically (Baumberger and Baulcombe 2005). In animals, however, miRNAs act mainly by promoting deadenylation of mRNAs, (Eulalio, Huntzinger, and Izaurralde 2008) and also by repressing their effective translation (Fabian, Sonenberg, and Filipowicz 2010). Ago proteins tethered artificially to the 3'UTR of target mRNAs suppress their translation without affecting the mRNA level, suggesting their involvement in miRNA-mediated translation inhibition (Pillai, Artus, and Filipowicz 2004). It was later suggested that miRNAs inhibit translation by interfering with the initiation step, and that the presence of 5' cap and 3' poly(A) structures in the target mRNA are both necessary for this inhibition (Humphreys et al. 2005). Ago2 binds to the 5'cap structure and was proposed to competitively inhibit interaction between the cap and eIF4E (Kiriakidou et al. 2007). eIF6, a protein that inhibits ribosome subunit joining, is also suggested to be important in miRNA-mediated repression, as eIF6-depleted cells have decreased ability to sustain post-transcriptional regulation by miRNAs (Chendrimada et al. 2007). It was also shown that miRNAs cosediment with polysomes, which may indicate a role in the inhibition of post-initiation steps, though the mechanism of regulation of these steps is still poorly understood(Janas et al. 2012). The extent of the overall contribution of translation

repression and mRNA decay to miRNA-mediated gene silencing has been studied. It was shown that the levels of mRNAs closely reflect protein expression of many miRNA targets in mammalian cells, indicating the predominance of destabilisation of mRNA transcripts in miRNA-mediated gene regulation (Guo et al. 2010).

Each miRNA may regulate multiple genes, while any given target mRNA may be regulated by more than one miRNA. The complicated interactions between miRNAs and mRNAs are tissue- and developmental time-specific, and these interactions also have clinical significance in many human diseases. The role of miRNAs in cancer is one of the most widely studied aspects of clinical miRNA research; miRNAs may have tumour suppressor or oncogenic roles in cancer biogenesis. *miR-15* and *miR-16* are either absent or down-regulated in most cases of chronic lymphocytic leukemia (CLL) (Calin et al. 2002). It has been found that *miR-15* and *miR-16* target the anti-apoptotic B-cell lymphoma protein (Bcl2), which is overexpressed in CLL (Cimmino et al. 2005). Similarly, down-regulation of *let-7* is a predictor of poor prognosis in many cancers (Takamizawa et al. 2004; Boyerinas et al. 2010a). The miRNAs functioning as tumour suppressors are thought to target mRNAs that favour cancer biogenesis, cell survival, or are anti-apoptotic, whilst miRNAs functioning as oncogenes target tumour supressors. Examples of the latter include the overexpression of *miR-372* and *miR-373* in human testicular germ cell tumours, and *miR-221*, *miR-222* and *miR-146* in papillary thyroid carcinoma (Zhang et al. 2007). Finally, it is important to note that alterations in miRNA expression may also lead to other diseases including those of inflammatory or neurodegenerative origin (Sonkoly and Pivarcsi 2009; Nelson, Wang, and Rajeev 2008).

1.2.2.2. AU-Rich Elements

The importance of certain elements in 3'UTRs for regulation of gene expression in response to environmental stimuli was initially discovered almost three decades ago (Treisman 1985). AREs were identified in the 3'UTRs of several mRNAs important in inflammation, including TNF mRNA (Caput et al. 1986). It was then revealed that the mRNAs encoding a number of proto-oncogenes, cytokines and transcription factors have AU-rich elements in their 3'UTRs that play regulatory roles in gene expression (Khabar 2005). Initially, AREs conferring instability to the mRNA were discovered (Shaw and Kamen 1986; Shyu, Belasco, and Greenberg 1991). Though the well-characterised function of AREs is to decrease the mRNA half-life, AREs may also positively regulate mRNA stability (Topisirovic et al. 2009; Fan and Steitz 1998). In addition, several genes contain AREs that exert their function through regulating translation either negatively (Qi et al. 2012) or positively (Mazan-Mamczarz et al. 2003).

AREs are usually 50-150 nucleotide long elements (Barreau, Paillard, and Osborne 2005). Historically, the AUUUA pentamer and general U-richness were thought to be the common components of a functional ARE. However, it was subsequently shown that these features were not sufficient for regulatory purposes in some genes (Chen and Shyu 1995). Some studies suggested that UUAUUUAUU or UUAUUUA(U/A)(U/A) nonamer motifs were the minimal functional *cis*-acting element, and that the number of copies of this element in the 3'UTR is a determining factor for the mRNA stability (Zubiaga, Belasco, and Greenberg 1995; Lagnado, Brown, and Goodall 1994). More recently, it has been shown that several other AU-rich elements are important as

recognition motifs for RBPs (Meisner et al. 2004; Anant and Davidson 2000). There is therefore no current consensus description of features common to all AREs.

AREs are *cis*-acting elements required for the binding of *trans*-acting proteins that have roles in gene expression regulation. AU-rich elements are recognized by RBPs, which regulate post-transcriptional stability and rate of translation. The ARE-specific interaction of RBPs with the 3'UTR may be important in gene regulation, and defects in these interactions may result in pathology (Khabar 2005). Regulation through the 3'UTR is not necessarily through interaction with a single RBP. On the contrary, many mRNAs containing AREs are regulated through multiple RBPs that may compete for binding to the 3'UTR. Although many RBPs bind 3'UTRs and regulate gene expression, only those which have been widely studied will be discussed in this chapter.

1.2.2.3. Regulatory RNA-binding proteins

1.2.2.3.1. AUF-1

The AU-rich element RNA binding factor 1 (AUF1) was initially characterised as an RNA-binding protein that mediates ARE-directed degradation of several mRNAs such as those of c-myc, c-fos and GM-CSF (Zhang et al. 1993; Brewer 1991). AUF1 is mainly nuclear, but it can shuttle between nucleus and cytoplasm to perform its function (Sarkar, Lu, and Schneider 2003). AUF1 mainly confers instability to its interacting mRNAs (Gratacos and Brewer 2010; Xu, Chen, and Shyu 2001). It has four different isoforms, which are generated by alternative splicing, and each isoform may have a different affinity for RNA targets (Wagner et al. 1998). For instance, several genes, such as *FGF9* and *COX-2*, are

regulated by the p42 isoform of AUF1 while the other isoforms have minimal or no effect on their regulation (Chen et al. 2010; Zhai et al. 2010).

1.2.2.3.2. Tristetraprolin

Tristetraprolin (TTP) is a 36 kDa protein, and as its name implies, it contains three repeats of tetraproline residues. It was initially identified as a gene whose expression is induced after stimulation of cells with insulin, growth factors, phorbol esters, or serum (DuBois et al. 1990; Lai, Stumpo, and Blackshear 1990). However, the physiological role of TTP as a regulatory protein was first suggested by the observation that TTP knockout mice exhibited a severe inflammatory syndrome including cachexia, dermatitis, arthritis, kidney involvement and autoantibodies against nuclear antigens. This phenotype is due to abnormally high levels of the proinflammatory cytokine tumour necrosis factor (TNF), as TTP-deficient mice became asymptomatic when treated with antibodies against TNF soon after birth, suggesting a negative regulation of TNF by TTP (Taylor et al. 1996). TTP binds to AREs in the 3'UTR of the mRNAs of TNFα and many other cytokines (Carballo, Lai, and Blackshear 1998, 2000; Van Tubergen et al. 2011). This binding confers a regulatory function on the target mRNAs, mainly decreasing their half-lives through promoting their deadenylation (Lai et al. 1999; Carballo, Lai, and Blackshear 2000). The targets of TTP are not limited to cytokine mRNAs. It also functions as a tumour suppressor (Ross, Brennan-Laun, and Wilson 2012). TTP was shown to inhibit tumour growth by suppressing IL-3 synthesis in tumours that express abnormally stable IL-3 mRNA, and are dependent on an IL-3 autocrine loop (Stoecklin et al. 2003). In addition, TTP was shown to decrease tumour angiogenesis by targeting and down-regulating vascular endothelial growth factor (VEGF) (Essafi-Benkhadir et al. 2007). Ectopic

overexpression of TTP resulted in decreased VEGF and IL-8 mRNA and protein levels in malignant glioma cells, and thereby inhibited tumour proliferation and increased apoptosis (Suswam et al. 2008). The role of TTP as a tumour suppressor has also been characterised in other tumours. In cervical squamous cell carcinomas, TTP immunoreactivity is strongly reduced in comparison with normal cervical tissue. TTP binds the mRNA encoding E6-associated protein and mediates its decay, thereby increasing p53 protein expression, and decreasing telomerase activity, which results in increased cellular senescence (Sanduja, Kaza, and Dixon 2009). Modulation of TTP expression in head and neck squamous cell carcinomas (HNSCC) resulted in differences in the expression of several genes prognostically important. TTP downregulation resulted in increased expression and secretion of IL-6, VEGF and prostaglandin E2 (PGE2). This regulation may be physiologically important, as levels of TTP expression are significantly lower in HNSCC compared to non-cancerous tissue samples (Van Tubergen et al. 2011). The level of TTP expression has prognostic value in breast cancer, such that tumours with lower TTP expression are more advanced, and are associated with reduced overall survival (Brennan et al. 2009). The role of TTP as a tumour suppressor has also been characterised in colon (Young et al. 2009) and liver cancer (Sohn et al. 2010). However, despite its well-characterised role as a tumour suppressor, TTP was also shown to downregulate the LATS2 tumour suppressor mRNA, and hence favour cell proliferation (Lee et al. 2010).

1.2.2.3.3. HuR

Human antigen R (HuR) is a widely expressed member of the embryonic lethal abnormal vision (ELAV)-like family of proteins, whose other members are known to be expressed

solely in neuronal tissues. HuR is an RBP which binds AREs and regulates various mRNAs encoding growth factors, cytokines, oncogenes and tumour supressors (Wang, Guo, et al. 2013). Though the interactions of HuR with AREs in 3'UTRs are well characterised, HuR can also bind to the 5'UTRs of several mRNAs (Kullmann et al. 2002; Yeh et al. 2008). HuR is well known for stabilising its target mRNAs. The expression of several genes important in cell cycle regulation is regulated by HuR. Cyclin A and B1 mRNAs are stabilised by HuR upon entry into S-phase, due to the relocalisation of HuR from the nucleus to the cytoplasm (Wang et al. 2000). Cyclin E1 overexpression in breast cancers is also mediated at least in part by HuR (Guo and Hartley 2006). HuR regulates cell survival by favouring the expression of anti-apoptotic proteins while supressing that of pro-apoptotic regulators. HuR binds the 3'UTR of anti-apoptotic Sirtuin 1 mRNA, and increases its expression by stabilising the transcript (Abdelmohsen et al. 2007). Translation of prothymosin α, an inhibitor of the apoptosome, is also increased by HuR (Lal et al. 2005). HuR regulates BCL2, another important anti-apoptotic molecule, both through stabilising and increasing translation of its mRNA (Ishimaru et al. 2009). On the other hand, the expression of MYC, a potentially pro-apoptotic transcription factor, is inhibited by HuR at both mRNA and protein levels, and this inhibition is dependent upon recruitment of *let-7* by HuR (Kim et al. 2009). Hypoxia-inducible factor 1-alpha (HIF-1α) is an important molecule in cancer cell survival, especially under hypoxic conditions. HuR was shown to interact with both the 3'UTR and the 5'UTR of its mRNA, and increase its expression (Galban et al. 2008; Sheflin, Zou, and Spaulding 2004). Expression of many other genes that favour cancer cell survival, tumour angiogenesis or proliferation is regulated positively by HuR, through increasing either their mRNA stability or their translation (Abdelmohsen and Gorospe 2010). In contrast, there is at least one instance

in which HuR has an anti-tumour activity by enhancing the translation of the tumour suppressor p53 in human colorectal carcinoma cells (Mazan-Mamczarz et al. 2003).

HuR may also confer reduced expression on its targets. BRCA1 is regulated post-transcriptionally through its 3'UTR, and overexpression of HuR results in decreased expression of BRCA1 protein (Saunus et al. 2007; Saunus et al. 2008). CD9 mRNA and protein levels are also negatively regulated by overexpression of HuR in the oestrogen receptor-negative MD-MB-231 cell line (Calaluce et al. 2010). In addition, decay accelerating factor (DAF) mRNA is also bound by HuR, which decreases its expression through destabilising the transcript (Gray, Hughes, and van den Berg 2010).

HuR is predominantly nuclear, however it can shuttle between nucleus and cytoplasm to perform its function (Abdelmohsen and Gorospe 2010). Though it was shown to enhance the translation of p53 in one study, HuR is a molecule whose excess expression correlates with tumour progression in clinical studies. Cytoplasmic immunoreactivity of HuR has been found to be significantly increased in the sequence of progression from adenoma to carcinoma in familial adenomatous polyposis patients (Brosens et al. 2008). Expression of HuR, particularly in the cytoplasm, but also to some extent in the nucleus, is linked with poor prognosis in non-small cell lung cancer (NSCLC), correlating with increased tumour progression and decreased patient survival (Tanaka et al. 2006). Cytoplasmic HuR expression is clinically important in bladder cancer patients, as it is associated with increased proliferation, microvessel density and poor prognosis (Miyata et al. 2013). Breast cancer is another cancer where the expression of HuR in the cytoplasm has prognostic value (Heinonen et al. 2007; Wang, Li, et al. 2013). Taking the potential clinical importance of HuR in cancer into consideration, the effects of

downregulation of HuR in cancer cells have been investigated. Knocking down HuR results in decreased invasion and motility potential of oral cancer cells (Kakuguchi et al. 2010), decreased cell proliferation in mammary epithelial cells (Yan et al. 2012), as well as reduced cell growth and invasion potential in breast cancer cell lines (Yuan et al. 2011). Finally, HuR knockdown results in increased chemosensitivity to tamoxifen in both tamoxifen-sensitive and resistant breast cancer cell lines (MCF-7 and BT474 respectively), while overexpressing HuR confers chemoresistance to this drug in MCF7 (Hostetter et al. 2008).

1.2.2.3.4. KH-type splicing regulatory protein (KSRP)

KSRP is a single-stranded nucleic acid binding protein that regulates RNA stability in addition to having roles in the regulation of transcription and splicing (Gherzi et al. 2010). KSRP has been shown to bind and regulate many mRNAs. iNOS mRNA is one target to which KSRP binds, decreasing its stability. As discussed previously, some mRNA 3'UTRs bind multiple RBPs, which compete for binding, and usually have opposing effects on the regulation of the gene. iNOS mRNA is capable of binding to both HuR and KSRP. KSRP binding to an ARE in the 3'UTR confers decreased stability to iNOS mRNA while the binding of HuR to the same ARE stabilises it. Interestingly, TTP is also involved in the regulation of iNOS, despite not binding directly to its 3'UTR. After cytokine treatment, TTP interacts more strongly with KSRP, causing its dislocation and favouring the binding of the competing RBP, HuR, to the iNOS mRNA (Rodriguez-Pascual et al. 2000; Linker et al. 2005). Pitx2, a transcription factor, is also regulated post-transcriptionally via KSRP, TTP and HuR. The Wnt/beta-catenin pathway confers increased stability on Pitx2 mRNA by decreasing its interaction with destabilising

proteins KSRP and TTP, and increasing its interaction with the stabilising HuR (Briata et al. 2003). The competition between HuR and KSRP in interacting with their common targets is usually dependent upon a signal. A further example of this is the regulation of IL-8 mRNA, where HuR binding strongly overcomes KSRP binding when the cell is stimulated with IL-1β (Suswam et al. 2005). Interestingly, IL-1α and IL-6 are regulated by KSRP. This regulation requires the interaction between KSRP and the 3'UTRs of these genes, and results in translational repression. In addition, IL-1 interferes with the ability of KSRP to regulate IL-6 (Dhamija et al. 2011). Finally, KSRP and its variants destabilise the mRNAs encoding phosphoglycerate kinase 2, parathyroid hormone, LDL receptor and many others (Xu, McCarrey, and Hecht 2008; Nechama et al. 2009; Li et al. 2009; Briata et al. 2013). KSRP also regulates miRNAs (discussed in section 1.2.2.5.).

1.2.2.3.5. Butyrate response factor 1 (BRF1)

BRF1 (also called ZFP36L1) is a member of the TIS11 family to which TTP also belongs (Sanduja, Blanco, and Dixon 2011). BRF1 is essential for normal development, and mouse BRF1 knockouts display embryonic lethality (Stumpo et al. 2004). BRF1 is an AU-rich element binding protein (AUBP), which was initially shown to bind VEGF mRNA, and to regulate its expression negatively under normal circumstances and after ACTH stimulation (Ciais et al. 2004). BRF1 null mouse embryonic fibroblasts were subsequently shown to have increased VEGF expression, mainly attributable to post-translational regulation under normoxic conditions, but also involving increased mRNA levels under hypoxic conditions (Bell et al. 2006). Interestingly, the Von Hippel-Lindau tumour suppressor protein (VHL), a well-known regulator of VEGF expression, performs its function in part by regulating BRF1 expression. However, VHL-mediated regulation of

BRF1 expression is complicated. VHL negatively regulates BRF1 expression through a posttranscriptional mechanism possibly involving *miR-29b* under normoxic conditions, but surprisingly it increases the stability of BRF1 mRNA, and hence positively regulates its expression under hypoxic conditions (Sinha et al. 2009). In addition to negatively regulating a growth factor, VEGF, BRF1 also negatively regulates an anti-apoptotic gene, human inhibitor of apoptosis protein-2 (cIAP2) through its 3'UTR in human HNSCC cell lines, in which BRF1 expression is associated with cisplatin sensitivity. Cisplatin-sensitive cells express more BRF1 than cell lines that are cisplatin-resistant (Lee et al. 2005).

1.2.2.4. Mechanism of regulation by ARE-Binding Proteins

AU-Rich Element Binding Proteins (AUBPs) are well known for recognizing and binding to AU-rich elements in 3'UTRs, though these proteins may also interact with 5'UTRs or even protein coding regions (Prechtel et al. 2006; Meng et al. 2005). ARE-mediated regulation of genes may involve modification of RNA decay and/or translation rates. Once an AUBP recognizes and binds an ARE, it may lead to rapid degradation of the target mRNA via the recruitment of deadenylases. c-Fos was one of the first genes in which AREs and instability elements in the coding region were shown to promote deadenylation of the message (Shyu, Belasco, and Greenberg 1991). One of the most widely studied destabilising AUBPs, TTP, was shown to induce mRNA deadenylation. TTP interacts with the ARE and promotes deadenylation by PARN (Lai, Kennington, and Blackshear 2003). On the other hand, ARE-binding proteins TTP and BRF1 were found to exist in a complex with the decapping machinery, and overexpression of hDcp2, the catalytic subunit of the decapping complex, increased ARE-mediated mRNA decay. The same study also revealed an interaction of TTP and BRF1 with 5'-3'exonuclease hXrn1 and deadenylase

hCcr4 (Lykke-Andersen and Wagner 2005). In addition, Not1, which is part of the Ccr4-Not complex, interacts with TTP, and is required for TTP-mediated mRNA decay (Sandler et al. 2011). KSRP was found to interact with PARN and the exosome complex. Furthermore, degradation of mRNAs mediated by KSRP requires the presence of Xrn1, Dcp2 and PARN, as well as exosome components such as RRP40 and MTR3 (Gherzi et al. 2004; Chou et al. 2006). Exosome-mediated decay of ARE-containing transcripts requires their recognition by AUBPs and the subsequent recruitment of the exosome complex (Chen et al. 2001). Interestingly, AUBPs such as TIA-1 are components of stress granules, and other AUBPs such as HuR and TTP can be recruited to stress granules and p-bodies, both of which are important subcellular structures in determining the fate of RNAs (Barreau, Paillard, and Osborne 2005).

miRNAs are also involved in ARE-mediated RNA decay. It was shown that TTP and *miR-16* are functionally interdependent for the decay of RNAs containing ARE (Jing et al. 2005). As discussed above, the requirement of *let-7* miRNA for HuR to negatively regulate c-Myc expression is another example for the involvement of miRNAs in AUBP-conferred regulation of gene expression (Kim et al. 2009). On the other hand, the role of HuR in relocation of CAT-1 mRNA from p-bodies to polysomes, where it is translationally active, can be explained by HuR-mediated derepression of *miR-122* suppression (Bhattacharyya et al. 2006). Though there are currently few examples where AUBPs promote or prevent miRNA-mediated gene silencing, considering the complex interaction of *cis*-acting elements in the 3'UTR with *trans*-acting factors such as AUBPs and miRNAs, it would not be surprising to see more examples of the involvement of miRNAs in ARE-mediated decay in the future.

Despite many studies focusing on molecular mechanisms of AUBP-mediated increase in RNA turnover, it is relatively poorly understood how AUBPs, especially HuR, positively affect mRNA half-life. The role of HuR in relieving *miR-122* suppression (Bhattacharyya et al. 2006) might be an example of its role as a positive regulatory element by preventing miRNA-mediated gene silencing. In fact, many of the known targets of HuR are also known to interact with other AUBPs that promote RNA decay. HuR is therefore believed to compete with negatively regulating AUBPs to stabilise its target transcripts (Yuan et al. 2010).

AREs and AUBPs not only regulate mRNA stability, but also repress or induce the translation of mRNAs. TIA-1 represses the translation of TNF-α and COX-2 and, though the mechanism of this repression is not fully clear, TIA-1 may exert this function through the promotion of stress granule assembly (Espel 2005). In addition to the previously discussed role of HuR in derepression of translation through miRNAs, it was also suggested that HuR increases translation through a mechanism that similarly results in derepression. However this mechanism involves lincRNA-p21, a long non-coding RNA, rather than miRNA. HuR mediates silencing of lincRNA-p21, which normally reduces the translation of several HuR targets (Yoon et al. 2012a). Tristetraprolin may also inhibit the polysomal association of its targets in addition to its well-known destabilising function. The inhibitory function of TTP on the ARE-3'UTR mRNAs requires a translation repressor called RCK (Qi et al. 2012).

1.2.2.5. Regulation of *let-7* biogenesis by RNA-binding proteins

Some of the RNA-binding proteins discussed above are also involved in regulating miRNA biogenesis, either by binding and regulating the precursor or mature forms of the

miRNAs, or indirectly, through affecting the expression of genes with roles in biogenesis or post-transcriptional regulation of miRNAs. *Let-7* biogenesis is regulated by various RBPs through direct interaction with the miRNA, but also indirectly. TTP regulates *let-7* expression. However, this is not at the transcriptional level, as pri-*let-7* and pre-*let-7* are not affected by the level of TTP in the cell. TTP downregulates Lin28A, and hence functions as a posttranscriptional regulator in *let-7* biogenesis. TTP binds the 3'UTR of the Lin28A mRNA and destabilises it. Many human cancer cell lines show inverse correlation between TTP and Lin28A expression (Kim et al. 2012). More recently, it has also been shown that TTP is a target of p53, and treating cells with doxorubicin, a DNA damaging agent, resulted in increased *let-7* levels by a p53-mediated pathway. Therefore, p53 was shown to be a key regulator of the previously identified TTP/Lin28A/*let-7* pathway (Lee et al. 2013). On the other hand, RNA binding protein KSRP is also a component of the Dicer complex, and interacts with pre-*let-7a-1*. KSRP favours *let-7* maturation, and down-regulation of KSRP strongly inhibits the interaction of *let-7* precursors with Drosha and Dicer (Trabucchi et al. 2009). The KH3 domain of KSRP binds to GGG triplets on pri-*let-7*, and is proposed to compete for binding to pri-*let-7* with hnRNP A1, another RNA-binding protein, which negatively regulates *let-7* processing (Trabucchi et al. 2009; Michlewski and Caceres 2010). On the other hand, HuR was recently shown to down-regulate c-Myc expression through an interdependent mechanism with *let-7*. HuR recruits *let-7* loaded RISC complex to *MYC* mRNA in order to reduce its mRNA levels as well as translation. Interplay between *let-7* and HuR functions is involved in the reduction of *MYC* expression (Kim et al. 2009).

1.2.2.6. Polyadenylation and alternative polyadenylation

In eukaryotes, pre-mRNAs, with the exception of histone mRNAs, acquire poly(A) tails following endonucleolytic cleavage to generate their mature 3' ends. Polyadenylation is vitally important in the life of an mRNA. Firstly, transcription and polyadenylation are coupled, and the presence of a poly(A) signal is important for proper transcription termination (Wilusz, Wormington, and Peltz 2001; Mandel, Bai, and Tong 2008). In addition, it is necessary for the mRNA export from the nucleus to the cytoplasm (Huang and Carmichael 1996), and as discussed previously, the poly(A) tail binds to PABP, which interacts with the 5' cap structure in order to circularise the transcript for efficient translation. Finally, PABP prevents 3' exonucleolytic degradation, thereby increasing the stability of the transcript. The processing of mRNA 3' ends requires a complex machinery, which contains many proteins including cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I (CFIm), and cleavage factor II (CFIIm), poly(A) polymerase (PAP), PABP, symplekin and the CTD domain of PolII, most of which are required for the cleavage reaction (Fig. 1.3) (Mandel, Bai, and Tong 2008). On the other hand, polyadenylation itself can be performed in vitro by CPSF, PAP and PABP (Zhao, Hyman, and Moore 1999). These *trans*-acting protein factors recognize and interact with certain *cis*-acting elements on mRNA 3' ends to determine and process the cleavage and polyadenylation site. In mammals, the polyadenylation signal is usually AAUAAA, while a relatively small proportion of transcripts instead have AUUAAA, and several other signal sequences also exist (Beaudoing et al. 2000). The cleavage site is found 10-30 nt downstream of the polyadenylation signal (Millevoi and Vagner 2010) and the length of this interval is conserved. When the nucleotides between these two sites are deleted, the mRNA is

polyadenylated at a new site that will preserve the length of this interval (Fitzgerald and Shenk 1981). In addition to the polyadenylation signal and the cleavage site, there is also a less conserved downstream element, which is usually GU-rich and approximately 10 nucleotides downstream from the cleavage site. Finally, an auxiliary element is often found upstream of the polyadenylation signal and enhances the efficiency of cleavage and polyadenylation (Mandel, Bai, and Tong 2008).

Cleavage and polyadenylation of an mRNA do not necessarily occur at a single position, and for a significant proportion of mammalian genes, mature mRNAs differ in length due to the engagement of alternative cleavage and polyadenylation sites (Tian et al. 2005). Regulation of genes post-transcriptionally through alternative polyadenylation is a widely used and important mechanism, considering that the 3'UTR is a dock for multiple *trans*-acting regulatory elements, and differences in the 3'UTR length may result in differential binding of some of the elements. Alternative polyadenylation was found to have important roles in major cellular events such as proliferation, differentiation, transformation and development (Akman and Erson-Bensan 2014). Longer transcripts usually contain more *cis*-acting elements such as miRNA binding sites or AU-rich elements recognized by regulatory AUBPs (Lutz and Moreira 2011). Proliferating cells in many cases express mRNAs that preferentially use upstream polyadenylation sites, thus resulting in expression of mRNAs with fewer miRNA target sites (Sandberg et al. 2008). Cyclin D1 mRNA, when expressed as a shorter isoform lacks the negative regulatory elements towards the 3' end, resulting in increased mRNA half-life and expression of cyclin D1, driving cell proliferation (Wiestner et al. 2007; Rosenwald et al. 2003). It was later revealed that mRNAs in cancer cells are often alternatively polyadenylated. This

phenomenon results in the expression of shorter transcripts escaping miRNA-mediated repression, and thereby activating the expression of genes that are important in oncogenic transformation (Mayr and Bartel 2009).

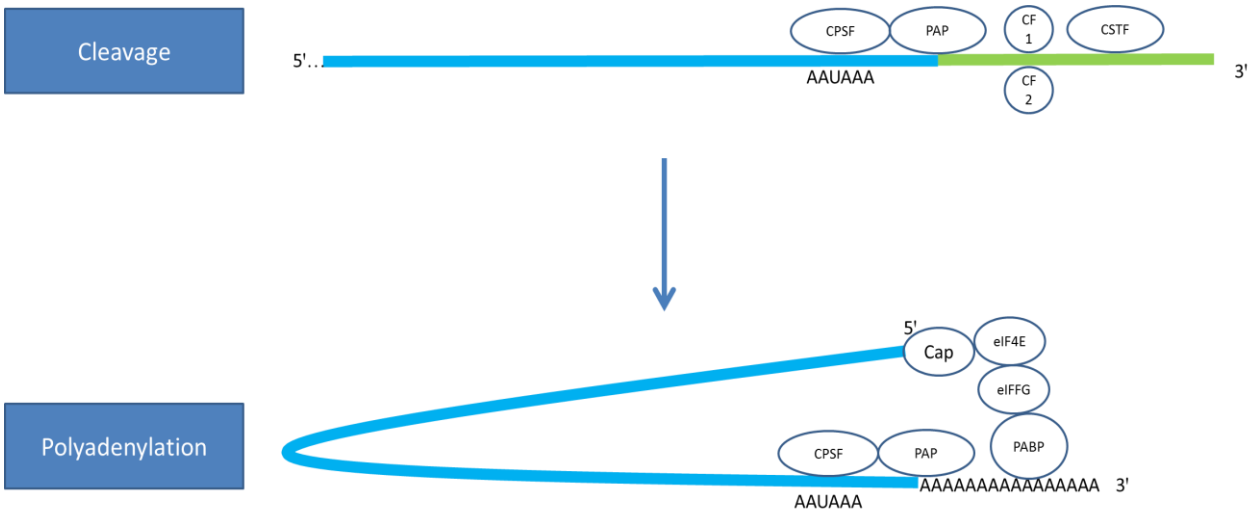


FIGURE 1.3. Schematic representation of cleavage and polyadenylation of mRNAs.
Several proteins are used for cleavage and polyadenylation processes. The cleavage site is 10-30 nucleotides downstream of the polyadenylation signal. After the cleavage, polyadenylation occurs. mRNA is then circularised through the interaction of PABP and eIF4G. Circularisation protects the mRNA from exonucleases.

1.3. Subcellular localisation of mRNA decay: p-bodies and stress granules

mRNA decay rates are regulated at multiple steps, as discussed in the previous sections. The translation and degradation machineries both have the mRNA as substrate, therefore initiation of translation and degradation are in a competition. External stimuli, as well as internal factors such as *cis*-acting elements and interacting *trans*-acting

factors, determine the half-life and translatability of an mRNA. Eukaryotic cells possess mRNP foci, called stress granules and P-bodies, where mRNA fate is thought to be determined. In these bodies mRNAs may be stored, directed to active translation or degraded. While stress granules, as the name implies, assemble in response to stress conditions, P-bodies also exist in unstressed cells. However, they may also form in response to cellular stress. Finally, exosomes are protein complexes that mediate 3' to 5' mRNA degradation.

1.3.1. Stress granules

Stress granules are non-membranous cytoplasmic structures assembled upon cellular stress, and comprise an aggregation of stalled translational preinitiation complexes. Adaptation to stress conditions is thought to involve re-prioritisation of the mRNAs translated by the cell. Stress conditions may therefore result in redistribution of mRNAs from polysomes, where they are translationally active, to stress granules. The key step in the formation of stress granules and translational arrest is common: the phosphorylation of translation initiation factor eIF2α, a mechanism that inhibits initiation of translation by decreasing the availability of ternary complex (Kedersha et al. 2005). Furthermore, destabilising or stabilising polysome formation results respectively in formation and dissolution of stress granules; this observation also suggests that there is an equilibrium and a close functional relationship between these structures in cells (Kedersha et al. 2000). Various environmental conditions, including oxidative stress, heat shock, hyperosmolarity, viral infections, UV radiation and drugs inhibiting translation cause stress granule formation. However, the components of stress granules are not necessarily constant and the presence of specific proteins within these structures may

be dependent on the type of stress inducing stress granule formation. For example, HSP27, a heat shock-induced stress granule constituent, is not a component of stress granules formed under oxidative stress (Kedersha et al. 1999). Similarly, in yeast, sodium azide (NaN_3)-induced stress granules differ in their protein composition from those induced by glucose deprivation (Buchan, Yoon, and Parker 2011).

Prion-like aggregation of TIA-1, a protein that binds to the 3'UTR of mRNAs and regulates their translation, has been suggested to be essential for spontaneous stress granule formation (Gilks et al. 2004). It was proposed that TIA-1 and TIA-1 related protein function as downstream mediators of eIF2α phosphorylation in the assembly of stress granules (Kedersha et al. 1999). TIA-1 was thought to be a part of dynamic structure of stress granules where components of the stress granules move in and out of these structures. On the other hand, TTP, which is also a component of stress granules, is excluded from stress granules by MK2-induced phosphorylation under arsenite stress, but not under energy deprivation by FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) induction (Stoecklin et al. 2004). Hence, posttranslational modification of RNA-binding protein components of stress granules may control mRNA traffic through stress granules and the dynamic nature of these foci.

It is not currently possible to describe a generally applicable role of stress granules in gene expression. They might function to stabilise or destabilise certain mRNAs or affect their translation. AUBPs such as HuR, TTP, BRF1, TTP, TIA-1 and many other proteins may be components of stress granules, each of which may be stabilising or destabilising. Several studies suggest the involvement of stress granules and p-bodies (see below) in ARE-AUBP-mediated gene regulation (von Roretz et al. 2011).

1.3.2. p-bodies

p-bodies, which contain many components of the mRNA turnover machinery, are distinct subcellular structures also thought to be important in determining mRNA destiny in the cell. Their discovery may be traced to a study showing that Xrn1, an important cytoplasmic 5' to 3' exoribonuclease, is localised in the cytoplasm to prominent, discrete foci (Bashkirov et al. 1997). Later studies suggested that the decapping enzyme Dcp2 and GW182, a protein later shown to have an important function in miRNA-mediated gene silencing (Braun, Huntzinger, and Izaurralde 2013), colocalised with Xrn1 in these foci. This suggests that these foci play a significant role in mRNA turnover (Eulalio, Behm-Ansmant, and Izaurralde 2007).

The bidirectionality of mRNA decay is ensured by distinct degradation machineries with different protein components. Protein complexes called exosomes, which will be discussed later in this chapter, play a major part in degradation of mRNAs from the 3' end. However, p-bodies contain components required for decapping and the subsequent 5' to 3' degradation pathway (Fillman and Lykke-Andersen 2005). In other words, p-bodies are sites for 5' to 3' decay of translationally repressed mRNAs. However, p-bodies, similar to stress granules, are not solely sites of mRNA decay. Translational repression may also occur in p-bodies, where repressors of translation such as Rap55, Dhhp1 and its orthologues are present. The mRNAs kept transiently in these structures can also be directed back to polysomes for active translation (Parker and Sheth 2007). Hence, although the specific presence of 5' to 3' decay machinery ascribes a unidirectional role in mRNA turnover to p-bodies, these subcellular structures, similar to stress granules, have important roles in storing mRNAs transiently or

repressing their translation. ARE-mediated gene regulation is also an aspect of p-body function. In addition, components of p-bodies may vary according to cell type or conditions such as cellular stress (Parker and Sheth 2007) and, p-body formation can be induced specifically under stress conditions (Iwaki and Izawa 2012). Many components of stress granules and p-bodies are common (Fig. 1.4), and these two structures are not isolated discrete structures; on the contrary, they are functionally and physically interrelated (Kedersha et al. 2005). The study showing these interactions proposed a model in which stress granules function as 'triage' centres, where mRNAs disassociated from polysomes are remodelled and directed to p-bodies, where they are degraded. However, the interaction between these two subcellular structures - their 'job-sharing' in mRNA regulation - is not yet fully understood and requires further investigation.

To date, there is no direct evidence that terminal uridyl transferases reside in or relocalise to stress granules and/or p-bodies. The only indirect connection between gene regulation by terminal uridylation and these subcellular structures comes from a study showing that Lin28A, the protein that recruits TUTases including ZCCHC11 to pre-*let-7*, localises to stress granules under cellular stress (Balzer and Moss 2007; Polesskaya et al. 2007) and also to p-bodies (Balzer and Moss 2007). However, to my knowledge no study has so far revealed the biological significance of this localisation of Lin28A, or any other protein that is involved in terminal uridylation, to p-bodies or stress granules in mammalian cells.

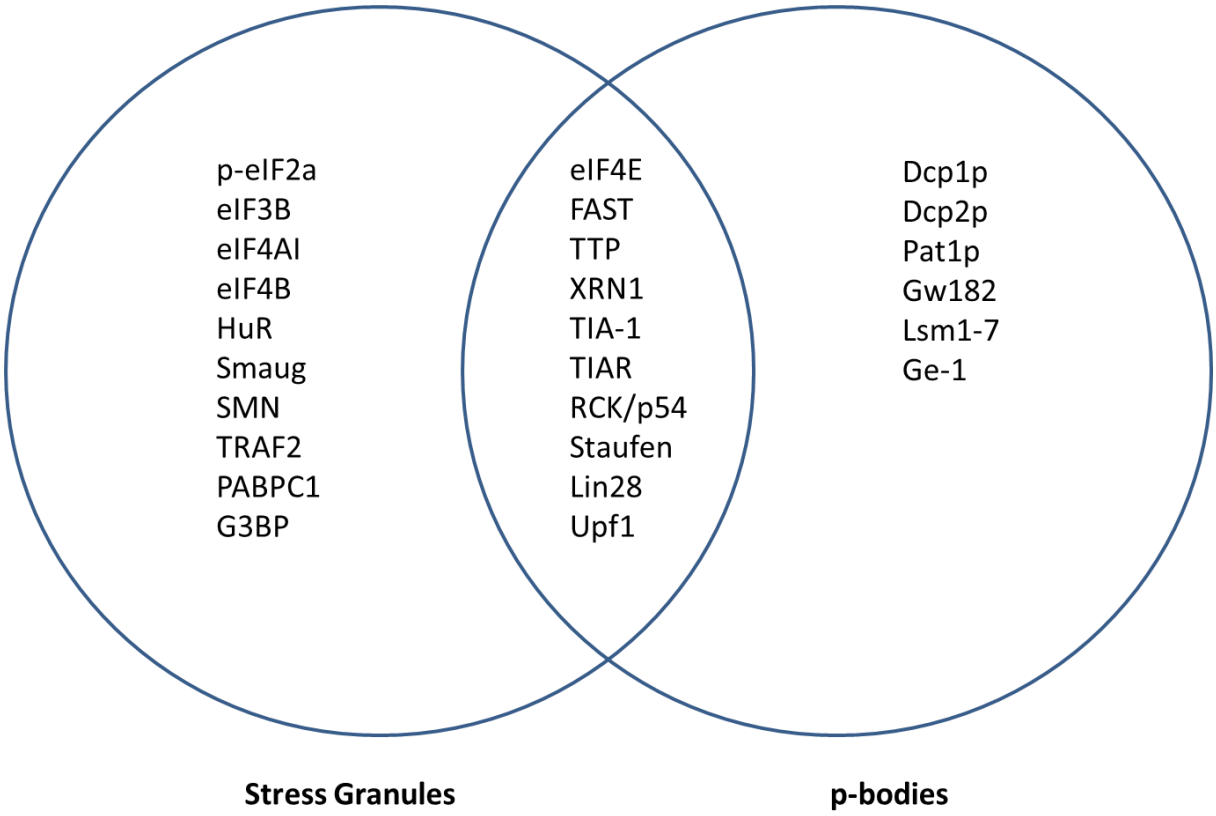


FIGURE 1.4. Key proteins of stress granules and p-bodies.
Stress granules and p-bodies are dynamic components that share protein components (Eulalio, Behm-Ansmant, and Izaurralde 2007; Parker and Sheth 2007; Brown et al. 2011).

1.3.3. Exosome complex

The exosome is a protein complex responsible for the 3' to 5' decay of mRNA and it is functional both in the nucleus and in the cytoplasm. In addition, the nuclear exosome plays an important role in degrading precursor mRNAs, pre-tRNAs and pre-rRNAs, while cytoplasmic exosomes are only known for mature mRNA turnover (Table 1.2) (Houseley, LaCava, and Tollervey 2006). Interestingly, although the role of the exosome in mRNA turnover is arguably better studied, it was identified as a multienzyme ribonuclease

complex required for 5.8 rRNA maturation (Mitchell et al. 1997). The exosome structure consists of six subunits, and in addition to these proteins, which form a ring structure, the complex contains three proteins that have RNA binding domains (Lebreton and Seraphin 2008). The exosome also contains proteins such as Dis3 and Rrp6 which are the catalytically active nucleases of the complex (Schneider and Tollervey 2013). In addition, several proteins including AUBPs interact with exosome complexes, and the exosome plays a crucial role in the turnover of many mRNAs through the involvement of AREs, as discussed in previous sections.

TABLE 1.2. The key features of nuclear and cytoplasmic exosomes (Lykke-Andersen, Brodersen, and Jensen 2009; Houseley, LaCava, and Tollervey 2006).

Nuclear Exosome	**Cytoplasmic Exosome**
• In addition to common components with the cytoplasmic exosome, contains Rrp6 and Rrp47 • has a role in the processing of snRNAs, snoRNAs, 5.8S rRNA • has a role in the degradation of aberrant rRNAs, tRNAs, snRNAs, snoRNAs, mRNAs • TRAMP and Nrd1 recruit it to aberrant RNAs	• In addition to common components with the cytoplasmic exosome, contains Ski7 • has a role in the degradation of aberrant mRNAs • is involved in nonsense-mediated decay and non-stop decay • has a role in the degradation of replication dependent histone mRNAs • is important in the degradation of ARE-containing mRNAs through the involvement of AUBPs

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

1 x TBE

10 mM Tris, 10 mM H_3BO_3, 2 mM EDTA pH 8.0

6 x DNA loading buffer

30% sucrose, 100 mM EDTA pH 8.0, 0.01% bromophenol blue

SOB

2% Bactotryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, autoclaved

TSB

10% PEG (6000), 5% DMSO, 20 mM $MgCl_2$, in LB

5 x KCM

0.5 M KCl, 0.15 M $CaCl_2$, 0.25 M $MgCl_2$, filter-sterilised

RIPA buffer

50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X100, 0.1% SDS, 0.5% deoxycholic acid, 10% glycerol

add to 1 ml buffer before use: 1 mM DTT, 2 mM PMSF, 1 mM benzamidine, 20 µl of one

proteinase inhibitor tablet (CompleteTM; Roche) dissolved in 1ml water

1x Tris-buffered saline (TBS)

50 mM Tris-HCl, pH 7.5

150 mM NaCl

4x Sample buffer

200 mM Tris-HCl pH6.8, 40% glycerol, 8% SDS, 4% β-mercapto ethanol, 0.02%

bromophenol blue

Separating gel

375 mM Tris-HCl pH 8.8, 0.1% SDS, 6-15% acrylamide:bisacrylamide (37.5:1, Fisher Scientific), 0.03% APS, 0.2% TEMED

Stacking gel

125 mM Tris-HCl pH 6.8, 0.1% SDS, 6% acrylamide:bisacrylamide (37.5:1, Fisher Scientific),

0.03% APS, 0.2% TEMED

SDS running buffer

25 mM Tris pH 8.3, 0.1% SDS, 190 mM glycine

PBS

0.14 mM NaCl, 3.4 µM KCl, 10 µM Na2HPO4, 1.8 µM KH_2HPO_4

HBS

40 mM HEPES pH 7.0, 200 mM NaCl

Elution buffer

20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 25 mM glutathione

2.1.2. Plasmids

In order to perform eGFP reporter assays discussed in this study, the pcDNA 3.1+ expression vector (Invitrogen) was modified. The eGFP open reading frame was subcloned into this plasmid using *Bam*HI and *Eco*RI. 3'UTR sequences were inserted at the 3' end of eGFP using the *Eco*RI and *Xba*I restriction sites (Chapter 3 and 5). These sequences include the 3'UTR of β-Actin or the 3'UTR of ZCCHC11 as wild-type, the full length ZCCHC11 3'UTR bearing individual mutations of AREs, or the fragments of the 3'UTR of ZCCHC11. The 5'UTRs of ZCCHC11 identified by cRACE were amplified from genomic DNA using two sets of primers in order to clone the 5'UTR without the intron present in the DNA. A first PCR product was obtained for each 5'UTR with a primer

including a *Hind*III restriction site and a sequence specific of the start of the 5'UTR and a reverse primer ending just before the intron. A second PCR product was amplified with a primer starting just after the intron and a reverse primer ending just before the AUG and including a *Bam*HI restriction site. The PCR products were digested *Hind*III and *Bam*HI respectively. The second PCR product was subsequently phosphorylated at the 5' end using T4 Polynucleotide kinase (NEB) to enable ligation with the first PCR product. Finally, a ligation of the two PCR products and the pCDNA eGFP plasmid *Hind*III-*Bam*HI digest was performed for each 5'UTR (Chapter 4). The ZCCHC11 coding sequence was inserted to pcDNA3.1+ using *Eco*RI and *Not*I. eGFP was inserted to its 3' end using *Not*I and *Xba*I in order to generate GFP tagged ZCCHC11 (GFP_ZCCHC11) that was used in immunofluorescence experiments in Chapter 5. pcDNA3.1_TAP was used as a transfection control in parallel with GFP reporter plasmids containing the desired sequences at their 3' end or 5' ends. It contains the TAP tag sequence inserted into pcDNA3.1+ using *Not*I and *Xba*I. ZCCHC6 coding sequence was inserted into pcDNA3.1+ using *Eco*RI and *Not*I and TAP tag was ligated to its 3' end between *Not*I and *Xba*I in order to generate TAP tagged ZCCHC6 (TAP_ZCCHC6). Schematic representations of the eGFP reporter plasmids are shown in Figure 2.1. Complete list of plasmids can be found in Table 2.1.

TABLE 2.1. Plasmids used in this study.

RE: reporter experiments IF: immunofluorescence

Plasmid	Use	Source
peGFP	RE/IF	Norbury group
peGFP[β-Actin_3'UTR]	RE	This study
peGFP[ZCCHC11_3'UTR]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr1]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr2]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr3]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr4]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr23]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr34]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr234]	RE	This study
peGFP[ZCCHC11_3'UTR_Fr1only]	RE	This study
peGFP[ZCCHC11_3'UTR_sf1a]	RE	This study
peGFP[ZCCHC11_3'UTR_sf1b]	RE	This study
peGFP[ZCCHC11_3'UTR_sf1c]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut1]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut2]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut3]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut4]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut5]	RE	This study
peGFP[ZCCHC11_3'UTR_AUmut6]	RE	This study
peGFP[ZCCHC11_3'UTR_PPS_mut]	RE	This study
peGFP[ZCCHC11_3'UTR_let7mut]	RE	This study
peGFP[ZCCHC11_3'UTR_*mir26*mut]	RE	This study
peGFP[ZCCHC11_3'UTR_*mir132/212*mut]	RE	This study

peGFP[β-Actin _5'UTR]	RE	S.Fleurdepine
peGFP[ZCCHC11_5'UTR_short]	RE	This study
peGFP[ZCCHC11_5'UTR_long]	RE	This study
peGFP[ZCCHC11_5'UTR_short_uAUG1_mutation]	RE	This study
peGFP[ZCCHC11_5'UTR_short_uAUG2_mutation]	RE	This study
peGFP[ZCCHC11_5'UTR_long_uAUG3_mutation]	RE	This study
peGFP[ZCCHC11_5'UTR_long_uAUG123_mutation]	RE	This study
pcDNA3.1_TAP	transfection control in RE	Norbury group
Flag_HuR	overexpression of HuR	W. Eberhardt
TAP_ZCCHC6	overexpression of ZCCHC6	This study
peGFP[ZCCHC11]	IF	This study
peGFP[ZCCHC11_CCHC2_mut]	IF	This study
peGFP[ZCCHC11_s104a]	IF	This study
peGFP[ZCCHC11_C2H2_mut]	IF	This study
Geneclip(scrambled)	Control for drosha knockdown	M.J. Schmidt
Geneclip(Drosha)	Drosha Knockdown	M.J.Schmidt

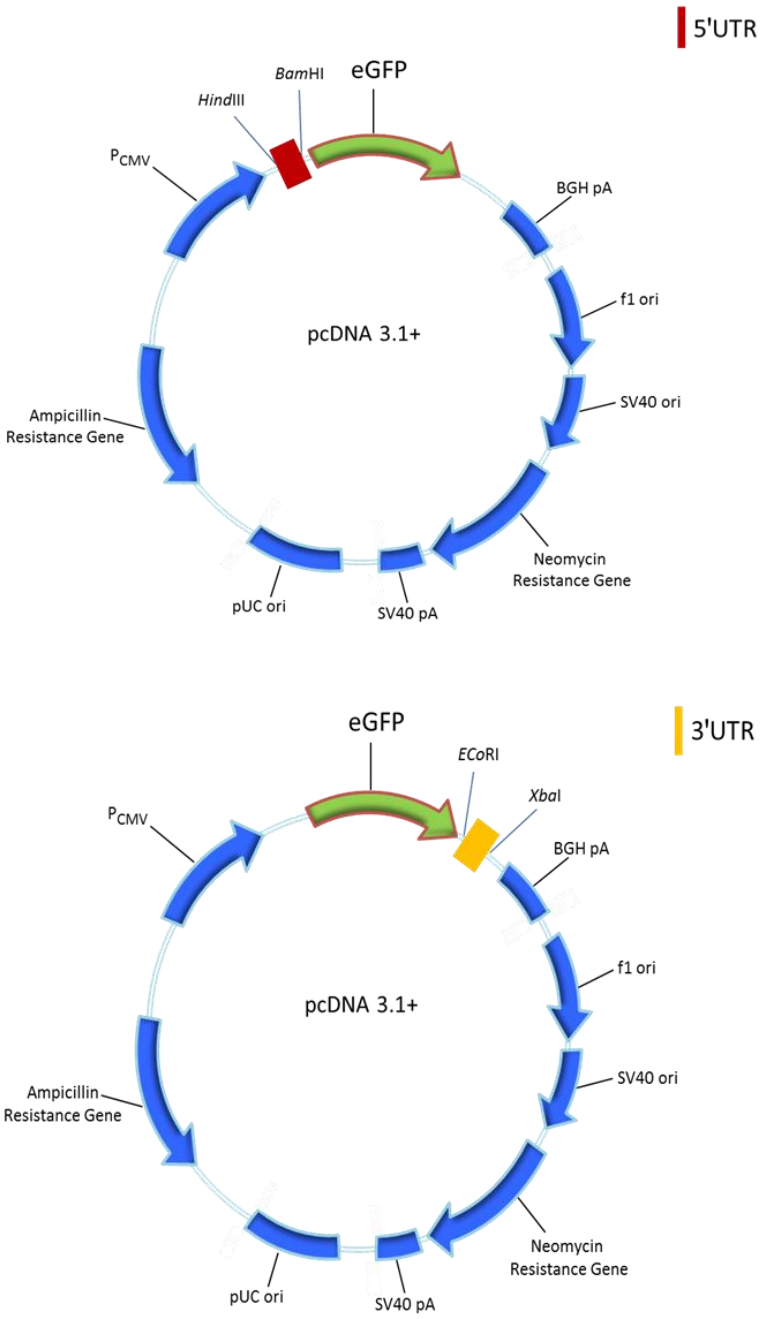


FIGURE 2.1. Schematic representation of eGFP reporter plasmids used in this study.
eGFP open reading frame was inserted to pCDNATM3.1+ (InvitrogenTM) for eGFP reporter experiments in this study. Upper panel shows the eGFP reporter plasmid ligated to the 5'UTR of the gene of interest. Lower panel shows the eGFP reporter plasmid ligated to the 3'UTR of the gene of interest.

2.1.3. Mammalian cell lines

HeLa (kind gift from Nick Proudfoot), HEK293T (kind gift from Jean-Yves Bleuyard and Fumiko Esashi), MDA-MB-231 and A204 cells (kind gift from Martin Pule from University College London, Research Department of Haematology, Cancer Institute) were used for this study.

2.1.4. Bacteria

XL1-Blue chemically competent *E. coli* (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (TetR)]*) were used to amplify plasmid DNA. BL21(DE3)pLysS (*F- ompT gal dcm lon hsdSB(rB- mB-) λ(DE3) pLysS(cmR)*) chemically competent *E. coli* were used for recombinant protein expression. Both strains were grown in LB (Luria Bertani) medium. The medium of BL21 cells was supplemented with 34 μg/ml chloramphenicol for maintenance of the pLysS plasmid.

2.2. Methods

2.2.1. DNA methods

2.2.1.1. Determination of DNA concentration

The concentration of dsDNA preparations was determined by measuring the absorbance at 260 nm (A_{260}). The elution buffer, usually 10 mM Tris, pH 8.5, used to dissolve the DNA was used as blank control. Each measurement was repeated four times and the mean values of the measurements were used to determine the concentration of the DNA, based on the assumption that a DNA solution of 50 μg/ml has an A_{260} of 1.0. Pure DNA had an A_{260}/ A_{280} ratio of 1.8. The purity, quantity and integrity of the DNAs were further confirmed by agarose gel electrophoresis.

2.2.1.2. PCR using a DNA template

2.2.1.2.1. PCR using Taq polymerase

PCR reactions comprised when not otherwise stated: DNA template (100 ng genomic DNA or 100 ng cDNA or 30 ng plasmid DNA), 0.5 mM dNTPs (Bioline), 0.5 units Simple Red Taq Polymerase (Fisher Scientific), 1x Taq buffer, 0.5 µM of each oligonucleotide (synthesised by Sigma Aldrich and Life Technologies) and made up to 50 µl with dH_2O. Reactions were carried out in a Veriti® Thermal Cycler (Applied Biosystems) as follows: 95°C for 2 min, followed by 25 cycles of (94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s per kb) and a final 7 min hold at 72°C, before chilling to 4°C. The annealing temperature (Tm) varied depending on the GC/AT ratio of the oligonucleotides and was calculated using the equation Tm=4(G+C)+2(A+T)°C. The Thermo Scientific website was also used for calculating and confirming Tm values.

2.2.1.2.2. Colony PCR

A single colony of bacteria was briefly resuspended directly in 25 µl red *Taq* reaction mixture containing ZCCHC11 specific primers (listed in Appendix C) and PCR was carried out as described in 2.2.1.2.1. In order to amplify the bacteria in parallel, a pipette tip was used to collect a small sample of the pellet. The tip was then placed in 2 ml LB supplemented with ampicillin (100 µg/ml) for growth overnight with shaking at 37°C.

2.2.1.3. Agarose gel electrophoresis

6x DNA loading dye was added to samples as 0.2 volumes and the samples were loaded on 1-2% agarose gels prepared with 1 x TBE buffer and containing 0.5 µg/ml ethidium bromide (Sigma). Electrophoresis was carried out at 2-10 V/cm in 1x TBE running buffer.

5 µl of a size marker (Hyperladder™ I or II, Bioline) was run alongside. DNA was visualised using a UV transilluminator (UVP).

2.2.1.4. Restriction enzyme digestion

Digestions were performed in a 30 µl reaction: 0.2 – 1 µg DNA, 1x restriction buffer, 5 units of restriction enzyme and 100 µg/ml of bovine serum albumin (BSA) if necessary. Reactions were incubated at the optimum temperature (usually 37°C) for 1-3 h and digestion was verified by agarose gel electrophoresis.

2.2.1.5. Gel Extraction of DNA

DNA purification from agarose gel slices was performed using the QIAquick gel extraction kit (QIAgen) according to the manufacturer's instructions. Briefly, the DNA band was visualised in agarose gel under UV light and small agarose gel slices were cut. These slices were melted in high-salt buffer, and silica-gel columns were used to bind DNA. Ethanol-containing wash buffer was used to clean DNA, which was then eluted in 30 µl dH_20.

2.2.1.6. Ethanol precipitation

To concentrate nucleic acids, 2.5 volumes of 100% ethanol and 0.1 volumes of 2 M sodium acetate pH 5.2 were added, mixed and incubated at -20°C for 1 h. Samples were centrifuged (16000 x *g*, 10 min, room temperature), and the pellet was then washed in 150 µl 70% ethanol, air-dried and resuspended in dH_2O.

2.2.1.7. pSuperior cloning systems

To create plasmid constructs for gene silencing in mammalian cells, the pSuperior™ System (Oligoengine) was used. The sequences of complementary oligonucleotides

containing the desired siRNA hairpin target sequence can be found in Appendix D. Complementary hairpin primers were annealed and ligated into the linearised pSuperior™ vector following the manufacturer's instructions. Plasmids containing the insert were transformed into *E.coli* as described (2.2.2.3). DNA sequencing was performed as described (2.1.10).

2.2.1.8. Site-directed mutagenesis

Transition mutations were generated where adenine is mutated to guanine and thymine is mutated to cytosine in the ZCCHC11 3'UTR. Histidine to alanine mutation was performed to generate tagged versions of ZCCHC11 that lack zinc finger or zinc knuckle integrity as described previously (Fields et al. 2008; Hensen et al. 2002). Guanine residues in upstream AUGs of the ZCCHC11 5'UTR were mutated to adenine. Site directed mutagenesis was performed by setting up a PCR reaction using 10 ng of template vector DNA, 250 nM of complementary oligonucleotides spanning the mutation site (30-40 nt in length) and 5 units of PfuTurbo DNA polymerase (Stratagene). Thermal cycling conditions were as follows: 95°C for 1 min, 18 cycles of 95°C for 50 s, 55°C for 50 s, 68°C for 1 kb/min of plasmid length and 7 min hold at 68°C. Following PCR the non-mutated parental DNA was digested by adding 10 units of *Dpn*I (New England Biolabs) to the reaction and incubating at 37°C for 1 h. Samples were then transformed into *E.coli* (XL1 Blue) as described.

2.2.1.9. DNA Ligation

Inserts that were purified following digestion and linearised plasmid DNAs were ligated as follows. Insert and vector DNA was combined in a reaction medium with a final 4:1 insert:vector molar ratio. This medium contains 1x T4 DNA ligase Buffer and 10 U T4

DNA ligase. This mixture was incubated at 16°C overnight and it was transformed into bacteria as described in 2.2.2.3.

2.2.1.10. DNA sequencing

Dideoxy-DNA sequencing was carried out using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems®). 1 µl BigDye reagent was mixed with 50-300 ng DNA template, 3.2 pmol oligonucleotide primer, 1.5 µl 5x buffer and made up to 10 µl with dH_2O. Reactions were carried out in a Veriti® Thermal Cycler (Applied Biosystems) as follows: 96°C for 4 min, followed by 25 cycles of (96°C for 30 s, 50°C for 10 s, 60°C for 4 min). Samples were ethanol precipitated and left to dry at room temperature for 30 min before being sent for analysis using a 3730xl DNA Analyzer (Applied Biosystems) carried out by the Geneservice (Source BioScience). The resulting data were analysed using ApE Universal software.

2.2.2. Bacterial Methods

2.2.2.1. Competent cell preparation of XL1-Blue

200 µl of an overnight LB culture was diluted into 50 ml of fresh SOB and incubated at 37°C for 2-2.5 h. At A_{600} of 0.3-0.5, the culture was cooled on ice for 30 min and harvested by centrifugation (3000 x *g*, 10 min, 4°C). The cell pellet was resuspended in 5ml cold TSB and incubated on ice for 10 min. Aliquots of 100 µl were dispensed, snap-frozen in liquid nitrogen and stored at -80°C.

2.2.2.2. Competent cell preparation of BL21(DE3)pLysS

An overnight culture was diluted 1:100 in LB + chloramphenicol. 45ml of the cells were harvested at A_{600} of 0.4-0.6 by centrifugation (3000 x *g*, 15 min, 4°C). The pelleted cells

were resuspended in 20 ml of cold 50 mM $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged as above and resuspended in 5 ml ice-cold $CaCl_2$. Following incubation on ice of at least 30 min, cells were ready for transformation.

2.2.2.3. Transformation of *E. coli*

2-4 µl of a DNA ligation reaction was mixed with 20 µl of 5 x KCM and dH_2O to a final volume of 100 µl and cooled on ice. The DNA was incubated with 100 µl of competent cells on ice for 20-30 min prior to heat shock (42°C, 90 s) and quick chill on ice. 700 µl of LB was added and cells were incubated at 37°C shaking for one h. Cells were centrifuged (3000 x *g*, 1 min, room temperature), resuspended in 200 µl LB, plated on LB-agar containing selective antibiotic and incubated overnight at 37°C.

2.2.2.4. Purification of Plasmid DNA

Large-scale plasmid DNA preperations were obtained using GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich) as described by the manufacturer. Plasmid Miniprep kit (QIAgen) was used to generate small-scale plasmid DNA preparations as described by the manufacturer. Briefly, an overnight culture of recombinant *E. coli* was harvested by centrifugation. The cells were then subjected to a modified alkaline-SDS lysis procedure. After addition of salts, the DNA was adsorbed onto silica, and contaminants were removed by a spin-wash step. DNA was eluted in 10 mM Tris, pH 8.5. DNA concentration was measured as described (2.2.1.1).

2.2.3. Protein methods

2.2.3.1. Protein quantification

Protein concentration of samples was measured using the Bradford Assay (Bio-Rad, UK). The assay was carried out according to the manufacturer's protocol. A range of bovine serum albumin (BSA; Sigma-Aldrich, UK) concentrations (0, 1.25, 2.5, 5, 10 µg/µl) were used to produce a standard curve and the concentrations of samples were determined accordingly.

2.2.3.2. Denaturing protein gel electrophoresis (SDS-PAGE) and Western Blotting

Mammalian cells were washed twice in cold PBS, detached using a disposable cell scraper and resuspended in 100 µl- 1000 µl RIPA lysis buffer supplemented with protease inhibitors (Roche EDTA free protease inhibitor tablet, 1 mM benzamidine, 2 mM PMSF). Lysates were incubated on ice for 20-25 min and then centrifuged at 16,100 x *g* at 4°C for 15 min. The supernatants containing protein samples were kept on ice afterwards. Protein quantification was performed as described (2.2.3.1). Protein samples in 1x SDS sample buffer were loaded onto SDS-PAGE gels (80 µg of protein per well) alongside a protein molecular weight marker (Colorplus). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes using a transblot semi-dry electrophoretic transfer cell (BioRad) or a mini-transblot electrophoretic transfer cell (BioRad) according to the manufacturer's instructions. Ponceau S stain was used to visualise transferred proteins before further processing the membranes. The membranes were blocked in 1x TBS- 0.1% Tween containing 5% dry milk for 1 h. The membranes were then incubated with relevant primary antibodies diluted in 1x TBS-T containing 5% dry milk at 4°C

overnight. Following 3-4 times 5 min washing with 1x TBS-T, the membranes were incubated with the secondary HRP-Conjugated antibody (Table 2.2.) diluted in 1x TBS-T containing 5% dry milk for 1 hr at room temperature. The membranes were then washed 3 times for 10 min each with 1x TBS-T before detection by ECL (Santa Cruz). Chemiluminescence was detected by exposure to X-Ray Films RX-NIF (Fujifilm).

2.2.3.3. Total protein staining

SDS-PAGE gels were stained with Imperial™ Protein Stain (Thermo scientific) and destained by washing the gel overnight in dH_2O.

2.2.3.4. Induction of recombinant proteins in *E.coli*

200 ml LB + 100 µg/ml ampicillin was used to dilute the overnight culture of BL21(DE3)pLysS cells transformed with pGEX6P 1:100 and it was further incubated at 37°C. Induction of protein expression was done by the addition of Isopropyl β-D-1-thiogalactopyranoside to the medium to a final concentration of 100 µM, when the culture reached an A600 of 0.5. Bacteria were grown for 3 more h at 37°C. Then, they were harvested by centrifugation (4000 x *g*, 15 min, 4°C).

2.2.3.5. GST purification of recombinant proteins

Harvested cells were washed once with cold PBS. Next, they were resuspended in BugBuster (Novagen) supplemented with 2 µl Benzonase (Novagen) per 50 ml bacterial culture. They were mixed at 4°C for 15 min in order to lyse. Centrifugation (16000 x *g*, 25 min, 4°C) was done to clear the lysate and the supernatant was incubated with 40 µl packed glutathione-Sepharose (pre-equilibrated in PBS, Amersham) for 1 h, rocking at 4°C. Prior to elution of the recombinant protein, beads were washed four times for 10

min in 1 ml cold HBS. Beads were incubated in 100 µl elution buffer for 10 min six times. The elution fractions were snap-frozen in liquid nitrogen and analysed on a 10% SDS-PAGE.

2.2.3.6. Affinity purification of antibodies by western blotting

This method was used in order to purify antibodies against ZCCHC11. Sample buffer to a final volume of 1 ml was used to dilute elutions from the GST purification containing the recombinant antigen. This solution was run on a large 10% SDS-PAGE gel (Protean II) for 5 h. Antigen localisation was performed using Coomassie staining of a vertical strip and the gel area containing the recombinant antigen was excised and transferred to an Immobilon-P PVDF membrane (Millipore). In order to prepare the PVDF membrane, it was soaked in 100% methanol prior to soaking in transfer buffer. Transfer was performed at constant 0.04 mA for 2 h. The membrane was then blocked for 2 h in PBS-T containing 5% low-fat dried milk. 2 ml of crude polyclonal rabbit antiserum raised against recombinant ZCCHC11 was diluted 1:1 in PBS-T containing 5% milk and membrane was incubated with this mixture while rotating at 4°C overnight. TBS-T was used to wash the membrane after this incubation step three times for 10 min. Antibody elution was performed by addition of 1 ml of 0.1 M glycine pH 4.2 to the membrane and incubation for 5 min at 4°C. Neutralisation of the solution was done with 120 ml of 1M Tris-HCl pH 8.0. The concentration of the antibody was quantified using different concentrations of BSA and running them on 10% SDS-PAGE in parallel. The antibody was stored at 4°C.

2.2.4. Cellular Methods

All cell lines were cultured in 37°C incubators with 5% CO_2 and grown in DMEM, supplemented with 10% foetal calf serum (FCS) and 0.1 mg/ml (1X) penicillin / streptomycin antibiotics (Gibco and PAA Laboratories). Cells were split and passaged by trypsinisation using PET solution (PBS, 1 mM EDTA, 0.125% Trypsin) to detach adherent cells. Cells were passaged every 3-4 days at a 1/8 dilution. Cells were prepared for storage by re-suspending trypsinised cells in 1 ml of 90% DMEM / 10% DMSO solution in cryo-vials and freezing in CoolCell containers (Biocision) at -80°C. Frozen cells were revived by rapid thawing at 37°C, washing and re-suspension in DMEM growth media.

2.2.4.1. Cell transfection

2.2.4.1.1. Cell transfection using plasmid DNA

Transfections of mammalian cells for reporter experiments discussed in this study were performed using jetPRIME® (Polyplus transfection) according to the manufacturer's instructions. Briefly, cells were split into 6-well plates. 24 h after splitting the cells, when the cells were 60%-80% confluent, transfection was performed as follows: 2 µg plasmid DNA was diluted in 200 µl of jetPRIME® buffer and 4 µl jetPRIME® reagent. The transfection mixture was incubated at room temperature for 10 min. The cell medium was renewed and transfection mixture was added to the cells. Cells were incubated for 48-72 h (72 h for the cells transfected with pSuperior plasmids and 48 h for other plasmids) before being collected.

Transfections of human cells for the immunofluorescence experiments discussed in Chapter 5 was performed using PEI transfection reagent (Sigma 408727) diluted at 0.6136 mg/ml in MES-HEPES buffered saline. Cells were seeded onto coverslips in 6-well

plates. 24 h after seeding the cells, when the cells were 50%-80% confluent, transfection was performed as follows: 2 µg plasmid DNA was diluted in a total of 1000 µl antibiotic- and serum-free DMEM and 4 µl PEI transfection reagent were added. The transfection mixture was incubated at room temperature for 20 min. Cells were washed with 2 ml antibiotic- and serum-free DMEM and the medium was replaced with 1 ml antibiotic- and serum-free DMEM. Transfection mixture was added onto the cells, and the cells were incubated for 4 h before the medium was replaced with full DMEM (as described previously in this chapter). The cells were incubated for 48 h before being fixed and prepared for microscopy.

2.2.4.1.2. Cell transfection using siRNA

*Trans*IT-X2® Dynamic Delivery System (Mirus Bio) was used to transfect cells with siRNA. Cells were seeded into 6-well plates the day before transfection in order to achieve around 80% confluence at the time of transfection. 250 µl of Opti-MEM I Reduced-Serum medium (Life Technologies) was placed in a tube. 6.8 µl of 20 µM siRNA stock solution were added to this medium in order to have a final concentration of 50 nM per well. Next, 7.5 µl of *Trans*IT-X2 reagent was added. After mixing thoroughly by pipetting, the mixture was incubated at room temperature for 20-30 min. Meanwhile, the cells were washed once with 2 ml DMEM and 2 ml of DMEM containing 10% FCS and 1x Penicillin-Streptomycin was added onto the cells. *Trans*IT-X2:siRNA complexes were added dropwise to the cells and the culture was gently rocked to distribute the mixture homogenously. Cells were incubated at 37°C for 48 h before being collected. Knock-down efficiency was investigated using qRT-PCR. Transfection of plasmids expressing shRNAs was performed as described in 2.2.4.1.1.

2.2.4.2. Drug treatment and cell cycle arrest induction

Cells were arrested in S-phase using hydroxyurea (2 mM) and in G2-phase using doxorubicin (150 nM) for 24 h.

2.2.4.3. Induction of cellular stress

Oxidative stress was induced using 20 µM sodium arsenite for 30 min or 2.5 µM sodium arsenite for 24 h. Genotoxic stress was induced using 5 mM hydroxyurea as described previously (Schmidt, West, and Norbury 2011). Cells were incubated for 30 min at 42°C to induce heat shock stress. Hyperosmolar stress was induced by incubating the cells in DMEM supplemented with 10% FCS and 150 mM NaCl overnight.

2.2.4.4. Flow cytometry

2.2.4.4.1. Acquisition of Flow Cytometry Data

Cells were washed once with 1x PBS, collected by centrifugation for 5 min at 4°C 300 x *g*, and then either analysed directly or fixed by mixing with 500 µl prechilled PBS and 500 µl cold 2% paraformaldehyde solution. They were then incubated at 4°C for 20 min before washing with 1x PBS. Cells were then permeabilized by adding 1 ml of 70% ethanol dropwise whilst vortexing. This mixture was then incubated overnight in 70% ethanol. The next day, cells were washed with 1x PBS and re-suspended in a 1 ml PBS solution containing 40 µg/ml propidium idodide and 100 µg/ml RNAse A. DNA content and GFP fluorescence data were acquired by flow cytometry (FACScalibur, Becton Dickinson).

2.2.4.4.2 Analysis of Flow Cytometry Data

Flow Cytometry Data was analysed using FlowJo software. Cells which were transfected with eGFP reporters were first analysed in Forward Scatter (FSC)-Side Scatter (SSC) graph in order to exclude the unviable cell populations from the analysis (Fig. 2.2.). eGFP expression of the cells was than analysed on a logarithmic scale by an histogram showing FL1H signal (Fig 2.2.) and it was attempted to include as many cells as possible on the logarithmic scale to the extent which the flow cytometry machine enabled. Mean values of FL1H signals were calculated in order to compare eGFP expression of the cells transfected with different plasmids encoding eGFP. Propidium Iodide staining of the cells was analysed in parallel by a histogram showing FL2H signal when required.

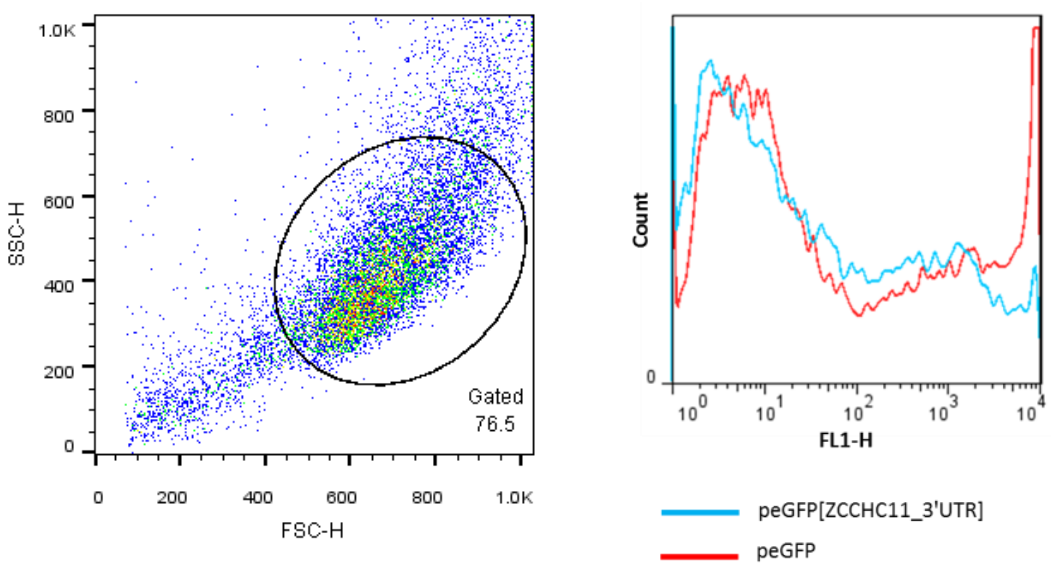


FIGURE 2.2. Sample flow cytometry graphs. The sample FSC-SSC graph on the left shows the distribution of the cells transfected with peGFP and selection of the viable cell population (gated). Histogram on the right shows the signal from the FL1H channel and hence eGFP expression on a logarithmic scale.

2.2.5. RNA methods

2.2.5.1. RNA extraction

Total RNA was isolated using Tri-Reagent (Sigma) followed by chloroform extraction and isopropanol precipitation of RNA. Total RNA was dissolved in 30-50 µl dH_20. The purity and concentration of the resuspended RNA was measured using a nanodrop spectrophotometer by measuring the absorbance at 260 nm (A_{260}). Each measurement was repeated three times and the mean values of the measurements were used to determine the concentration of the RNA, based on the assumption that an RNA solution of 40 µg/ml has an A_{260} of 1.0. Pure RNA had an A_{260}/ A_{280} ratio of 2.1.

2.2.5.2. DNAse treatment of total RNA

3 µg of total RNA was treated with 1.5 µl DNase (Promega) in 1x RQ1 DNAse buffer for 2 hours at 37°C (with an extra 1.5 µl of DNase added after the initial hour). After the 2 hours incubation, samples were incubated at 70°C for 15 min to inactivate the DNAse.

2.2.5.3. Reverse Transcription

cDNA was synthesised using Im-Prom-II™ Reverse Transcriptase (Promega). An RNA sample (usually 2 µg) was combined with 10 pmol random hexamers (Invitrogen) or gene-specific primers as stated in a volume of 25.4 µl (12.7µl for RT+ and RT- samples) and incubated at 70°C for 2 min before immediate cooling on ice. After this incubation, the remaining reagents were added in a total volume of 20 µl to the following final concentrations: ImProm-II Reaction Buffer(1x); $MgCl_2$ (3 mM); dATP/ dCTP/ dGTP/ dTTP mix (0.5 mM each) and ImProm-II Reverse Transcriptase (4 U/µl). Reactions were incubated as follows: 23°C for 5 min; 42°C for 1 h; 70°C for 15 min; then chilled to 4°C.

For each sample, an additional reaction was performed in the absence of reverse transcriptase (replaced with H_2O) to control for contaminating DNA.

2.2.5.4. Quantitative RT-PCR (*qRT-PCR*)

RT+ cDNAs and RT- control were diluted by a factor of two to 40 µl with dH_20. qRT-PCR primers for each gene of interest and endogenous housekeeping genes (GAPDH and β-actin) were designed using the Real-Time PCR Primer Design Tool (GenScript). qRT-PCR reactions were prepared for both RT+ and RT- samples, both for the gene of interest as well as the housekeeping control gene. Three technical replicates of qRT-PCR reaction for RT+ samples and one technical replicate for RT- samples were prepared. 1 µl of cDNA was used for each technical replicate and the cDNAs were combined with 500 nM final concentrations of forward and reverse qRT-PCR primers and 1x SYBR Green master mix (Bioline). Quantitative PCR was carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems®) running the following program: 95°C for 10 min; 40 cycles of (95°C for 15 s, 60°C for 1 min). A melt curve was also performed for each sample (1°C rise, from 60°C to 95°C). Data were analysed using the StepOne™ Software.

2.2.5.5. Circularised rapid amplification of cDNA ends (cRACE)

(Couttet et al. 1997) described this method. Briefly, RNA samples were prepared and quantified as in 2.2.5.1 and 2.2.5.2. In order to remove uncapped transcripts and capture capped transcripts, 12 µg of total RNA were incubated successively with Shrimp Alkaline Phosphatase (Roche) and Tobacco Acid Pyrophosphatase (Cambio) for one hour each respectively at 37°C. 12 µg RNA was combined with 1x T4 RNA ligase I buffer and 1 U T4 RNA ligase (NEB) in 400 µl. This mixture was incubated overnight at RT. After intramolecular ligation, circularised RNA products (cRNA) were precipitated using

ethanol and the pellet was resuspended in 20 µl dH_20. Reverse transcription was performed on 4 µl of this cRNA with a gene-specific primer as described in 2.2.5.3. 4 µl of the resulting cDNA was amplified using primers that span the intramolecular ligation site of both ends of ZCCHC11 as described in 2.2.1.2.1, except with 23 cycles and a 72°C elongation time of 50 s. Nested PCR with a second set of inner primers was used to amplify the product of this first PCR using the same PCR settings. Nested PCR products were cloned directly into PCR2.1 (TA cloning kit, Invitrogen). Screening of these inserts in E. *coli* transformants was performed using colony PCR as described in 2.2.1.2.2 with the nested PCR divergent primers. Insert-containing plasmids were then purified and sequenced.

2.2.5.6. RNA half-life determination

In order to measure the half-life of specific mRNAs, cells were treated with the transcription inhibitor actinomycin-D (5 µg/ml) as described previously (Herrick and Ross 1994). Cells were collected at 0, 2, 4, 6 and 8 h after actinomycin-D treatment. RNA isolation and qRT-PCR were performed as discussed (2.2.5.1-4.).

2.2.6. Microscopy Methods

2.2.6.1. Cell fixation and preparation

HeLa cells growing on sterile glass coverslips were transfected with plasmids encoding GFP-tagged WT or mutant ZCCHC11, and were exposed to cellular stress as described (details in Chapter 5) if necessary. Cells were washed with PBS twice before being fixed and permeabilised in 100% methanol at -20°C for 10 min. Cells were then washed twice with PBS and blocked with PBS + 10% FCS for 30 min at 37°C. After washing once, they were incubated with relevant primary antibody (Table 2.2.) in PBS + 10% FCS

+ 0.1% Tween-20 at 37°C for 1 h. They were then washed 3x 5 min with PBS +0.1% Tween-20. Cells were then incubated with secondary antibodies conjugated to Cy3 or FITC (Table 2.2.). Finally, cells were washed 3x 5 min with PBS +0.1% Tween-20. Coverslips were stained with DAPI and stored at -20°C if not used immediately for microscopy.

TABLE 2.2. Antibodies used in this study.

Antibodies used for western Blot

	Source	**Concentration**
Anti-ZCCHC11	Bethyl(A302-635A)	1/300
Anti α-Tubulin	Sigma Aldrich (T9026)	1/1000
Anti GFP	Sigma Aldrich (G1544)	1/1000
Anti-Mouse-HRP	Sigma Aldrich (A0545)	1/10000
Anti-Rabbit-HRP	Sigma Aldrich (A4416)	1/10000

Antibodies used for Immunofluorescence

	Source	**Concentration**
Primary		
Anti-ZCCHC11	Bethyl(A302-635A)	1/250
Anti α-Tubulin	Sigma Aldrich (T9026)	1/1000
Anti-TIA-1	Santa Cruz (sc-1751)	1/500
Anti-GFP	Source Bioscience(ABX325)	1/500
Anti-GW182	Santa Cruz (sc-47034)	1/250
Anti-eIF3b (N-20)	Santa Cruz (sc-16377)	1/200
Anti-ZCCHC11 (CN91)(Rabbit polyclonal)	Lab stock	1/1000
Secondary		
Anti-Rabbit IgG (Cy3)	Sigma Aldrich(C2306)	1/500

Anti-Goat IgG (Cy3)	Jackson Immunoresearch(705-165-147)	1/300
Anti-Rabbit IgG (Alexa Fluor® 488)	Life Technologies (A21206)	1/500
Anti-Mouse IgG (FITC)	Sigma Aldrich (F6257)	1/300

2.2.6.2. Immunofluorescence microscopy

Wide-field fluorescence microscopy (Zeiss Aioskop) was used to visualise the cells stained with fluorescent antibodies and DAPI.

2.2.6.3. Confocal microscopy

Olympus FluoView™ FV1000 Confocal Microscopy was used to visualise cells stained with fluorescent antibodies and DAPI. Analysis of colocalisation was performed using a custom ImageJ macro written by Richard Wheeler (Fig 2.3.).

```
threshold=500;
bins=64;

slice1=2;
slice2=3;
maxPixel=4096;

src=getImageID();
print("\\Clear");
histo=newArray(bins+1);
for (x=0; x<getWidth(); x++) {
        showProgress(x/getWidth());
        for (y=0; y<getHeight(); y++) {
                setZCoordinate(slice1-1);
                if (getPixel(x, y)>threshold) {
                        setZCoordinate(slice1-1);
                        v1=floor(log(getPixel(x, y)+1)*(maxPixel/log(maxPixel))/(maxPixel/bins));
                        setZCoordinate(slice2-1);
                        v2=floor(log(getPixel(x, y)+1)*(maxPixel/log(maxPixel))/(maxPixel/bins));
                        histo[v2]=histo[v2]+getPixel(x, y);
                }
        }
}

for (i=0; i<lengthOf(histo); i++) {
        print(histo[i]);
}
```

FIGURE 2.3. The script of the custom ImageJ macro written by Richard Wheeler.

CHAPTER THREE

THE 3'UTR OF ZCCHC11

3.1. Introduction

Recent studies have shown that terminal uridylation is required for the regulation of several human RNAs whose aberrant expression leads to diseases including cancer. The human terminal uridyl transferase ZCCHC11 has been found to play important roles in regulation of the expression of tumour supressor *let-7* miRNAs (Hagan, Piskounova, and Gregory 2009b; Piskounova et al. 2011), replication-dependent histone mRNA (Schmidt, West, and Norbury 2011), *miR-1* (Rau et al. 2011), and also in the regulation of key genes in inflammation through the function of *miR-26* (Jones et al. 2009). Knocking down ZCCHC11 results in decreased proliferation of cancer cells through increased cell cycle arrest (Blahna et al. 2011), and decreased tumour volume and tumourigenic capacity in xenografts (Piskounova et al. 2011). ZCCHC11 was shown to be overexpressed in human cancers (Fu et al. 2014), and its high expression found to be associated with poor prognosis in human breast cancer (Hallett and Hassell 2011). Therefore, understanding the regulation of ZCCHC11 carries a significant importance. Very little is known about the regulation of this regulatory protein. Our group has previously shown that ZCCHC11 mRNA and protein levels decrease upon entry to S phase and return to their normal levels at the end of S phase (Schmidt, West, and Norbury 2011). Considering the role of ZCCHC11 in degrading replication-dependent histone mRNAs, this decrease in ZCCHC11 level suggests its possible involvement in the precise control of histone accumulation during S-phase. Nevertheless, the molecular mechanism underlying this regulation or any other potential cell cycle-independent regulation of ZCCHC11 is not known. Post-transcriptional regulatory mechanisms could explain the decrease in both mRNA and protein levels.

3'UTRs may contain many elements, such as AREs and miRNA binding sites, which are essential for interactions with *trans*-acting factors. In addition, other types of regulation such as alternative polyadenylation may be exerted through elements in 3'UTRs. A major goal of this study was to determine whether ZCCHC11, a post-transcriptional regulatory protein regulating gene expression through modification of RNA 3' ends, is itself regulated at the post-transcriptional level.

3.2. Aims

- To investigate whether the 3'UTR of ZCCHC11 has a regulatory role.
- To investigate the involvement of the 3'UTR of ZCCHC11 in its S phase-dependent regulation.
- To understand sequence motifs and molecular mechanisms involved in the regulation of ZCCHC11 through its 3'UTR.
- To investigate the involvement of the alternative polyadenylation in the regulation of ZCCHC11.
- To investigate the potential regulation of ZCCHC11 by its orthologue ZCCHC6.

3.3. Results

3.3.1. The ZCCHC11 3'UTR negatively regulates its expression

The expression levels of many genes are determined by their 3'UTRs, and the regulatory elements they bear. AUBPs in particular, which interact with the 3'UTRs of mRNAs encoding various oncoproteins, cytokines and cell cycle regulators, have either positive or negative influences on the expression of these genes. To my knowledge, there is no evidence suggesting the involvement of AUBPs in regulation of any TUTase. Nevertheless, several recent studies indicated the importance of AUBPs, such as TTP and KSRP, in regulating *let-7* expression through mechanisms involving the regulation of Lin28A, a key component in ZCCHC11-mediated pre-*let-7* uridylation (Kim et al. 2012; Trabucchi et al. 2009; Michlewski and Caceres 2010). The 3'UTR of ZCCHC11 contains a number of AU-rich elements and miRNA target sites that are potentially important in determining the level of its expression (Fig. 3.1a, Appendix A). Therefore, it was hypothesised that ZCCHC11, which regulates gene expression through uridylating RNA 3' ends, might itself be regulated post-transcriptionally through its own 3'UTR. To test this hypothesis, the biological activities of the ZCCHC11 3'UTR were assayed using eGFP reporter plasmids. I chose to work with the longest ZCCHC11 mRNA encoding the full-length protein available in annotated genome databases (Fig. 3.1a). The 3'UTR of ZCCHC11 was inserted downstream of the eGFP open reading frame cloned in pCDNA^{TM}3.1+ (Invitrogen^{TM}) expression vector to form the plasmid peGFP[Zcchc11_3'UTR]. Plasmids containing either the 3'UTR from the β-actin transcript (Khabar et al. 2002) (peGFP[β-Actin_3'UTR]), or the minimal 3'UTR from the plasmid peGFP were used as controls (Fig. 3.1b). Both peGFP[Zcchc11_3'UTR] and peGFP[β-

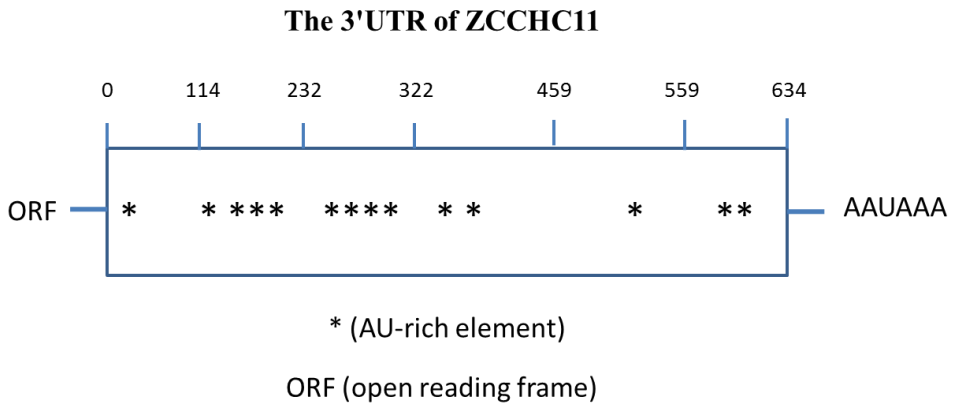
Actin_3'UTR] comprised 3'UTR sequences from the translation stop codon to the point immediately before the hexanucleotide cleavage/polyadenylation signal (AAUAAA), so the AAUAAA sequence in each case was derived from the plasmid backbone. In this study, eGFP reporter constructs were co-transfected with pcDNA_TAP plasmid, so that any variation in transfection efficiency could be controlled by amplifying the TAP by qRT-PCR for each sample. After harvesting HeLa cells at 48 h post-transfection, GFP expression was analysed by flow cytometry. Average mean FL1-H values, representing eGFP expression of the cells, are represented as bar graphs. The 3'UTR of ZCCHC11 conferred decreased eGFP expression compared to the 3'UTR of ß-actin or the minimal pCDNA3.1 3'UTR between the stop codon and the BGH cleavage/polyadenylation site in the plasmid (Fig. 3.2a). Similar results were also obtained in comparison to peGFP control in A204 and HEK293T cell lines (Figure 3.2b). Western blotting confirmed the impact of the ZCCHC11 3'UTR in decreasing the expression of the reporter construct (Fig. 3.2c).

3.3.2. The ZCCHC11 3'UTR destabilises mRNA

Regulation through the 3'UTR can impact on mRNA stability, mRNA translation or both. A qRT-PCR approach was used to investigate further the effect of the ZCCHC11 3'UTR on reporter mRNA levels. HeLa cells were transfected with the plasmids and collected as described in 3.3.1. Primers amplifying the open reading frame of eGFP common to all the plasmids were used in these experiments. The ZCCHC11 3'UTR was found to decrease expression of the eGFP reporter at the mRNA level (Fig. 3.3a). Next, the effect of the ZCCHC11 3'UTR on mRNA stability was investigated using actinomycin D to inhibit mRNA transcription as described previously (Herrick and Ross 1994). The ZCCHC11 3'UTR

decreased mRNA half-life of eGFP reporter in comparison to peGFP control group (Fig 3.3b). These results suggest that the ZCCHC11 3'UTR confers a negative effect on gene regulation, at least in part through mRNA destabilisation.

a.



b.



FIGURE 3.1. Schematic representation of the 3'UTR of ZCCHC11 and eGFP reporter plasmids. **a.** Distribution of AU-rich elements in the ZCCHC11 3'UTR. **b.** eGFP reporter system used to investigate the effect of the ZCCHC11 3'UTR. The 3'UTR of ZCCHC11 or Beta Actin was ligated to the 3' end of the eGFP ORF. peGFP[β-Actin] or peGFP were used as controls in this study (not to scale).

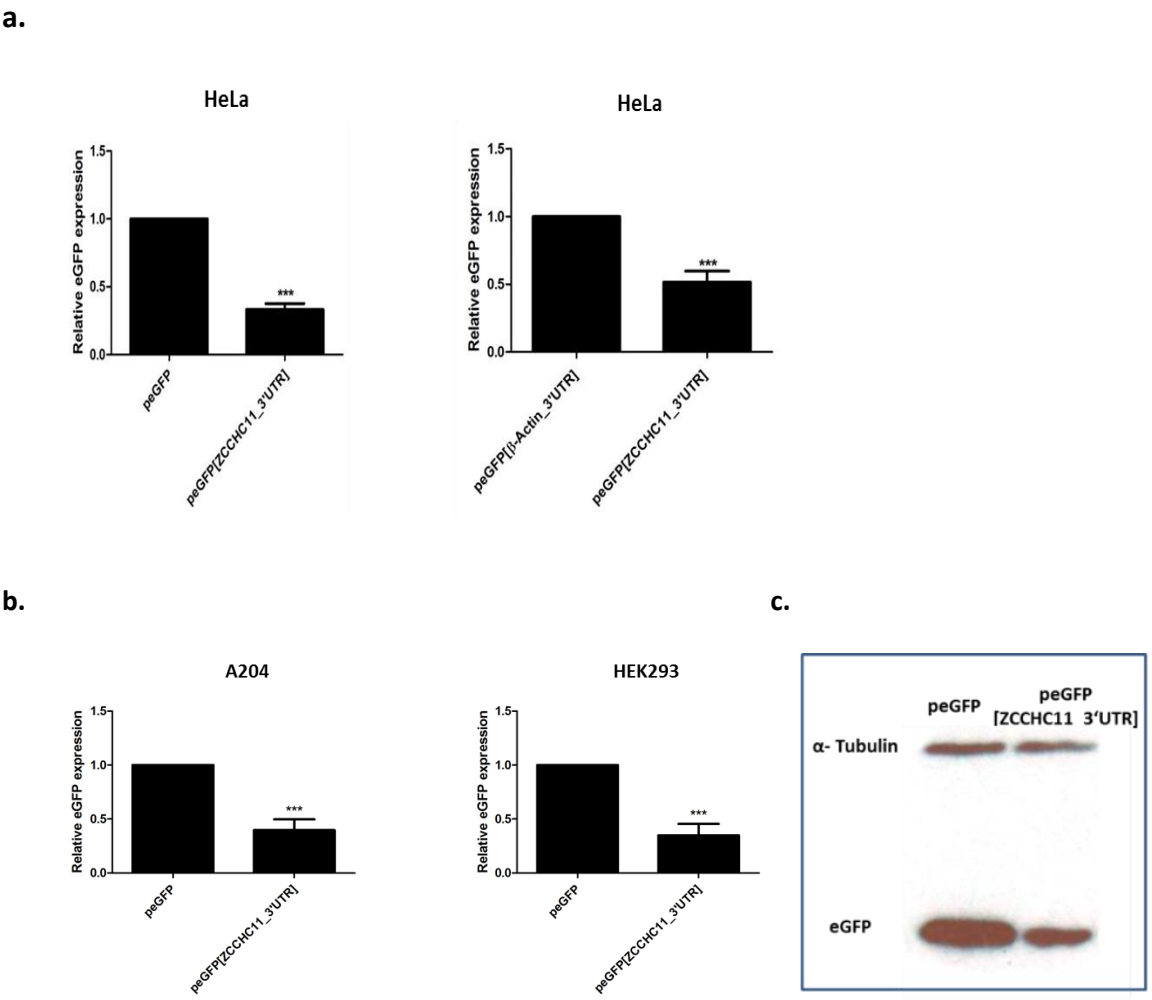
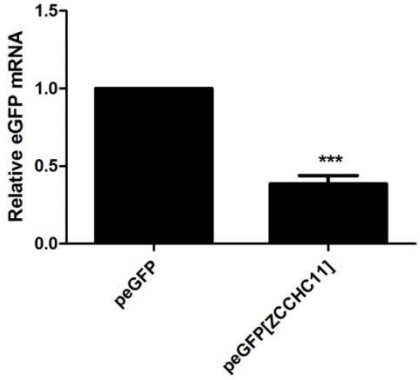


FIGURE 3.2. Gene expression conferred by the 3'UTR of ZCCHC11.

a-b. HeLa **(a),** HEK293T **(b)** and A204 **(b)** cells were transfected with peGFP[ZCCHC11_3'UTR] or a control plasmid peGFP or peGFP[β-Actin_3'UTR] **(a)** Expression of the eGFP was determined in live cells using flow cytometry (mean of at least three biological replicates ± SD). The eGFP expression conferred by the 3'UTR of ZCCHC11 was compared to that conferred by the control, the value of which is normalised to one . Student's t-test was applied and p values were determined using the control peGFP (*** $p<0.001$) as a reference. Co-transfecting a pcDNA_TAP (Tandem Affinity Purification) plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency. **c.** The effect of the 3'UTR of ZCCHC11 on the protein level in HeLa cells was investigated using immunoblotting. peGFP was used as control group, and α-tubulin was used as loading control. The immunoblot is representative of at least three independent experiments.

a.



b.

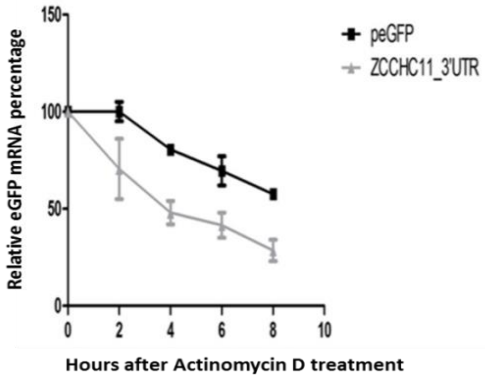


FIGURE 3.3. The effect of the ZCCHC11 3'UTR on mRNA level.

HeLa cells were transfected with peGFP[ZCCHC11_3'UTR] or peGFP as a control. The expression of eGFP at the mRNA level was measured by qRT-PCR. The data represent the mean of at least three biological replicates ±SD. Data were normalised to GAPDH mRNA levels. **a.** eGFP expression conferred by the ZCCHC11 3'UTR was compared to the expression conferred by the eGFP control, the value of which is normalised to one. Student's t-test was applied and p values were determined using the control peGFP as a reference (*** $p<0.001$). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample controlled the evenness of transfection efficiency. **b.** The effect of the 3'UTR of ZCCHC11 on mRNA stability was investigated. Cells were treated with 5 µg/ml actinomycin D to inhibit transcription. RNA isolation was performed at the indicated time points, and the level of mRNA remaining was measured by qPCR.

3.3.3. The proximal and distal halves of the ZCCHC11 3'UTR have opposite effects on gene expression

Next, I aimed to identify the minimal *cis*-acting element required for the negative influence conferred by the ZCCHC11 3'UTR. A strategy of dissecting the 3'UTR into overlapping fragments, as described previously (Qihui Zhou et al 2011), was followed. On the basis of the distribution of ARE sequences (Fig. 3.1a, Appendix A) the ZCCHC11 3'UTR was dissected into four overlapping fragments each of which contained at least one NNUUNNUUU sequence in common. It was shown previously that the presence of a NNUUNNUUU sequence is essential for the recognition of 3'UTRs by the AUBP HuR (Meisner et al. 2004). This was chosen as the basis for the dissection strategy as it is relatively evenly dispersed throughout the 3'UTR (Fig. 3.4, Appendix A). Each of these overlapping fragments was inserted downstream from the eGFP ORF in pCDNA3.1+ expression vector. The design of the experiment was similar to that described in section 3.3.1. Only the proximal half of the ZCCHC11, spanned by Fragment 1 (peGFP[ZCCHC11_Fr1]), conferred a level of reporter expression similar to that seen with the full-length 3'UTR (peGFP[ZCCHC11_3'UTR]; Fig. 3.4). This result suggests that the *cis*-acting negative regulatory elements of the 3'UTR of ZCCHC11 reside within its proximal half. In the dissection strategy as explained above, the lengths of the fragments were unequal. In order to rule out the possible contribution of the lengths of the fragments in determining the level of expression conferred on the reporter construct, Fragments 2, 3 and 4 were combined to generate a longer construct (Fig. 3.4). The combination of all of these fragments (peGFP[ZCCHC11_Fr234]), covering the distal half of the 3'UTR and overlapping with Fragment 1, was similar in length to Fragment 1. However none of the

combinations conferred a level of eGFP expression similar to that seen with Fragment 1 (Fig. 3.4). These results suggest that the negative effect of the 3'UTR of ZCCHC11 on gene expression was exerted by its proximal half, and is not simply a function of the length of this fragment. Interestingly, in contrast to its proximal half, the level of expression conferred by the distal half of the 3'UTR, especially by Fragment 2, was even higher than that seen with the peGFP control (Fig. 3.4). This suggested that although, under normal conditions, the 3'UTR destabilises ZCCHC11 mRNA through its proximal half, the contribution of the 3'UTR to gene expression is more complex, as the distal half conferred increased expression. These are the first indications that the 3'UTR of ZCCHC11 is potentially influenced by more than one factor, affecting gene expression either negatively or positively.

3.3.4. Individual AU-rich elements are not sufficient to determine the expression level mediated by the ZCCHC11 3'UTR

There are nine AU-rich elements in the sequence covered by Fragment 1; the distal three are also contained within Fragment 2, which confers increased gene expression. The proximal six AU-rich elements were therefore considered to be potentially responsible for the reduced gene expression conferred by Fragment 1. Site-directed mutagenesis experiments were performed, and these six elements were mutated individually within the full length ZCCHC11 3'UTR by mutating adenines to guanines and uracils to cytosines through transition mutations. The mutants were named AU-mut1 to AU-mut6, reflecting their proximity to the ORF (Fig. 3.5a). None of the individual mutations affected the level of gene expression conferred by the ZCCHC113'UTR, suggesting either that they are not involved in governing mRNA stability or that there is

redundancy in their function (Fig. 3.5b). Previous studies suggested that AREs may function in a redundant manner, and that the sequences flanking the AREs can be important for their function (Winzen et al. 2004). Therefore, a fragmentation approach was considered a more reasonable way of determining the minimal *cis*-acting element required for the negative regulatory function of ZCCHC11.

3.3.5. The common region between proximal and distal fragments of the ZCCHC11 3'UTR is dispensable for the negative effect on gene expression

Further attempts to identify the minimal *cis*-acting element destabilising the ZCCHC11 mRNA were carried out by sub-fragmentation of Fragment 1. Three overlapping sub-fragments were generated and inserted downstream from the eGFP ORF (Fig. 3.6). Flow cytometry experiments, performed as described previously in section 3.3.1, suggested that sub-fragment 1b conferred a level of gene expression similar to the one conferred by fragment 1. However sub-fragments 1a and 1c also exhibited a certain level of decrease in reporter gene expression, and the negative influence conferred by each of the sub-fragments was less than that seen with the full-length 3'UTR (Fig. 3.6). These results suggest that the *cis*-acting element(s) are not clustered in a particular region of Fragment 1; rather, the integrity of a wider region is required for the negative effect. Next, I investigated the importance of the common region of the 3'UTR of ZCCHC11 spanned by both negatively regulating Fragment 1 and positively regulating Fragment 234. I generated a construct where the eGFP ORF was ligated to the region which is only present in Fragment 1 (peGFP[ZCCHC11_Fr1only]). This relatively shortened Fragment 1

fully maintained the negative function observed with the Fragment 1 and the full-length 3'UTR, revealing the minimal *cis*-acting negative element in the ZCCHC11 3'UTR (Fig 3.6).

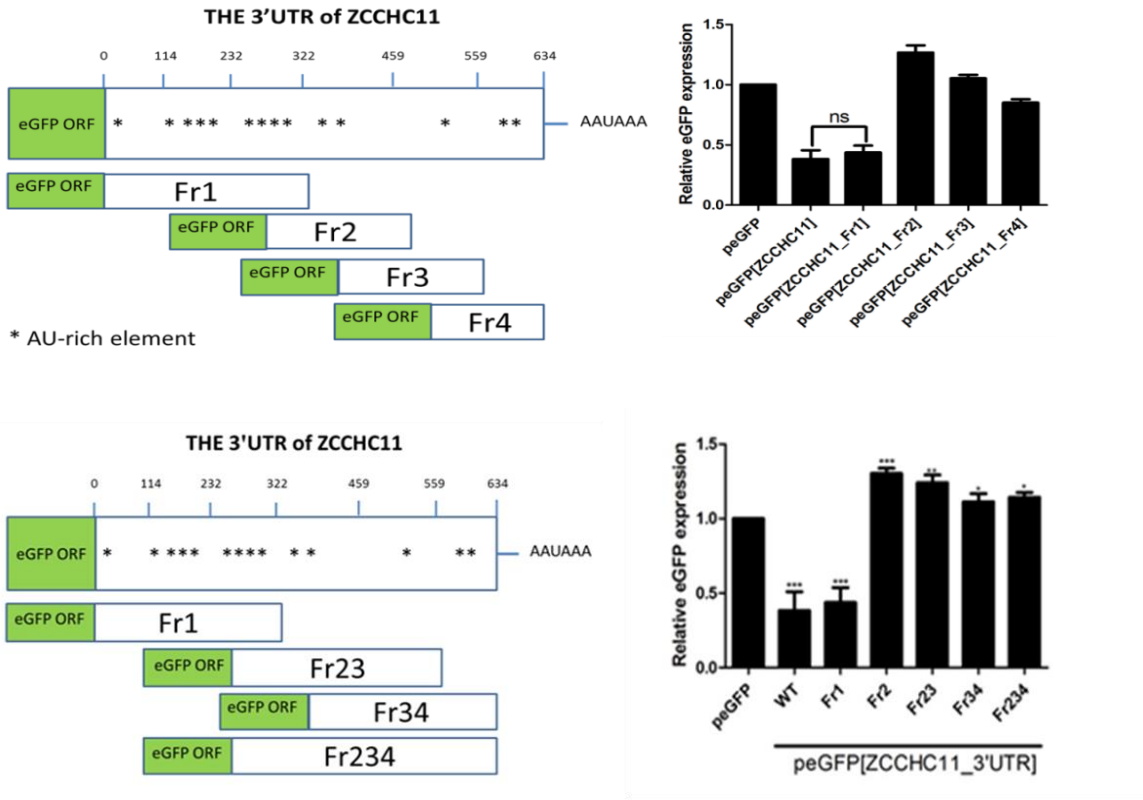
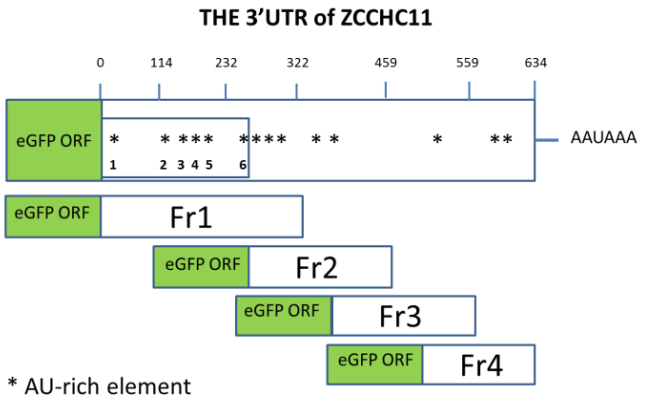


FIGURE 3.4. Fragmentation of the ZCCHC11 3'UTR.
Left panels are the schematic representation of the four fragments of the 3'UTR of ZCCHC11 or their combinations. Each fragment was ligated to the 3' end of eGFP ORF. eGFP ORFs are not to scale. Right panels are the eGFP expression of cells transfected with peGFP, peGFP[ZCCHC11_3'UTR], individual or combined fragments of the 3'UTR of ZCCHC11 ligated to eGFP ORF. The graphs represent the mean of the flow cytometry results of at least three independent experiments ±SD. eGFP expression conferred by the 3'UTR of ZCCHC11 or its fragments were compared to the expression conferred by the eGFP control, which value is normalised to one. Student's t-test was applied and p values were determined using peGFP as a reference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

a.



b.

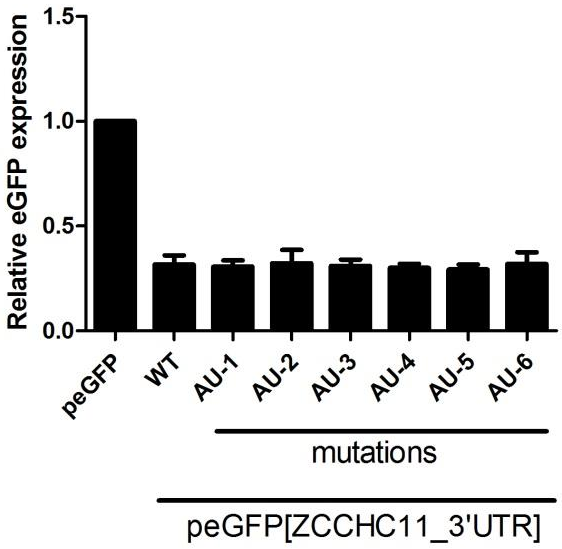


FIGURE 3.5. Individual mutations of AU-rich elements of the ZCCHC11 3'UTR.
a. Schematic representation of the ZCCHC11 3'UTR and the AREs mutated in this study. AREs mutated are only present in the region spanned by Fragment 1. Each ARE in this region was numbered from one to six (AU1-6). eGFP ORF is not to scale. **b.** HeLa cells were transfected with peGFP, wild type peGFP[ZCCHC11_3'UTR] or individual ARE transition mutations of the full length 3'UTR of ZCCHC11 ligated to the eGFP (AUUUA-> GCCCG, AUUUUA-> GCCCCG, AUUUU -> GCCCC). Expression of eGFP was estimated in live cells using flow cytometry (mean of at least three biological replicates ± SD). eGFP expression conferred by the 3'UTR of ZCCHC11 was compared to the expression conferred by the control, which value is normalised to one.

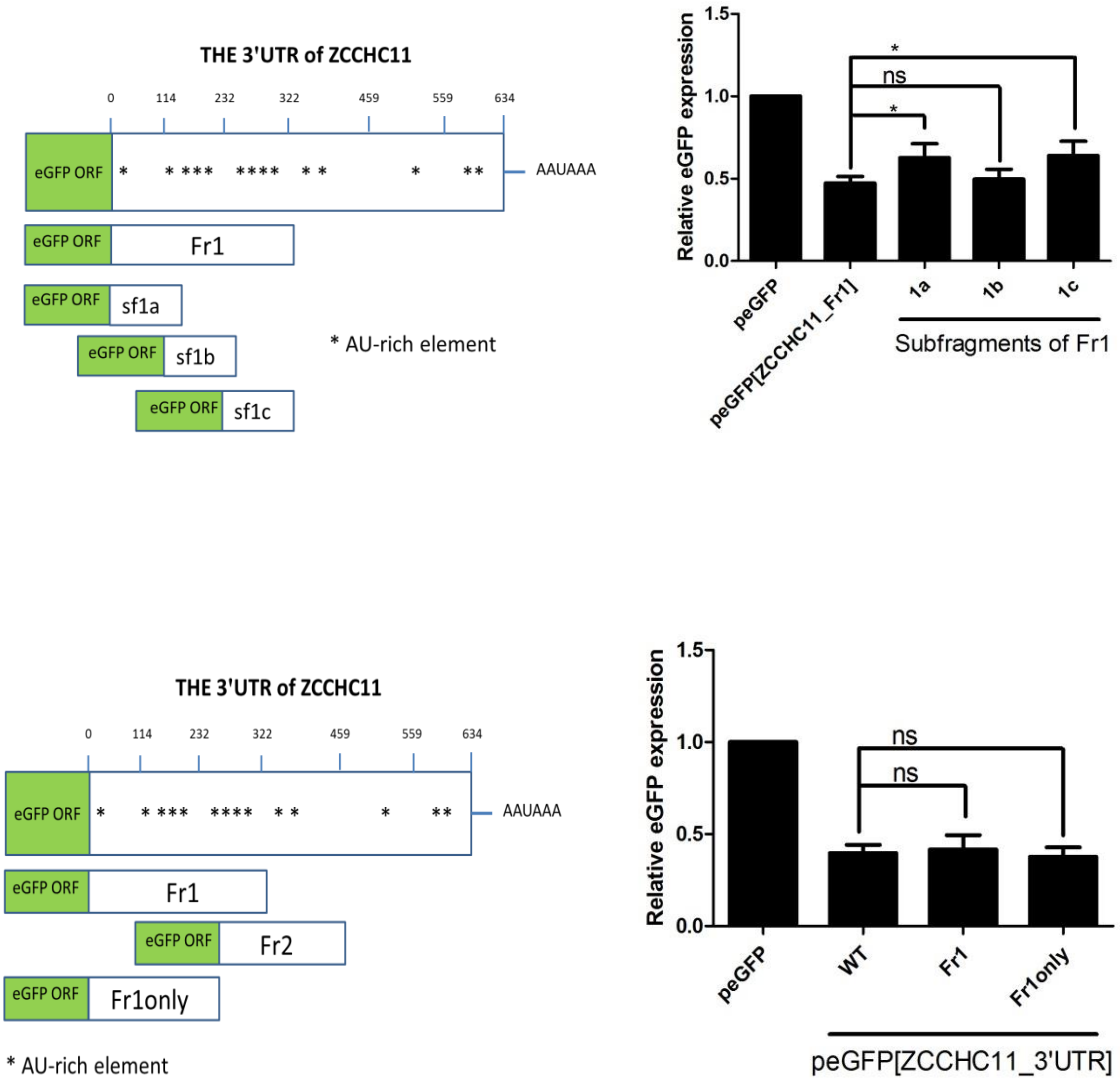


FIGURE 3.6. Investigation to identify the minimal *cis*-acting element that confers the 3'UTR-mediated decrease in gene expression.

Left hand panels are schematic representations of the three sub-fragments of the Fragment 1 of ZCCHC11 3'UTR and a shorter Fragment 1 (Fr1only). Each fragment was ligated to the 3' end of eGFP ORF. eGFP ORFs are not to scale. Right hand panels show eGFP expression of cells transfected with peGFP, peGFP[ZCCHC11_3'UTR], Fragment 1 (Fr1), Fr1 only and subfragments of Fragment 1 ligated to eGFP ORF. The graphs represent the mean of the flow cytometry results of at least three independent experiments ±SD. eGFP expression conferred by the 3'UTR of ZCCHC11, its fragments or subfragments was compared to the expression conferred by the eGFP control, which value is normalised to one. Student's t-test was applied and p values were determined using the peGFP control as a reference (ns= non-significant, * $p<0.05$,). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

3.3.6. miRNAs are not primarily responsible for mediating the decreased expression conferred by the ZCCHC11 3'UTR

miRNAs regulate gene expression mainly through the 3'UTRs of target genes. In order to understand if any miRNA is primarily responsible for mediating the negative effect of the ZCCHC11 3'UTR on gene expression, the region covered by Fragment 1 was analysed using the TargetScan miRNA database [http://www.targetscan.org]. A *miR-132/212* target site and a relatively less conserved *let-7* binding site were identified in this sequence (Fig 3.7a). Each site was mutated individually, but neither mutation was sufficient to alter reporter expression (Fig 3.7b). To investigate the influence of miRNAs more generally, cells were deprived of their capacity to produce mature miRNAs by knocking down Drosha, which is required for miRNA maturation (Fig. 3.7c). However, Drosha knock-down did not abrogate the negative effect of the ZCCHC11 3'UTR on reporter gene expression. On the contrary, perturbation of miRNA biogenesis led to a reduction in the level of GFP expression conferred by the 3'UTR. In order to understand if this further decrease in GFP expression is dependent on the presence of the 3'UTR, an analogous experiment was carried out in which the cells were transfected with peGFP, containing the minimal 3'UTR from the plasmid. Surprisingly, when Drosha was knocked down the peGFP-transfected cells showed an increase in eGFP expression, suggesting that the decrease in eGFP expression of peGFP[ZCCHC11_3'UTR]-transfected cells is specific to the presence of the ZCCHC11 3'UTR and the presence of *trans*-acting factor(s), which are regulated by miRNAs (Fig 3.7d).

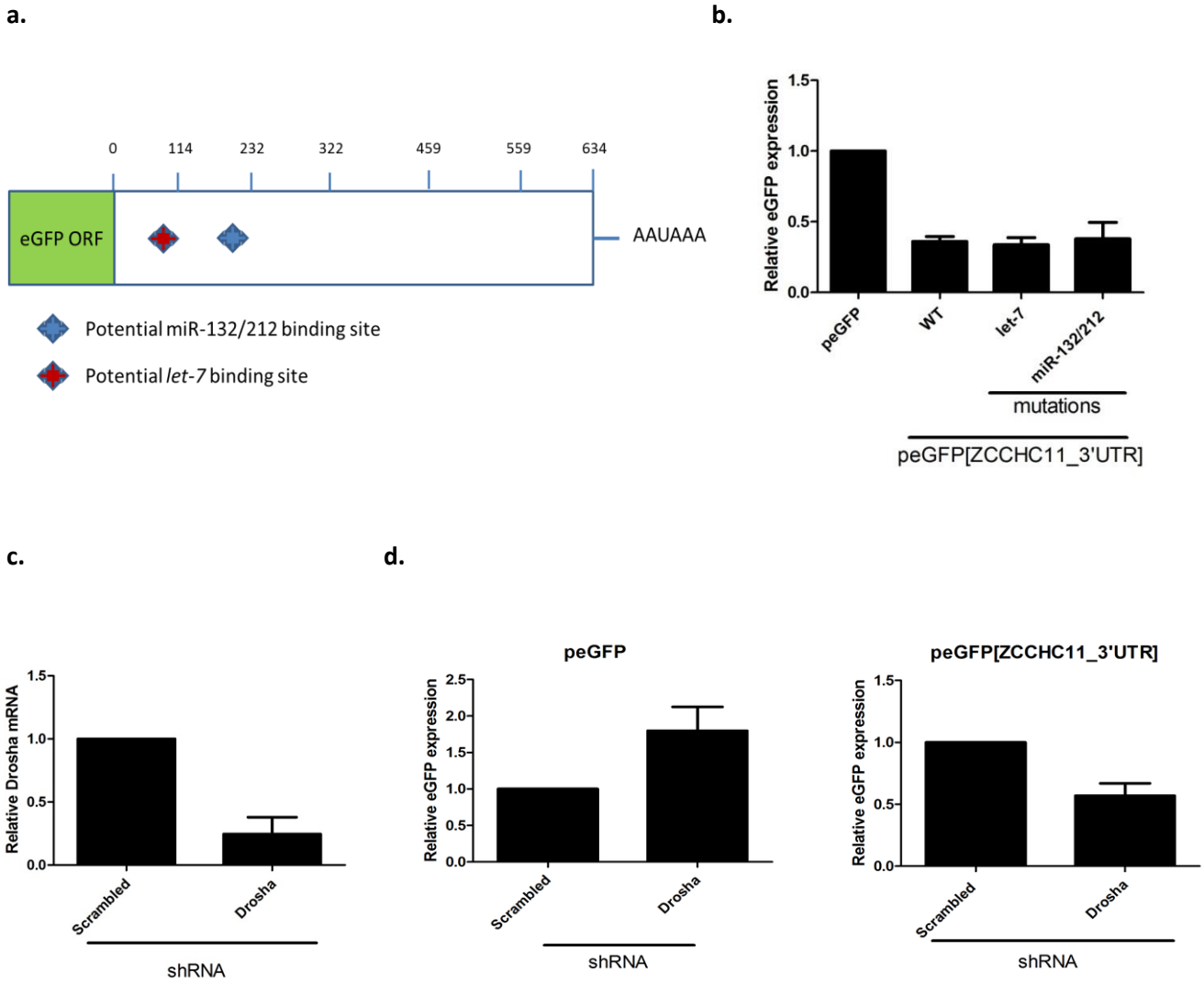


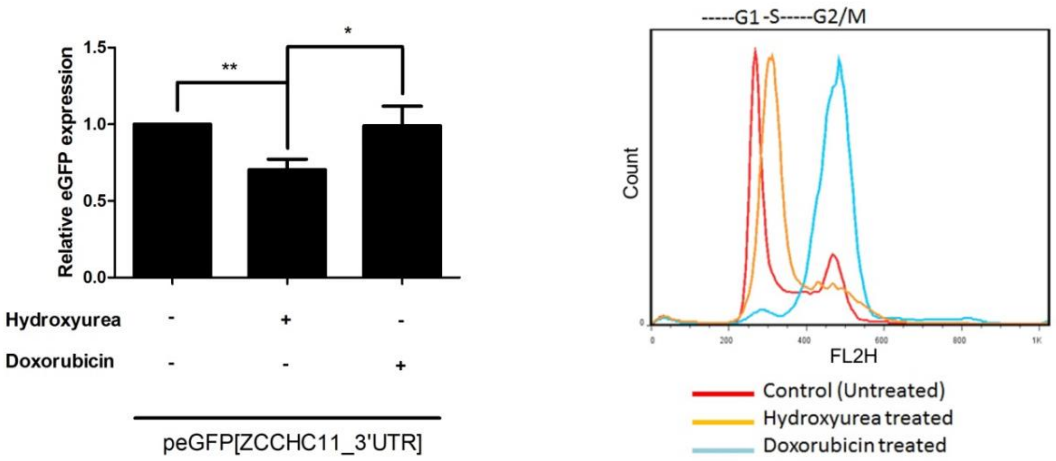
FIGURE 3.7. Investigation of the effect of miRNAs on ZCCHC11 expression.

a. Schematic representation of potential miRNA binding sites in the 3'UTR of ZCCHC11. eGFP ORF is not to scale. **b.** HeLa cells were used in this study. The graph represent the mean of the flow cytometry results of at least three independent experiments ±SD. eGFP expression conferred by the 3'UTR of ZCCHC11 and its mutants lacking the integrity of potential miRNA binding sites (*let-7*: UACCUCA -> UAUUCUG, *miR -132/212*: GACUGUU -> GAUCACC) were compared to the expression conferred by the eGFP control, which value is normalised to one. **c, d.** HeLa cells were transfected with plasmid encoding scrambled shRNA or shRNA against Drosha. The level of Drosha knockdown was quantified at mRNA level 72 hours after transfection **(c).** 36 hours after transfection cells were re-transfected with either peGFP or peGFP[ZCCHC11]. The graphs **(d)** represent the flow cytometry results of two independent experiments. eGFP expression in Drosha knock-down cells was compared to the expression in scrambled control cells, the value of which was normalised to one.

3.3.7. Hydroxyurea further decreases the expression conferred by the ZCCHC11 3'UTR

ZCCHC11 expression, as previously shown in our laboratory, fluctuates during S phase (Schmidt, West, and Norbury 2011) in a manner that might be important in histone mRNA regulation. To understand whether the ZCCHC11 3'UTR affects expression of eGFP differentially during S phase, cells that were transfected with peGFP[ZCCHC11_3'UTR] or peGFP[β-Actin_3'UTR] were arrested in S phase using 2mM hydroxyurea or in G2 using 150 nM doxorubicin for comparison (Fig. 3.8a). β-actin is constitutively expressed throughout the cell cycle, and its mRNA stability is not modified between cell cycle phases. Therefore, while comparing ZCCHC11 3'UTR-mediated GFP expression levels in S phase-arrested, G2-arrested or asynchronous cell populations, the values were divided by normalised values of eGFP expression from peGFP[β-Actin_3'UTR] from S phase-arrested, G2-arrested, or asynchronous cells. Flow cytometry results suggest that the 3'UTR of ZCCHC11 conferred decreased stability on the GFP reporter mRNA in hydroxyurea-treated cells compared with doxorubicin-treated cells and asynchronous cells. However when untreated (asynchronous) and doxorubicin-treated cells were compared, the 3'UTR of ZCCHC11 did not confer any significant differential GFP expression in either group when normalised to peGFP[β-Actin]-transfected cells (Fig. 3.8a). The effect of arresting cells in S-phase on endogenous ZCCHC11 levels was also investigated at the protein level. Treating HeLa cells with 2 mM hydroxyurea resulted in a modest decrease in ZCCHC11 protein (Fig. 3.8b). In addition, similar results were obtained by flow cytometry in an analogue experiment, in which the results were normalised to peGFP-transfected cells instead of peGFP[β-Actin_3'UTR] (Fig. 3.8c).

a.



b.

c.

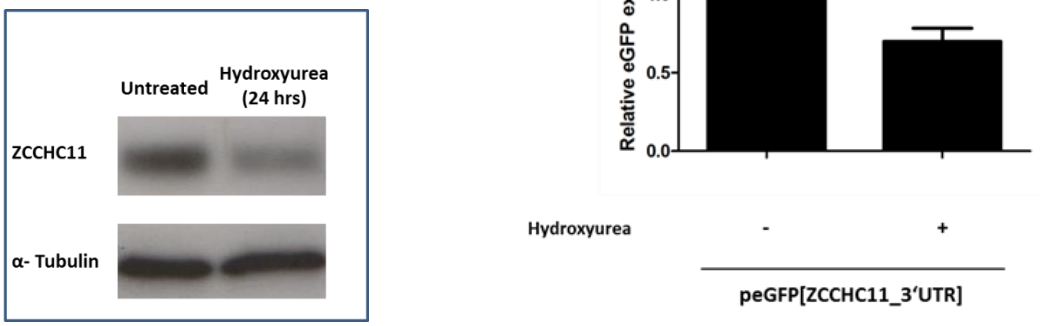


FIGURE 3.8. S-phase arrest-dependent regulation through the 3'UTR of ZCCHC11.
a. HeLa cells were transfected with peGFP[β-Actin_3'UTR] and peGFP[ZCCHC11_3'UTR]. One day after transfection, the cells were treated with 2 mM Hydroxyurea to arrest in S phase, 150 nM doxorubicin to arrest in G2, or were left untreated. 48 hours after transfection, cells were fixed with paraformaldehyde, stained with PI and analysed using flow cytometry. Upper graph represents the ratio of peGFP[ZCCHC11_3'UTR] expression of cell-cycle arrested groups to untreated group after normalisation against the same ratio in peGFP[β-Actin_3'UTR] expressing cells. This result is the mean of at least three independent biological replicates. Error bars represent standard deviations. Right panel is the PI staining of a demonstrative experiment (FL2-H). It shows the cell cycle distribution of the cells used in this study. **b.** Immunoblot showing ZCCHC11 expression after 2 mM hydroxyurea treatment for 24 hours. The immunoblot is representative of at least three independent experiments. **c.** Same experiment as a. with pEGFP and peGFP[ZCCHC11_3'UTR] (n=2)

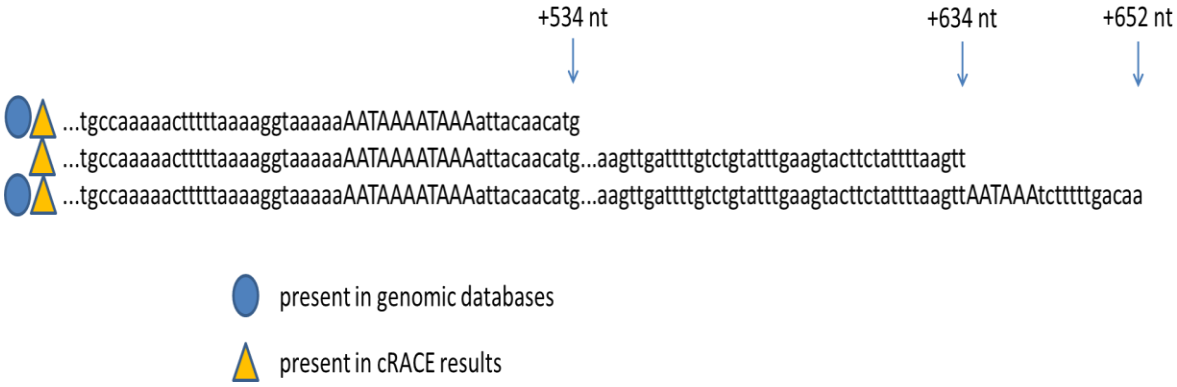
3.3.8. Alternative polyadenylation of ZCCHC11

In addition to the regulation through the 3'UTR by AUBPs or miRNAs, gene expression may also be regulated by alternative cleavage / polyadenylation. The plasmid constructs containing the ZCCHC11 3'UTR excluded the mRNA endogenous distal hexanucleotide cleavage signal (AAUAAA). Therefore, the AAUAAA sequence presumed to be used was derived from the plasmid backbone. However, proximal to this signal, the 3'UTR of ZCCHC11 has another tandem polyadenylation signal, AAUAAAAUAAA (Fig. 3.9). Genomic databases also reveal the active involvement of this proximal sequence in polyadenylation. Therefore, ZCCHC11 is potentially regulated through alternative polyadenylation. In order to characterise the 3' ends of the ZCCHC11 mRNAs, a cRACE approach was used (this method also aimed to investigate the 5' ends of the message, which will be discussed in Chapter 4). In the cRACE method, the 5' and 3' ends of an mRNA are ligated together following enzymatic removal of the 5' cap, and the sequences at the ends of the message are identified following selective PCR amplification of the region spanning the ligation site. This experiment aimed to finely characterise the 5' and 3' of ZCCHC11 mRNA in the cells used in this study. The results showed that endogenous ZCCHC11 has different mRNA isoforms, which differ in their 3' ends, suggesting the active involvement of alternative polyadenylation in generating ZCCHC11 transcripts. Interestingly, three different mRNA 3' ends were identified. Two of them match the isoforms annotated in current genomic databases (Fig. 3.9a). The proximal polyadenylation signal (PPS), AAUAAAAUAAA sequence was mutated to AAUCGCGUAAA in peGFP[ZCCHC11_3'UTR] to generate peGFP[ZCCHC11_3'UTR_PPS_mut], and a qRT-PCR approach was used to analyse mRNA

isoforms after transfection of these plasmids into HeLa cells. Two pairs of primers were used: the proximal primer pair spans a region in the eGFP ORF and the distal pair spans a region covering sequences distal to the proximal AAUAAAAUAAA (Fig. 3.9b). This experiment aimed to understand whether alternative polyadenylation affects ZCCHC11 gene expression. The levels of eGFP mRNAs that either comprise wild-type 3'UTR of ZCCHC11 or a mutant version lacking the proximal AAUAAAAUAAA sequence were quantified by using the proximal pair of primers to quantify both shorter and longer isoforms. Mutating the proximal AAUAAAAUAAA sequence resulted in a modest decrease in eGFP mRNA level, as shown by using the proximal pair of primers, and a relatively significant increase in the proportion of the longer isoform to the shorter isoform, as shown comparing the ratio of mRNAs amplified by the distal pair of primers to in comparison with the proximal pair. Given that the distal pair of primers also amplifies the endogenous ZCCHC11 mRNA, peGFP-transfected cells were analysed in parallel and the small value of endogenous mRNA amplified by the distal primer pair was subtracted before comparison (Fig. 3.10a). These results suggest that the proximal polyadenylation signal is functionally active and that a *cis*-acting element distal to this signal actively down-regulates ZCCHC11 mRNA levels. miRNA binding sites distal to proximal AAUAAAAUAAA signals were previously suggested to be responsible for the down-regulation of alternatively polyadenylated genes (Mayr and Bartel 2009). The Targetscan miRNA database shows a potential miRNA binding site of *miR-26* in this region (Fig. 3.9b). In order to understand whether *miR-26*, whose function is inhibited by ZCCHC11 (Jones et al. 2009), inhibits the expression of ZCCHC11, peGFP[ZCCHC11_3'UTR_*mir26*mut] plasmid including a disrupted potential binding site of *miR-26* was used. However, peGFP[ZCCHC11_3'UTR_*mir26*mut] did not confer

increased eGFP expression in comparison to the 3'UTR of ZCCHC11, suggesting that *miR-26* is not primarily responsible for the down-regulation of ZCCHC11 through its 3'UTR, at least under normal conditions (Fig. 3.10b). Further studies are required to determine the negative *cis* and *trans* regulatory factors acting distally to the proximal AAUAAAAUAA sequence.

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b.

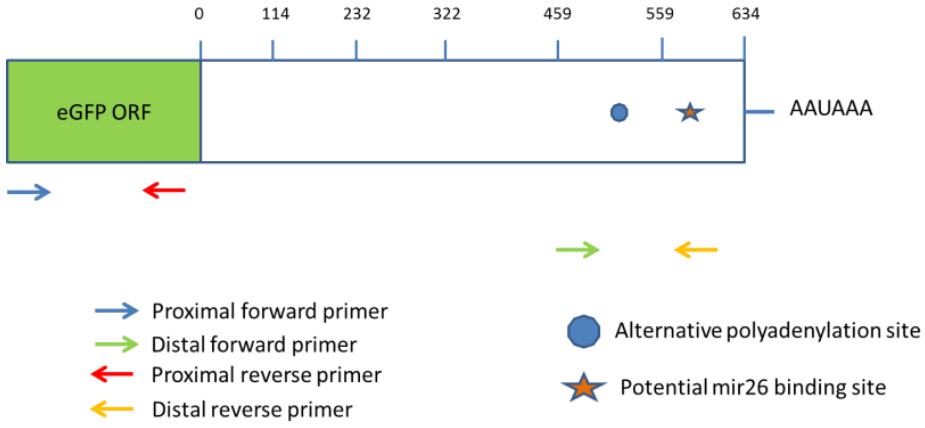
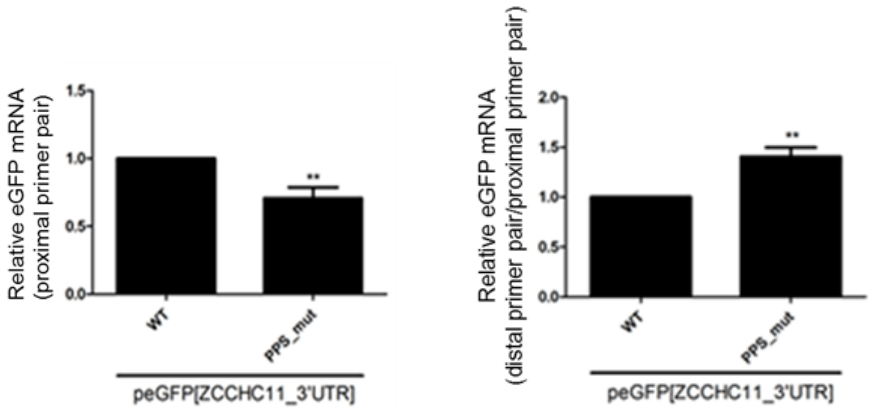


FIGURE 3.9. 3' end of ZCCHC11.
a. The sequence of the variants of 3' end of ZCCHC11. The mRNA isoforms annotated in genomic databases and those found in this study are shown. The number above the sequences represent the distance in nucleotides from the stop codon. **b.** Schematic representation of the 3'UTR of ZCCHC11 including the alternative polyadenylation site, forward and distal primer pairs used in qRT-PCR and potential *mir-26* binding site. eGFP ORF is not to scale.

a.



b.

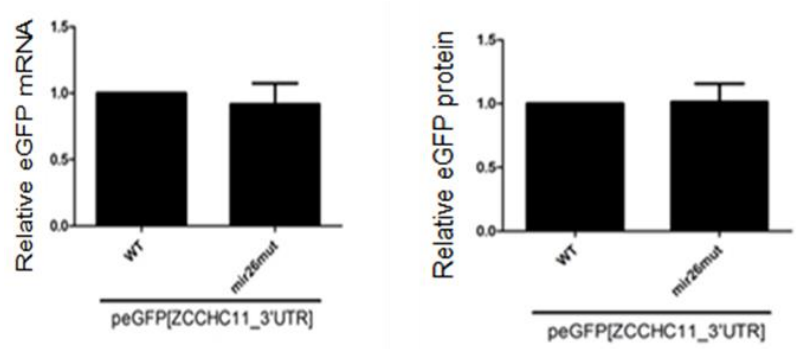


FIGURE 3.10. Investigating the effect of mutating the proximal polyadenylation site and potential *mir-26* binding site on ZCCHC11 expression.

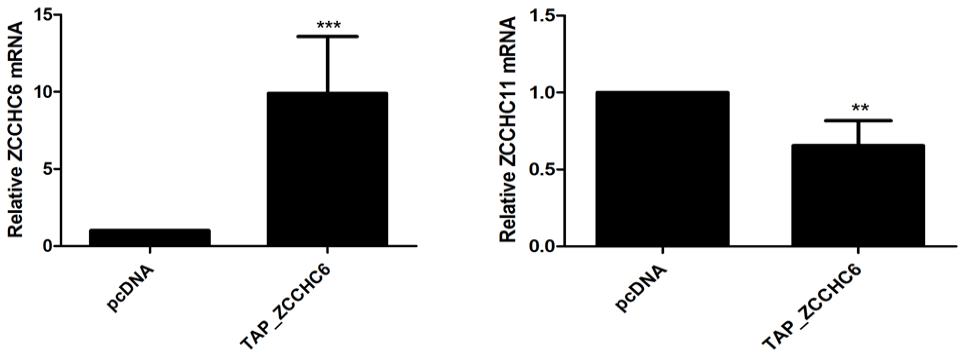
a. The graphs represent the qRT-PCR values of the cells transfected with either peGFP[ZCCHC11_3'UTR] or peGFP[ZCCHC11_3'UTR_PPS_mut] The graphs represent the mean of at least three independent experiments ±SD. The eGFP mRNA expression of peGFP[ZCCHC11_3'UTR_PPS_mut] amplified by the proximal primer pair (left) or proportion of the mRNA amplified by proximal primer pair to distal pair (right) were compared to the corresponding expression values of peGFP[ZCCHC11_3'UTR] , which value is normalised to one. **b.** eGFP expression quantified using qRT-PCR (left) and flow cytometry (right) conferred by peGFP[ZCCHC11_3'UTR_*mir26*mut] was compared to peGFP[ZCCHC11_3'UTR], which values are normalised to one. Student's t-test was applied and p values were determined using the peGFP[ZCCHC11_3'UTR] control as a reference (ns= non-significant, ** $p<0.01$,). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

3.3.9. Functional interplay between ZCCHC6 and ZCCHC11

The human terminal uridyl transferase ZCCHC6 is an orthologue of ZCCHC11; the two proteins share extensive sequence similarity (Fig. 1.1.), and have partially redundant functions in regulating pre-*let-7* levels. Considering its role as a regulatory protein and its intimate function with ZCCHC11, I investigated whether ZCCHC6 regulates ZCCHC11 expression. In order to find out if such regulation exists, I overexpressed TAP-tagged ZCCHC6. Cells overexpressing ZCCHC6 exhibited a decrease in endogenous ZCCHC11 mRNA (Fig. 3.11a). ZCCHC6 is likely to regulate gene expression through modification of RNA 3' ends. I therefore hypothesised that the effect of ZCCHC6 on ZCCHC11 expression could involve its 3'UTR. To test this hypothesis, I used peGFP[ZCCHC11_3'UTR]. peGFP, which has a minimal 3'UTR was used as control in this study, and the results were normalised to the peGFP-transfected group. Cells overexpressing ZCCHC6 exhibited even further decrease in eGFP expression in peGFP[ZCCHC11_3'UTR] transfected cells. This result suggested the importance of the 3'UTR of ZCCHC11 in the regulation involving ZCCHC6 (Fig. 3.11b). Interestingly, when the cells were transfected with peGFP[ZCCHC11_3'UTR], endogenous ZCCHC6 mRNA expression decreased (Fig 3.11b). Thus, the functional interaction between ZCCHC6 and ZCCHC11 is complex. To explain and further investigate this interplay, I developed a hypothetical model in which ZCCHC6 regulates ZCCHC11 through a mechanism involving the 3'UTR of ZCCHC11. In this model, an unknown protein with positive regulatory function regulates ZCCHC11 through its 3'UTR, and the same protein also regulates ZCCHC6 expression. When the cells are transfected with peGFP[ZCCHC11_3'UTR], the high copy-number of constructs expressing the 3'UTR of ZCCHC11 results in the titration of this unknown protein, hence

its positive effect on ZCCHC6 is diminished. This causes a decrease in ZCCHC6 expression (Fig 3.12).

a.



b.

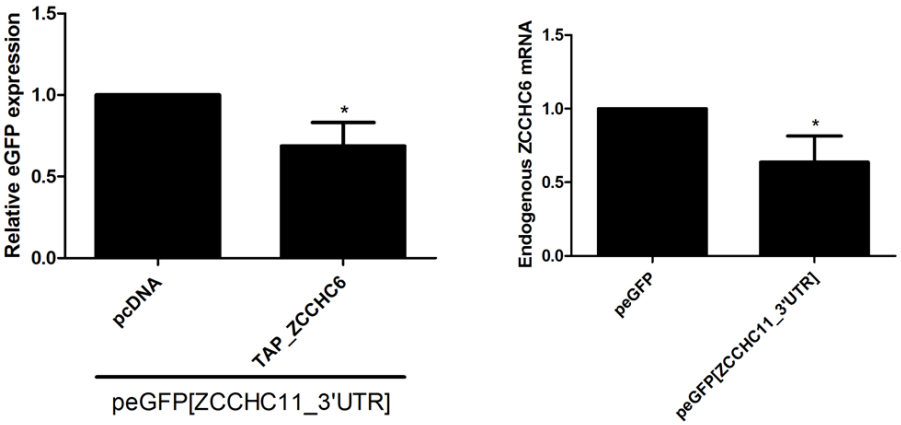


FIGURE 3.11. The interplay between ZCCHC6 and ZCCHC11.

HeLa cells were transfected with pcDNA or pcDNA[ZCCHC6-TAP]. a. Overexpression of ZCCHC6 was confirmed by qRT-PCR (left panel). The effect of ZCCHC6 overexpression was investigated using qRT-PCR. b. HeLa cells transfected with pcDNA or pcDNA TAP_ZCCHC6 were further transfected with peGFP or peGFP[ZCCHC11_3'UTR] 24 hours later. Left panel shows relative eGFP expression at the mRNA level after normalisation to the control peGFP transfected cells. Right panel shows the effect of peGFP[ZCCHC11_3'UTR] transfection on endogenous ZCCHC6 mRNA levels. The value of peGFP transfected group was normalised to one. Student's t-test was applied and p values were determined using the control group in each figure as reference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

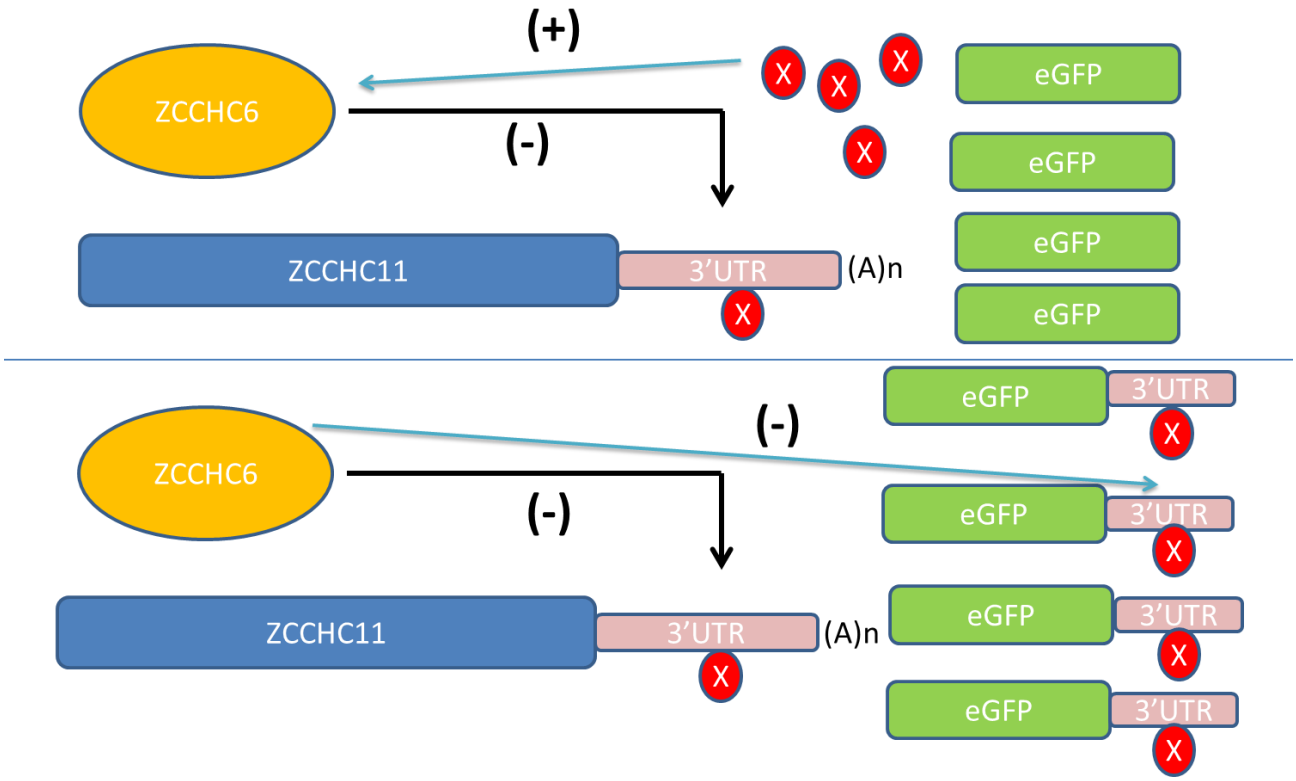


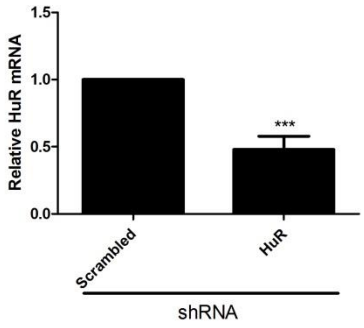
FIGURE 3.12. Hypothetical model of the interplay between ZCCHC6 and ZCCHC11.
ZCCHC6 negatively regulates ZCCHC11 through its 3'UTR. Hypothetical protein 'X' regulates both ZCCHC6 and ZCCHC11 positively. When the cells are transfected with peGFP[ZCCHC11_3'UTR] and high levels of ZCCHC11 3'UTR are present, protein 'X' is titrated away and cannot regulate ZCCHC6, resulting in its down-regulation.

3.3.10. The AUBP HuR positively regulates ZCCHC6 and ZCCHC11 mRNAs

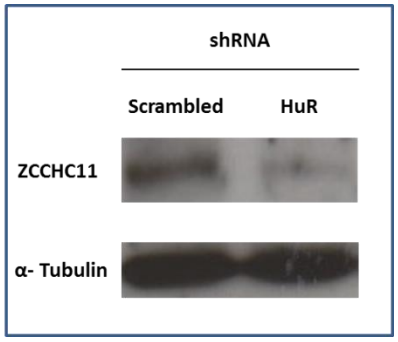
My previous experiments suggested that although the ZCCHC11 3'UTR conferred decreased expression under normal conditions and S phase arrest, the distal half of the 3'UTR of ZCCHC11 positively regulates gene expression. Furthermore, the analysis of ZCCHC6 and ZCCHC11 functional interplay hinted at the existence of a protein with a positive regulatory impact on the ZCCHC11 3'UTR. Taking these points into consideration, I investigated potential proteins that might bind and regulate ZCCHC11 expression positively. As discussed previously, most of the literature regarding ARE-AUBP interactions concerns AUBPs that negatively regulate gene expression. HuR is arguably the most widely studied protein in terms of positive regulation of gene expression through 3'UTRs. I therefore considered HuR as a candidate positive regulator of ZCCHC11 and ZCCHC6. HuR was knocked down with an shRNA (Fig. 3.13a). Decreasing the expression of HuR in HeLa cells resulted in a decrease in both ZCCHC6 and ZCCHC11 mRNA levels (Fig 3.13b). The impact of HuR knock-down on c-myc expression was used as control in this study, as c-myc is one of the relatively few genes known to be negatively regulated by HuR (Kim et al. 2009). Knocking down HuR resulted in an increase in c-myc mRNA expression as expected (Fig. 3.13b). I investigated the effect of HuR on the regulation of ZCCHC11 in further detail by studying the influence of decreased HuR expression on ZCCHC11 protein levels. ZCCHC11 protein levels were decreased following HuR knock-down (Fig 3.13c). Next, the cells were transfected with Flag-HuR to investigate the effect of overexpression of HuR on ZCCHC6 and ZCCHC11 (Fig 3.14a). A similar experimental design was followed. In a confirmatory manner, ZCCHC6

and ZCCHC11 mRNA levels increased and c-myc expression decreased in cells overexpressing HuR (Fig. 3.14b). In addition, ZCCHC11 protein levels were increased under these conditions (Fig. 3.14c). These results suggest that HuR affects both ZCCHC6 and ZCCHC11 expression positively and the positive effect of HuR on ZCCHC11 gene expression is exerted, at least in part, through its effect on ZCCHC11 mRNA levels.

a.



c.



b.

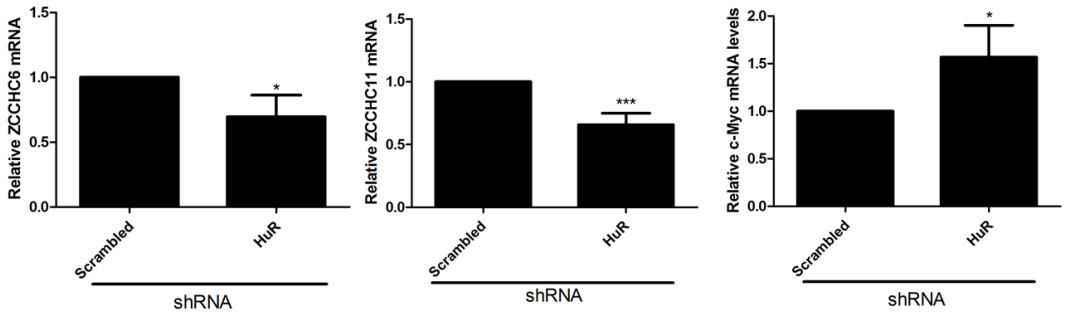


FIGURE 3.13. The effect of HuR knockdown on ZCCHC6 and ZCCHC11 expression.
HeLa cells were used to investigate the effect of HuR on ZCCHC11 levels. p values were determined using the control groups in each figure as reference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, $n\geq3$) **a.** Knockdown efficiency of HuR was confirmed at mRNA level by qPCR. **b.** The effect of HuR knockdown on ZCCHC6 (left panel), ZCCHC11(middle panel) and c-myc(right panel) mRNA levels. **c.** Immunoblot showing the effect of knockdown of HuR on ZCCHC11 expression. α-tubulin was used as a loading control. The immunoblot is representative of at least three independent experiments.

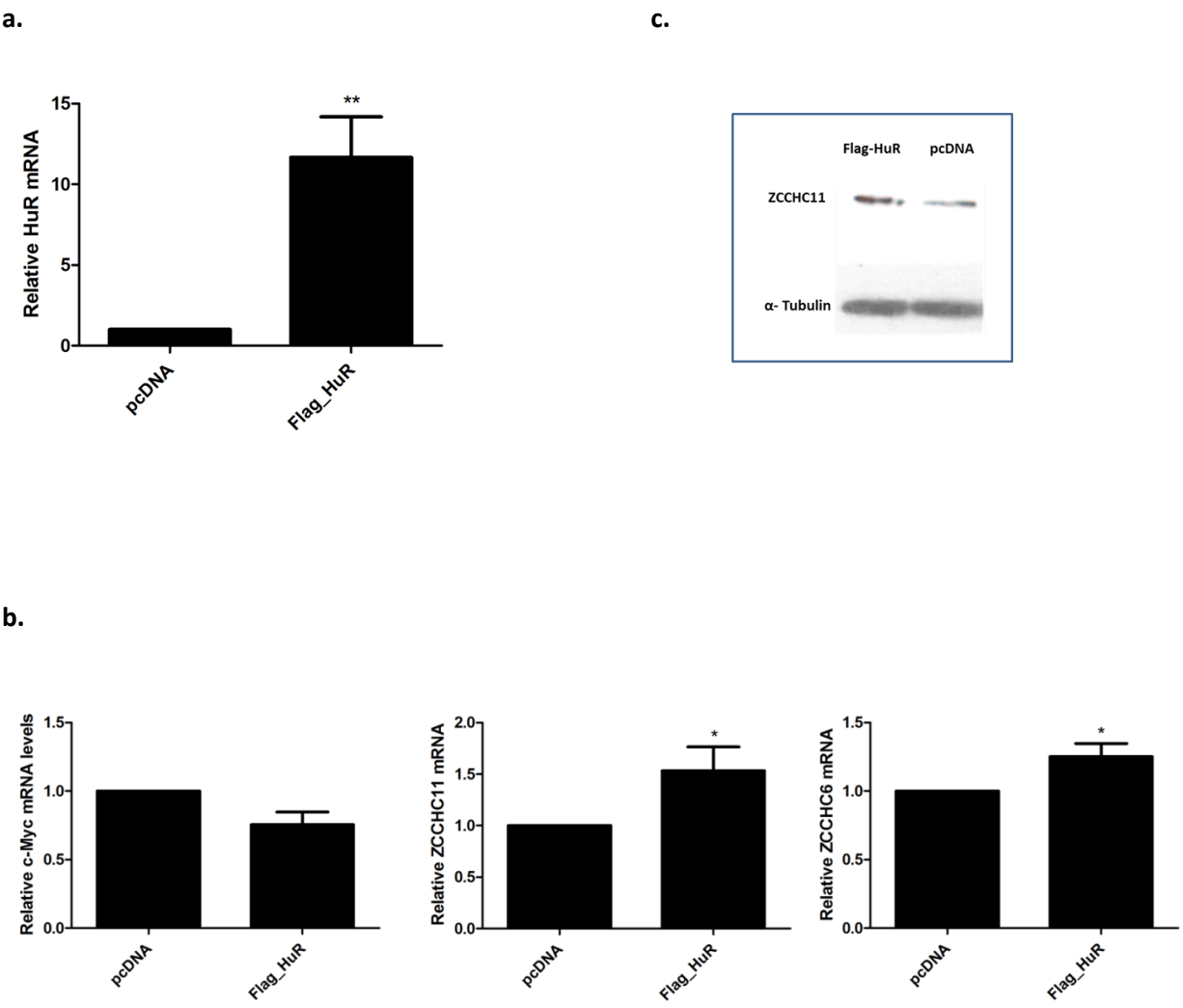
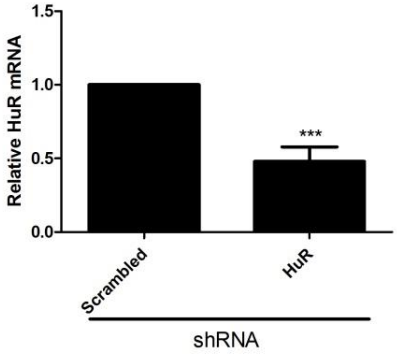


FIGURE 3.14. The effect of HuR overexpression on ZCCHC6 and ZCCHC11 expression.
HeLa cells were used to investigate the effect of HuR on ZCCHC11 levels. p values were determined using the control groups in each figure as reference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, $n\geq3$) **a.** Overexpression of HuR was confirmed at mRNA level by qPCR. **b.** The effect of HuR overexpression on ZCCHC6 (left panel), ZCCHC11(middle panel) and c-myc(right panel) mRNA levels. **c.** Immunoblot showing the effect of overexpression of HuR on ZCCHC11 expression. α-tubulin was used as a loading control. The immunoblot is representative of at least three independent experiments.

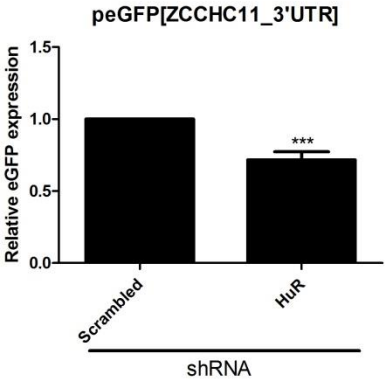
3.3.11. Regulation of ZCCHC11 by HuR involves the ZCCHC11 3'UTR

Most studies of HuR suggest that it regulates gene expression through interaction with mRNA 3'UTRs. I have shown that ZCCHC11 is regulated positively by HuR at the mRNA and protein levels. In order to understand if this regulation is exerted by the effect of HuR on the 3'UTR of ZCCHC11, HuR was knocked down (Fig. 3.15a). Cells transfected with either scrambled control shRNA or shRNA against HuR were further transfected with peGFP or peGFP[ZCCHC11_3'UTR]. Flow cytometry showed that knocking down HuR resulted in a further decrease in eGFP levels conferred by the ZCCHC11 3'UTR, when the data were normalised to those obtained using peGFP-transfected cells (Fig. 3.15b). This result suggested that HuR affects ZCCHC11 expression through its effect on the ZCCHC11 3'UTR. My previous findings suggested that, although the overall effect of the 3'UTR of ZCCHC11 on gene expression is negative, the distal half of the 3'UTR of ZCCHC11 regulates gene expression positively. In order to understand if the effect of HuR on the ZCCHC11 3'UTR is through a particular region of the sequence, a similar experimental design was followed. Knocking down HuR resulted in a decrease in eGFP expression conferred by the distal half of the 3'UTR of ZCCHC11 (peGFP[ZCCHC11_Fr234]). Interestingly, depletion of HuR also resulted in a further decrease in eGFP expression under control of the proximal half of the 3'UTR (peGFP[ZCCHC11_Fr1only]; Fig. 3.15c). These results suggest that the effect of HuR on the 3'UTR of ZCCHC11 involves *cis*-acting elements in both halves of the 3'UTR. The biological mechanisms underlying the regulation through each half of the 3'UTR may well be different and require further study.

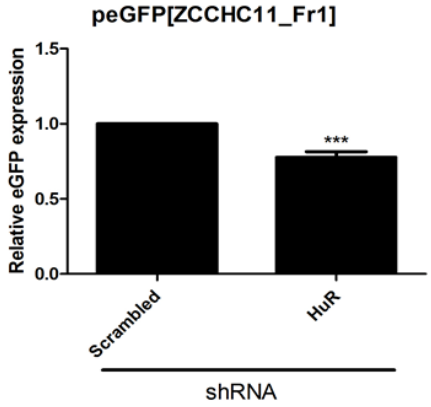
a.



b.



c.



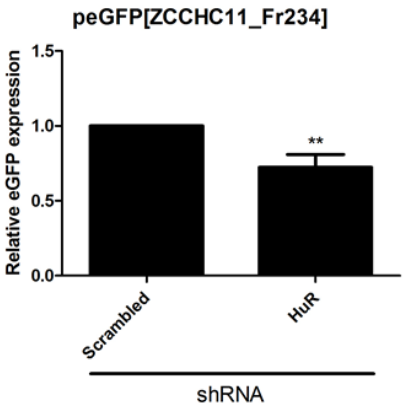


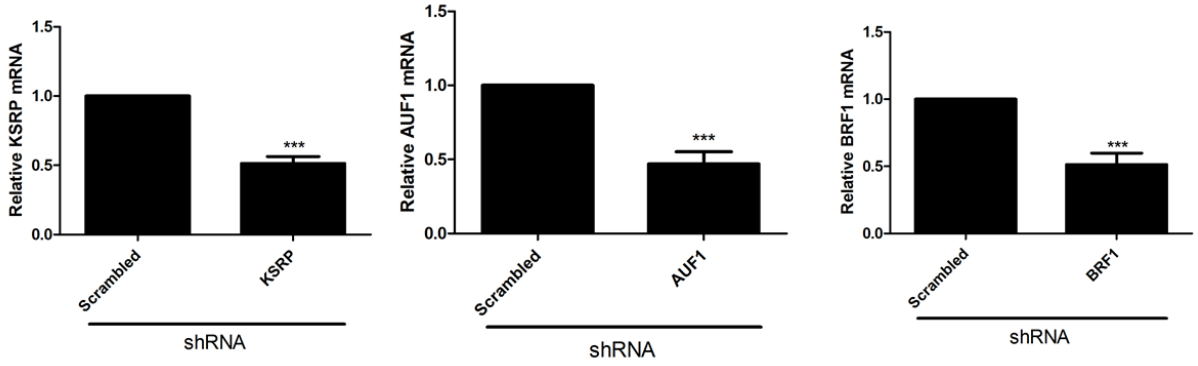
FIGURE 3.15. The effect of HuR on the 3'UTR of ZCCHC11 or its fragments.
HeLa cells were transfected with scrambled control shRNA or shRNA against HuR. 48 hours after transfection they are retransfected with peGFP, peGFP[ZCCHC11_3'UTR] (**b**), peGFP[ZCCHC11_3'UTR_Fr1] (**c**) or peGFP[ZCCHC11_3'UTR_Fr234](**c**) **a**. Knockdown of HuR was confirmed by qRT-PCR . **b-c.** The graphs show relative eGFP expression of cells retransfected with peGFP[ZCCHC11_3'UTR] (**b**), peGFP[ZCCHC11_3'UTR_Fr1] (**c**) or peGFP[ZCCHC11_3'UTR_Fr234](**c**), quantified by flow cytometry after normalisation to the control peGFP transfected cells. The eGFP expression value of HuR knockdown groups were then normalised to the values obtained with scrambled control transfected cells. Student's t-test was applied and p values were determined using the control group in each figure as reference (** $p<0.01$, *** $p<0.001$). Co-transfecting a pcDNA_TAP plasmid with eGFP reporter plasmids, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

3.3.12. Attempts to find the negative regulatory factor affecting the ZCCHC11 3'UTR

Although the effect of HuR is to regulate ZCCHC11 positively through its 3'UTR, the overall impact of the 3'UTR of ZCCHC11 on gene expression, at least under normal circumstances, is mRNA destabilisation. I therefore aimed to identify the *trans*-acting factor that confers this mRNA instability. Considering the results presented in section 3.3.5, it is unlikely that a miRNA is primarily responsible for a direct regulation of ZCCHC11 through its 3'UTR. ZCCHC11, having many AU-rich elements in its 3'UTR, is a candidate for regulation by ARE-AUBP interaction. I therefore followed a strategy of knocking down AUBPs identified previously as proteins mainly interacting with 3'UTRs and destabilising them. The pSuperior knock-down system was used to generate shRNAs impacting these proteins, using when possible siRNA sequences published previously (Appendix D) as target sequence. AUF1, KSRP and BRF1 were targeted, as they are arguably the most widely studied in terms of regulating gene expression negatively through ARE-AUBP interactions (Fig 3.16a). Each of these proteins was knocked down individually. The effect of knock-down of these proteins on endogenous ZCCHC11 expression was investigated by qRT-PCR. However, none of the individual knock-downs resulted in an increase in endogenous ZCCHC11 mRNA levels (Fig. 3.16b), suggesting that none of the proteins investigated is primarily responsible for the destabilising effect of the ZCCHC11 3'UTR, at least individually. Knocking down TTP was not efficient using the pSuperior system, so a siRNA-mediated knock-down approach was adopted. Although the knock-down by siRNA was not very efficient (Fig 3.17a), it resulted in a modest but significant increase in ZCCHC11 mRNA levels (Fig 3.17b). Next, it was aimed to

understand if the effect of TTP was through the ZCCHC11 3'UTR. Knocking down TTP resulted in increased eGFP expression conferred by the 3'UTR of ZCCHC11, suggesting that TTP contributes to the negative regulation of ZCCHC11 through its 3'UTR (Figure 3.17c).

a.



b.

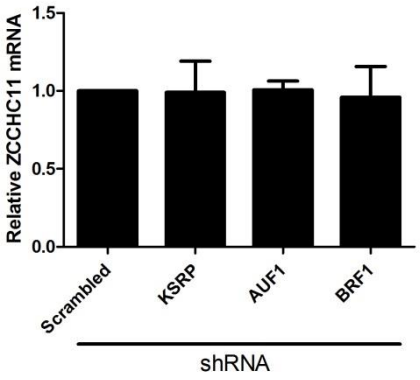
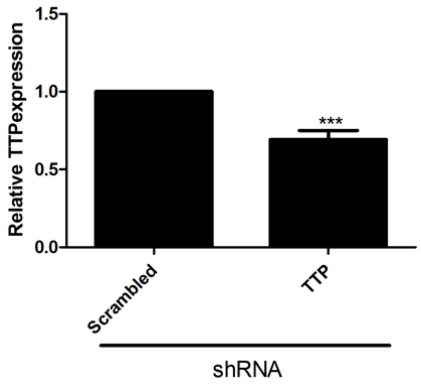
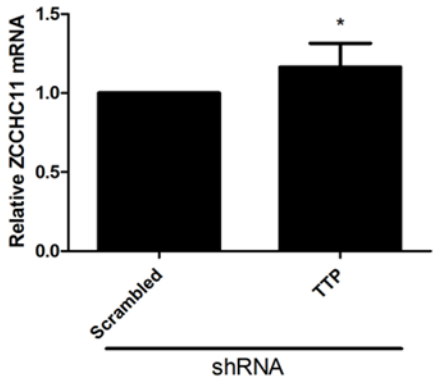


Figure 3.16. Investigation of AUBPs that regulate ZCCHC11 expression negatively.
HeLa cells were either transfected with plasmids encoding shRNA against AUBPs KSRP, AUF1 or BRF1, or with a scrambled control shRNA. Cells were incubated for 72 hours. The results were normalised to the group transfected with scrambled control shRNA, the value of which is to one. Student's t-test was applied and p values are determined with respect to the scrambled control ($n \geq 3$, *** $p<0.05$,). **a**. Quantification of knock down efficiency was performed using qRT-PCR. **b**. The effect of the knockdown of AUBPS on ZCCHC11 expression was investigated at the mRNA level.

a.



b.



c.

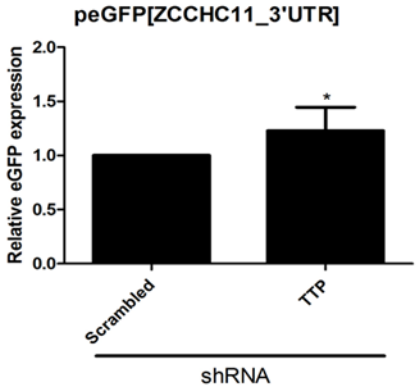


Figure 3.17. The effect of TTP on the regulation of ZCCHC11 expression

HeLa cells were transfected with siRNA against TTP or with a scrambled control siRNA. The cells were incubated for 48 hours. The results of the TTP knock-down group were normalised to the scrambled control group. p values were determined with respect to the scrambled control (ns= nonsignificant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). **a**. The efficiency of knock-down was measured using qRT-PCR. **b**. The effect of TTP knockdown on ZCCHC11 mRNA levels was measured using qRT-PCR. **c**. 24 hours after siRNA transfection cells were transfected with peGFP and peGFP[ZCCHC11_3'UTR]. The graph demonstrates the effect of TTP knockdown on peGFP[ZCCHC11_3'UTR] after normalisation against the peGFP transfected group.

3.4. Discussion

In recent years, ZCCHC11 has been identified as a key protein in the regulation of several RNAs crucial for human physiology and disease. Nevertheless, very little is known about the regulation of this regulatory protein. Understanding the mechanisms of its regulation is especially important considering its role in cancer biology and its potential importance as a therapeutic target in the future. The ZCCHC11 3'UTR contains conserved miRNA binding sites and a number of AREs, and is therefore a potentially important element in the regulation of ZCCHC11. The results discussed in this chapter reveal the role of the 3'UTR of ZCCHC11 in regulating its expression.

Regulation of the elements in *let-7* biogenesis through their 3'UTRs has been a focus of scientific interest recently. TTP was shown to destabilise Lin28A mRNA by binding to its 3'UTR. Furthermore, there is an inverse correlation between the expression of Lin28a and TTP (Kim et al. 2012). The expression of Lin28A, which supresses *let-7* biogenesis, is itself supressed by *let-7* through a feed-back loop (Rybak et al. 2008).

In this study, eGFP reporter constructs were used in order to understand whether ZCCHC11 is also regulated through its 3'UTR. The use of eGFP reporters is a widely used method to understand the impact of the 3'UTRs of genes (Benjamin et al. 2006; LaJevic et al. 2010). Using an eGFP reporter system allowed me to study the effect of the ZCCHC11 3'UTR, its fragments and mutant versions on mRNA levels as well as protein levels analysed respectively by qPCR and by western blotting and/or flow cytometry. This approach also made it possible to observe the expression patterns of the constructs used on an individual cell basis.

The results presented here suggest that the ZCCHC11 3'UTR restricts expression, and that destabilisation of the message through its 3'UTR plays an important role in this suppression. Although the overall impact of the 3'UTR is destabilising, its two halves act differently in terms of gene regulation. The proximal half confers a decrease in expression, while the distal half, particularly the region spanned by Fragment 2, increases it. The functional distinction between the two halves can be explained by differences in the *trans*-acting factors with which they interact. Though many 3'UTRs are known to confer reduced expression, there are examples of *trans*-acting factors, such as HuR, that affect gene expression mainly positively.

The proximal half of the 3'UTR was functionally dominant, at least under normal circumstances, conferring the overall inhibitory activity. Two different strategies were followed in order to understand whether a shorter sequence is solely responsible for the inhibitory effect. The first aimed to sub-fragment Fragment 1 and the second generated individual mutations of AU-rich elements. The fact that no smaller fragment or particular sequence maintaining the decreased level of expression could be identified can be explained by the presence of one *cis*-acting element dispersed over a wide region or redundant functions of multiple *cis*-acting elements. In summary, the minimal *cis*-acting element that limits expression lies within the proximal half of the ZCCHC11 3'UTR, and the intact proximal half of the 3'UTR is sufficient to maintain this function. The potential contribution of miRNAs to ZCCHC11 3'UTR function was also investigated. Mutating individual potential miRNA binding sites, identified using the TargetScan database, in peGFP[ZCCHC11_3'UTR] did not relieve the repression of expression. A further decrease in ZCCHC11 3'UTR-conferred expression was observed following the global inhibition of

miRNA biogenesis obtained by knocking down Drosha. This led me to suggest that, within the limitations of this experimental design, miRNAs are not primarily responsible for supressing ZCCHC11 expression. Nevertheless, their effect may be indirect through one or more *trans*-acting factor(s) which in turn leads to a positive effect of certain miRNAs on the regulation of ZCCHC11.

In this study, several proteins were targeted by an shRNA knock-down approach. These included KSRP, AUF1 and BRF1, arguably the most widely studied AUBPs suppressing gene expression. TIA-1 was not included in this study as a candidate protein destabilising ZCCHC11 mRNA, as it is mainly known for its function in inhibiting translation following its interaction with AREs (Lopez de Silanes et al. 2005). In order to investigate the potential effects of these proteins on endogenous ZCCHC11 mRNA, the AUBPs mentioned above were knocked down individually. However, among the proteins knocked down, only TTP was found to decrease ZCCHC11 mRNA levels. These observations have several potential explanations. Firstly, these proteins might affect ZCCHC11 expression but the knock-down efficiency of these individual proteins may not have been sufficient, with the remaining AUBP being sufficient to generate a physiological response. Secondly, in line with previous studies suggesting redundancy between AUBPs, the absence of one AUBP that negatively regulates ZCCHC11 expression might be compensated by the activity of another (Hodson et al. 2010; Gherzi et al. 2004; Lin et al. 2011). Finally, AUBPs other than those investigated in this study, or non-coding RNAs that are yet to be identified, might be primarily responsible for the negative regulation of ZCCHC11 through its 3'UTR.

The redundant functions of ZCCHC6 and ZCCHC11 in regulating *let-7* led me to investigate the potential functional interactions between these two proteins, especially to understand whether the regulation of ZCCHC11 through its 3'UTR involves the function of ZCCHC6, which regulates gene expression through RNA 3' ends. The results discussed in this chapter suggest that ZCCHC6 down-regulates ZCCHC11 expression through its 3'UTR. Although the down-regulation of ZCCHC11 did not result in increased ZCCHC6 levels (Appendix B), the interaction between these two proteins does not seem unidirectional. The overexpression of peGFP[ZCCHC11_3'UTR] would result in a high copy number of the 3'UTR of ZCCHC11, thereby potentially titrating away the hypothetical positive regulatory protein that regulates both ZCCHC6 and ZCCHC11. In this model, failure of the knock-down of ZCCHC11 to impact on ZCCHC6 expression could be explained if the resulting decrease in the copy number of ZCCHC11 3'UTR was not sufficient to generate a response through the hypothetical protein. Technical difficulties prevented knocking down ZCCHC6 and overexpressing ZCCHC11 experimentally. The investigation of the *trans*-acting factor that regulates ZCCHC6 and ZCCHC11 focused on HuR, an AUBP that positively regulates genes mainly through their 3'UTRs. Here, I have shown that HuR regulates both ZCCHC6 and ZCCHC11 positively. The effect on ZCCHC11 was mediated, at least in part, through its 3'UTR. My findings suggest that HuR affects both halves of the ZCCHC11 3'UTR. The effect of HuR on the distal half of the 3'UTR is in line with my previous findings that the distal half confers positive expression on an eGFP reporter. The further decrease in reporter expression obtained with the proximal half of the 3'UTR of ZCCHC11 after the depletion of HuR can be explained by the fact that different AUBPs might have opposite effects on the regulation of the same gene, as shown previously in the literature (Gummadi, Taylor, and Curthoys 2012; Chen, Shyu,

and Shneider 2011). There are indications that AUBPs might compete for binding to a given 3'UTR. Such a competition can be observed between the positive regulatory factor HuR and negative regulatory factor AUF1 (Topisirovic et al. 2009; Lal et al. 2004). Other AUBPs, such as TTP and KSRP were also shown to compete with HuR for binding to target 3'UTRs (Linker et al. 2005; Tiedje et al. 2012).

The results discussed up to this point concern the impact of the ZCCHC11 3'UTR on expression under normal circumstances. One reason for investigating the post-transcriptional regulation of ZCCHC11 was to understand whether potential regulation through its 3'UTR played a part in its cell cycle-dependent regulation. Previous findings suggested that ZCCHC11 mRNA and protein levels decreased during S-phase progression (Schmidt, West, and Norbury 2011). My findings showed that the ZCCHC11 3'UTR confers decreased gene expression in asynchronous cells. Hydroxyurea treatment was used in order to arrest cells in S phase in this study as described previously (Alvino et al. 2007; Karnani and Dutta 2011). The ZCCHC11 3'UTR conferred a further decrease in reporter expression in S phase-arrested cells in comparison with asynchronous and G2-arrested cells. Hydroxyurea treatment, which results in intra-S phase arrest (Alvino et al. 2007; Karnani and Dutta 2011), was used to induce S phase arrest, because the decrease in ZCCHC11 expression as shown by our group previously was most prominent in mid-S phase (Schmidt, West, and Norbury 2011). Because of the relatively long half-life of eGFP protein, it would have been technically difficult to observe any change if I had followed the double thymidine block and release method to analyse the S phase-related differences in eGFP expression. In this study, the effect of hydroxyurea induced S phase arrest on ZCCHC11 expression was further analysed by investigating endogenous

ZCCHC11 protein levels. The decrease in ZCCHC11 protein level was in line with previous findings in this laboratory, where the S phase-dependent changes were analysed using the double thymidine block and release method.

My results suggested that alternative polyadenylation also plays a role in ZCCHC11 regulation. Mutating the proximal AAUAAAAUAAA signal resulted in an increase in the proportion of the longer isoform to shorter isoform and a decrease in the overall level of ZCCHC11 mRNA. However, *miR-26*, which has a potential binding site in the region distal to the proximal AAUAAAAUAAA was not found to be primarily responsible for the alternative polyadenylation-mediated down-regulation of ZCCHC11. This is contrary to the conclusions of a recent study in which *miR-26* was suggested to down-regulate ZCCHC11 (Fu et al. 2014). This study attributed a negative effect of the ZCCHC11 3'UTR on gene expression to the function of *miR-26*, as shown by overexpression experiments, and the mutation of the *mir-26* binding site completely abolished this negative effect on gene expression. However it must be noted that the role of *mir-26* on ZCCHC11 expression was studied only under conditions in which cells overexpress *mir-26*. Although the findings in this thesis indicate that *mir-26* does not regulate ZCCHC11 expression through its 3'UTR under normal conditions, the difference in experimental designs of the two studies must be taken into consideration.

The results in this thesis concerning the involvement of the alternative polyadenylation site in ZCCHC11 gene expression relate to unperturbed cells, and the impact of several other conditions such as cell cycle arrest and cellular stress on the regulation of ZCCHC11 might involve alternative polyadenylation mechanisms; further investigation is required in order to clarify this point. It must also be noted that in this study it was not

possible to accurately quantify the longer/shorter isoform of mRNAs which differ in their 3' ends, as two different pairs of oligonucleotide primers were used and the efficiency of these primers are not necessarily equivalent. Nevertheless, according to the publicly available databases the preference of the usage of polyadenylation signal differs between tissues. The distal site is the preferred polyadenylation signal in brain, while the proximal site is used in kidney. On the other hand, ZCCHC11 mRNAs in liver have similar levels of proximal and distal polyadenylation signal usage (https://genome.ucsc.edu/).

In summary, ZCCHC11 is negatively regulated through its 3'UTR via a mechanism that is not currently fully clear. The proximal part of the 3'UTR is responsible for mediating this regulation, whereas the distal half confers a positive effect, at least in part through the AUBP HuR. TTP contributes to down-regulation through the ZCCHC11 3'UTR. The data presented here indicate that miRNAs are not primarily responsible for the regulation of ZCCHC11. ZCCHC6 regulates ZCCHC11 negatively, and HuR is a common positive regulator of these proteins. The decrease in gene expression conferred by the ZCCHC11 3'UTR is even more pronounced when cells are arrested in S phase by hydroxyurea treatment, which also decreases endogenous ZCCHC11 levels. Finally, alternative polyadenylation is also important in the regulation of ZCCHC11, however, the factors that play a part in this regulation, as well as the potential importance of such regulation under circumstances such as stress and cell cycle arrest, require further investigation.

CHAPTER FOUR

STRUCTURE AND FUNCTION OF THE ZCCHC11 5'UTR

4.1. Introduction

The findings presented in the previous chapter suggest that ZCCHC11 is regulated through its 3'UTR both in asynchronous and in S phase-arrested cells. The regulation involves *cis* acting elements of the 3'UTR bearing AU-rich elements, and *trans* acting factors such as HuR and ZCCHC6, the orthologue of ZCCHC11. Although gene regulation through 3'UTRs is well described, gene expression may also be controlled post-transcriptionally through mechanisms involving 5'UTRs. 5'UTRs may contain uORFs, which can inhibit translation, being scanned by the translation machinery to generate short polypeptides, with correspondingly decreased translation of the authentic ORF. In addition to their function in inhibiting translation, uORFs may also function to decrease mRNA stability through nonsense-mediated RNA decay. In contrast to their down-regulatory functions, 5'UTRs may favour gene expression in certain conditions such as cellular stress. Such an effect of the 5'UTR of ZCCHC11 will be discussed in the following chapter.

In this chapter, the characterisation of the 5' end of the ZCCHC11 mRNA and the potential influence of the 5'UTR on ZCCHC11 expression will be discussed.

4.2. Aims

- To characterise the 5' end of the ZCCHC11 mRNA.
- To investigate the potential effect of the 5'UTR on the expression of ZCCHC11.

4.3. Results

4.3.1. ZCCHC11 has different mRNA isoforms differing in their 5' ends

The characterisation of the 5' end of ZCCHC11 is an essential initial step in order to study the properties of the 5'UTR. Three different isoforms are found in public genome databases (Fig. 4.1). cRACE method, which was also used to characterise the 3' end of the ZCCHC11 mRNA (Chapter 3) enabled the precise characterisation of the 5' end of ZCCHC11 mRNAs in the cells used in this study. The results suggested the presence of two mRNA isoforms that differ by at least a couple of nucleotides. More interestingly, these two isoforms have two non-overlapping uORFs in common, while the longer isoform has a third uORF, which overlapped with the other two uORFs (Fig. 4.1). These mRNA isoforms have potential role in gene regulation as a consequence of their uORFs. The cRACE data did not provide evidence for the existence of the shortest annotated isoform of ZCCHC11 mRNA in HeLa cells, which has only one uORF. This mRNA isoform, if it is expressed at all, seems unlikely to be relevant to any of the observations made in these cells.

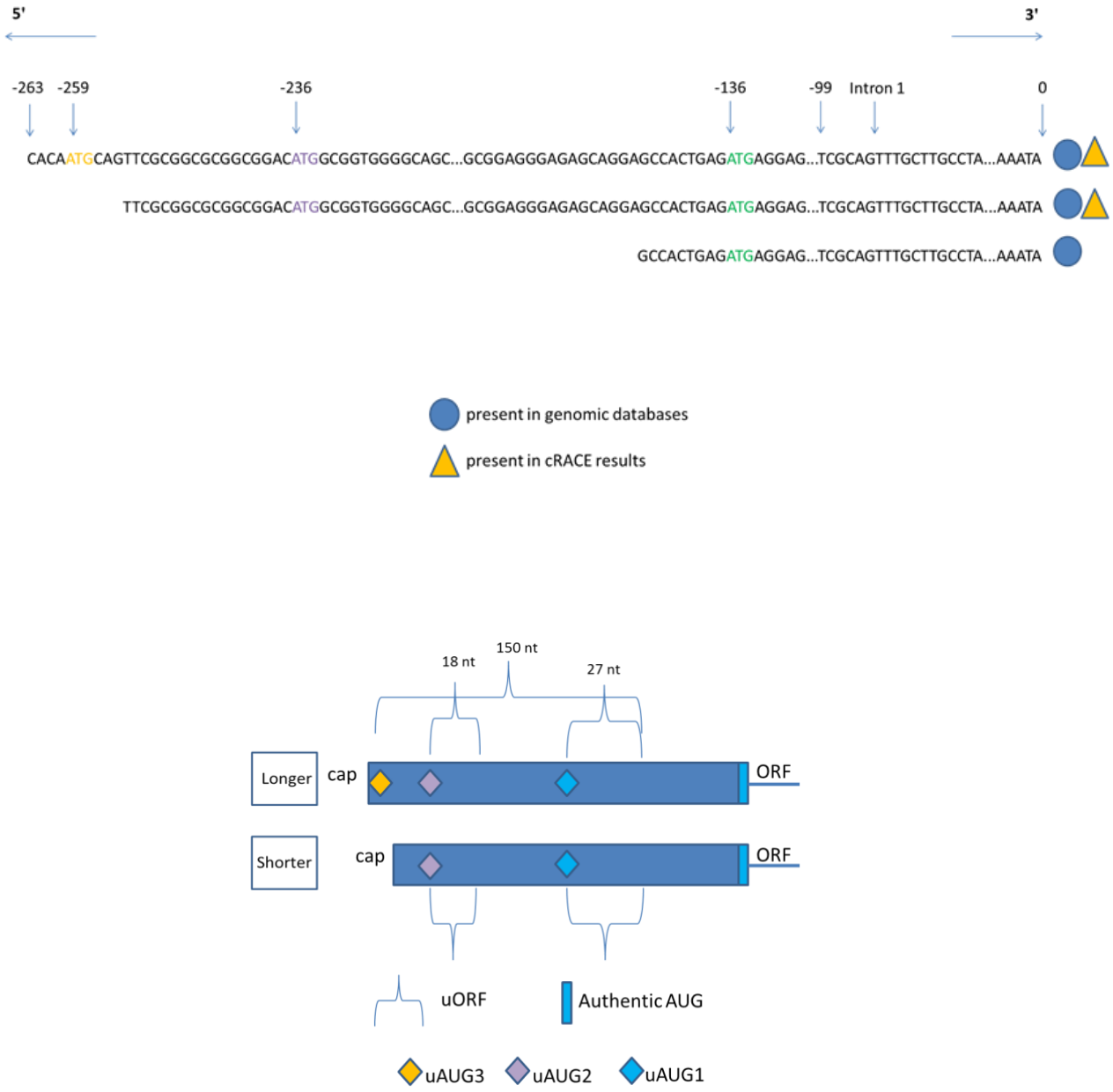


Figure 4.1. The 5'UTRs of ZCCHC11.

Upper panel shows the variants of the 5' end of the ZCCHC11 mRNA, which can be found in genomic databases (blue circle) and which were identified by cRACE (yellow triangle). Lower panel is a schematic representation of the isoforms of the 5'UTR of ZCCHC11, identified by cRACE and used in this study. Each of them was ligated to the 5' end of eGFP ORF. The same colours were used to represent the uAUGs.

4.3.2. The ZCCHC11 5'UTR represses expression of a downstream ORF

Next, the potential regulatory function of the ZCCHC11 5'UTR was investigated using eGFP constructs. Two variants of the 5'UTR were chosen for this study: one is shorter and contains the two distal uORFs (peGFP[ZCCHC11_5'UTR_short]) while the longer one contains three uORFs(peGFP[ZCCHC11_5'UTR_long]). These 5'UTRs were inserted upstream of the eGFP ORF in the pCDNA3.1+ expression vector. Analogous plasmids containing instead the 5'UTR of β-actin (peGFP[β-Actin_5'UTR) or encoding eGFP with a minimal 5'UTR (peGFP) were used as controls in this study. HeLa (Fig. 4.2a) and HEK293T cells (Fig. 4.2b) were transfected with these plasmids, and comparison of the eGFP expression levels was performed by flow cytometry. The results showed that the shorter 5'UTR isoform containing two uORFs decreased gene expression relative to the controls. In addition, the longer isoform containing an additional uORF at the 5' end conferred even stronger down-regulation to the eGFP reporter, suggesting that 5'UTRs of ZCCHC11 decrease gene expression (Fig. 4.2).

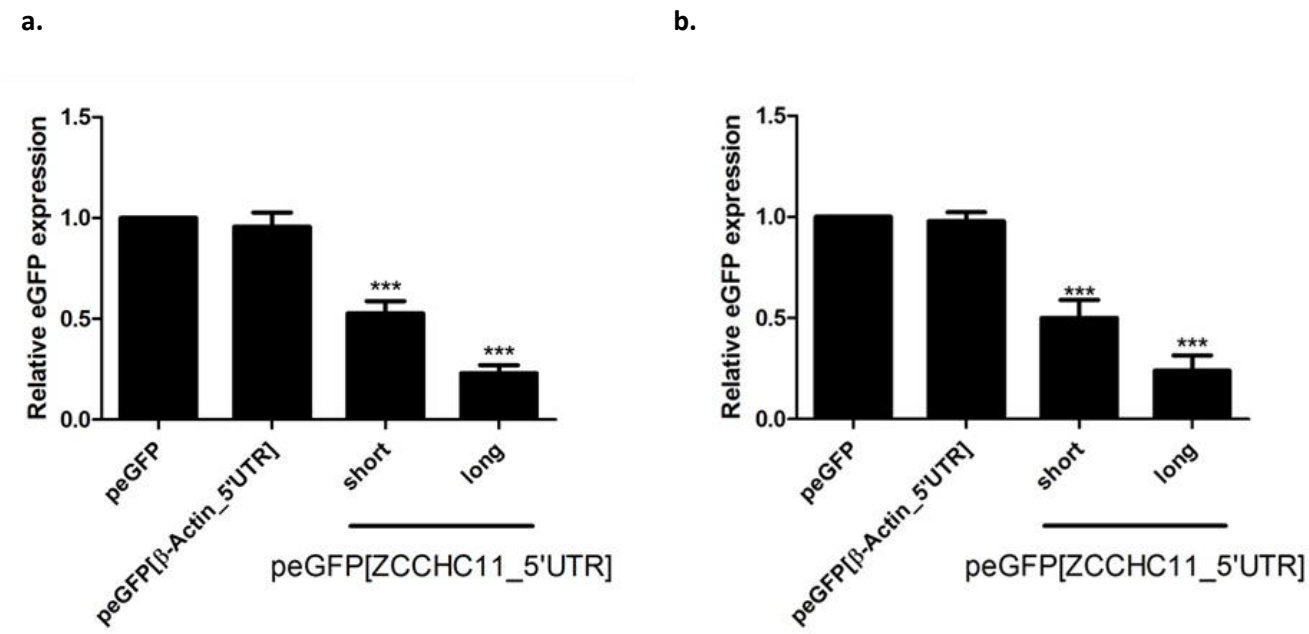


Figure 4.2. eGFP expression conferred by the 5'UTR of ZCCHC11.
HeLa **(a)** and HEK293T **(b)** cells were transfected with peGFP, peGFP[β-Actin_5'UTR], peGFP[ZCCHC11_5'UTR_short], peGFP[ZCCHC11_5'UTR_long]. The expression conferred by the 5'UTRs of β-Actin and ZCCHC11 was compared to the expression conferred by eGFP control, whose value is normalised to one. The graphs represent the mean of the flow cytometry results of at least three independent experiments ±SD. Student's t-test was applied and p values were determined using the peGFP control as a reference (*** $p<0.001$). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

4.3.3. uORFs are responsible for the 5'UTR-mediated repression

Given that the ZCCHC11 5'UTR was found to limit gene expression, the potential role in this process of the uORFs contained within the 5'UTR was tested by site-directed mutagenesis of the uAUGs, both individually and in combination. Although each mutation resulted in a partial increase in gene expression, mutating all of the uAUGs resulted in complete reversal of the inhibitory effect conferred by the 5'UTR (Fig 4.3). These results suggest that the uORFs are responsible for the decreased gene expression conferred by the ZCCHC11 5'UTR.

4.3.4. The decreased expression conferred by the ZCCHC11 5'UTR is solely translational

The results described in the previous section suggest that the ZCCHC11 5'UTR, like its 3'UTR, serves to limit the expression of ZCCHC11. The effects of 5'UTRs on translation are well studied. However, there are also indications that 5'UTRs may exert regulatory effects on gene expression through mechanisms such as nonsense-mediated decay (NMD). One such effect has been reported to involve uORFs (Barbosa, Peixeiro, and Romao 2013b). Therefore, the possible involvement of an analogous mechanism regulating ZCCHC11 mRNA quantity was investigated. Following transfection of the plasmids described in the previous section into HeLa cells, eGFP mRNA levels were quantified using qRT-PCR. The results showed that the decrease in eGFP expression conferred by the ZCCHC11 5'UTR is not reflected at the mRNA level (Fig 4.4). This result suggests that the 5'UTR decreases gene expression through its uORFs, in a manner that involves only the repression of translation.

1 peGFP
2 peGFP[ZCCHC11_5'UTR_short]
3 peGFP[ZCCHC11_5'UTR_long]
4 peGFP[ZCCHC11_5'UTR_short_uAUG1_mutation]
5 peGFP[ZCCHC11_5'UTR_short_uAUG2_mutation]
6 peGFP[ZCCHC11_5'UTR_long_uAUG3_mutation]
7 peGFP[ZCCHC11_5'UTR_long_uAUG123_mutation]

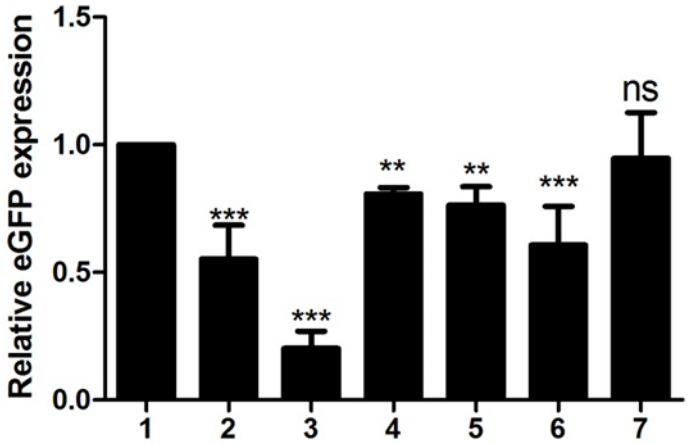


Figure 4.3. Effects of mutation of the ZCCHC11 uORFs on 5'UTR-mediated repression.
HeLa cells were transfected with peGFP, peGFP[ZCCHC11_5'UTR_short], peGFP[ZCCHC11_5'UTR_long], peGFP ligated to the short and long 5'UTRs whose uAUGs are mutated individually or in combination. Upper panel shows the transfected plasmids that correspond to the graph. The expression conferred by the WT or mutant 5'UTRs of ZCCHC11 was normalised to the expression conferred by the eGFP control. The graphs represent the mean of the flow cytometry results of at least three independent experiments ±SD. Student's t-test was applied and p values were determined using the peGFP control as a reference (* $p<0.05$,** $p<0.01$, *** $p<0.001$). Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

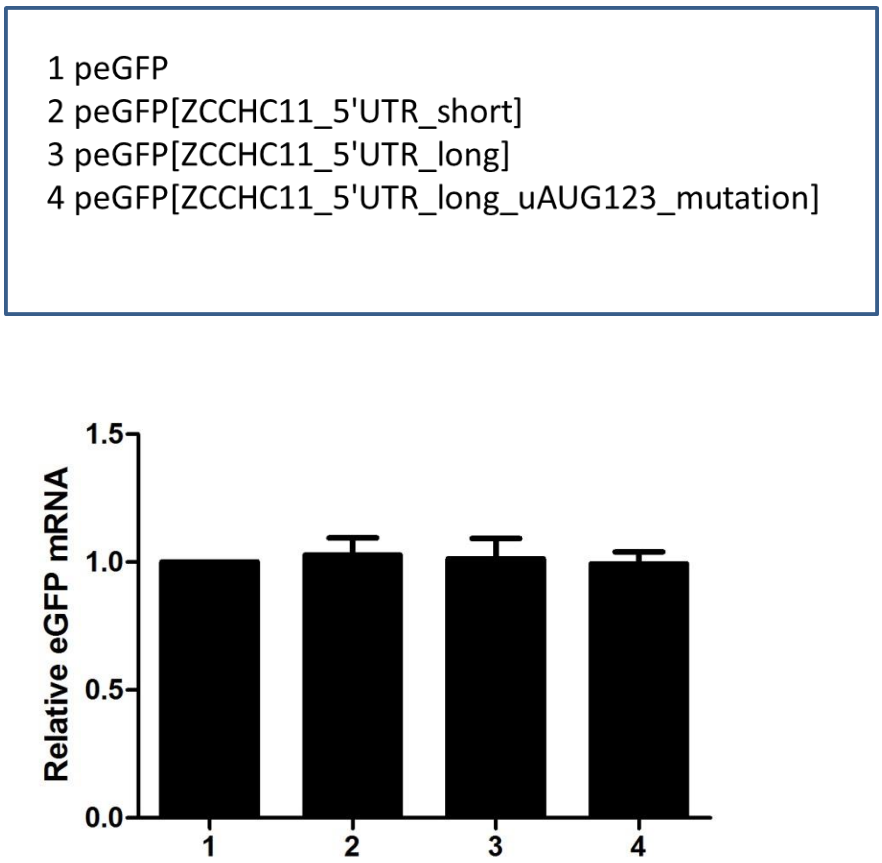


Figure 4.4. Investigation of mRNA expression levels conferred by the ZCCHC11 5'UTR.
HeLa cells were transfected with peGFP, peGFP[ZCCHC11_5'UTR_short], peGFP[ZCCHC11_5'UTR_long], and peGFP ligated to the long 5'UTR whose uAUGs are mutated. Upper panel shows the transfected plasmids. The expression conferred by the WT or mutant 5'UTRs of ZCCHC11 was normalised to the expression conferred by eGFP control group. The graphs represent the mean of the qRT-PCR results of at least three independent experiments ±SD. Co-transfecting a pcDNA_TAP plasmid, and amplifying the TAP by qRT-PCR for each sample served as a control for transfection efficiency.

4.4. Discussion

The previous chapter revealed a role of the 3'UTR in the regulation of ZCCHC11 in a cell cycle-dependent manner. This regulation appears complex, involving *cis* and *trans*-acting factors, some of which have opposite effects on gene regulation. 5'UTRs of genes may also contain *cis*-acting elements that mediate gene regulation. Arguably, the best studied *cis*-acting elements of 5'UTRs are uORFs, which can down-regulate gene expression through decreasing the efficiency of ribosomal scanning of the mRNA for the authentic AUG, as well as by triggering RNA decay mechanisms. Results in this chapter characterised the 5' ends of ZCCHC11 mRNAs. These include isoforms that differ in length, and contain either two or three uORFs. Both types serve to limit gene expression, although the longer isoform with three uORFs is substantially more potent than the shorter. Both of the mRNA isoforms identified using cRACE in this study comprised at least two uORFs. The abundance of these isoforms has not been studied, and RNA seq databases did not shed light on the relative expression of these isoforms in human cells. Their relative abundance might be different depending on the cellular situation.

5'UTRs may affect mRNA levels through mechanisms involving mRNA decay. However, the decreased expression conferred by the longer 5'UTR isoforms was seen only at the protein level; mRNA levels were not affected by the ligation of either shorter or the longer isoform of the 5'UTR of ZCCHC11 to eGFP in comparison with the plasmid encoding eGFP.

The results discussed in this chapter reflect the effect of the 5'UTR isoforms of ZCCHC11 only in normal conditions. 5'UTRs that contain uORFs may impact on gene expression

selectively under stress conditions. The influence of the 5'UTR on ZCCHC11 expression during cellular stress will be explored in the following chapter.

CHAPTER FIVE

STRESS-DEPENDENT REGULATION OF ZCCHC11

5.1. Introduction

ZCCHC11 is a terminal uridyl transferase whose substrates identified so far are cytoplasmic. However, published studies differ in their findings about the subcellular localisation of ZCCHC11. A previous study suggested that ZCCHC11 is mainly localised in the nucleus and relocalised to the cytoplasm after LPS treatment in the murine macrophage-like cell line RAW 264.7, as shown by immunofluorescence microscopy (Minoda et al. 2006). A previous study in our group suggested, on the basis of western blotting of fractionated HEK293T cells, that ZCCHC11 localises to the cytoplasm (Schmidt, West, and Norbury 2011). It must be taken into consideration that two different cell lines were used in these studies, which may explain the different results obtained. In addition, ZCCHC11 was also shown to be functional in regulating immune responses and cell cycle progression in a manner that was independent of its uridyl transferase activity (Blahna et al. 2011; Minoda et al. 2006). Considering this potential functional diversity and the variety of RNA substrates of ZCCHC11, it is important to understand the subcellular localisation of ZCCHC11 in more detail. There is good evidence that ZCCHC11 functions in regulating histone mRNA following genotoxic stress, and that ZCCHC11 is regulated in a cell cycle-dependent manner. In this study, I showed that ZCCHC11 is regulated post-transcriptionally through both of its UTRs. One other way in which it might be regulated, especially under conditions such as cellular stress and cell cycle arrest, is through alteration in its subcellular localisation. Thus, investigating the subcellular localisation of ZCCHC11 is not only important for understanding its functional interactions but also important for a more complete view of its regulation.

Stress granules and p-bodies are subcellular centres in which mRNAs are stored, followed either by their degradation or redirection to translation. Currently, there is no evidence of stress granule or p-body association of terminal uridyl transferases in mammalian cells. Nevertheless, Lin28A, the protein that recruits ZCCHC11 to pre-*let-7*, was shown to form distinct cytoplasmic foci that colocalised with p-bodies. In addition, Lin28A relocalises to stress granules following cellular stress.

Cellular stress can be generated by exposure to many environmental factors such as hyperosmolarity, heat shock, oxidative stress, viruses, protein synthesis inhibitors and proteasome inhibitors. Under stress conditions, cells undergo rapid adaptation of their protein synthesis machinery. In contrast to a general inhibition in gene expression, expression of several genes important for cellular survival under these abnormal conditions is favoured. These genes play roles in the response of the cells to the environmental stress. Transcriptional, post-transcriptional and translational mechanisms are important in generating such a response. uORFs in mRNA 5'UTRs can inhibit gene expression. However, under cellular stress conditions, the presence of uORFs may favour the expression of some genes (Vattem and Wek 2004; Lee, Cevallos, and Jan 2009). Similarly, regulation through the 3'UTR can be affected by stress conditions. The presence of AUBPs such as HuR and TTP in stress granules suggests the involvement of ARE mediated gene regulation in cellular responses to stress. There are also examples of the differential regulation of ARE-containing transcripts by AUBPs (von Roretz et al. 2011). Regulation by miRNAs can also be important in stress-related gene regulation (Bhattacharyya et al. 2006). In summary, both untranslated regions of the mRNA may be involved in determining the level of gene expression under cellular stress.

Proteins may be localised to stress granules or p-bodies under certain conditions, such as cellular stress. In addition, several proteins change their subcellular localisation depending on the cell cycle. For example, E2F-4, a member of the E2F family of transcription factors involved in the regulation of cell cycle-related genes (Stevens and La Thangue 2003), localises differentially to the nucleus and cytoplasm in a cell cycle-dependent manner (Verona et al. 1997; Lindeman et al. 1997). Cell cycle-related proteins may also localise to either cytoplasm or nucleus during specific cell cycle phases as a part of the regulation of their activity.

5.2. Aims

- To investigate the subcellular localisation of ZCCHC11 in different cell lines using immunofluorescence microscopy.
- To understand the localisation of ZCCHC11 in relation to subcellular compartments such as vesicles, mitochondria, processing bodies and stress granules.
- To determine the effect of several cellular stress conditions on the localisation of ZCCHC11 in stress granules.
- To investigate the effect of cellular stress on the regulation of ZCCHC11 expression.
- To investigate whether the subcellular localisation of ZCCHC11 changes during the cell cycle.

5.3. Results

5.3.1. ZCCHC11 localises predominantly to the cytoplasm

Despite several studies, including one from our group, of the subcellular localisation of ZCCHC11, there is no consensus agreement whether ZCCHC11 is a cytoplasmic or a nuclear protein. Therefore, I aimed to investigate the localisation of ZCCHC11 in different cell lines including HeLa, HEK293T, A204 and MDA-MB-231 using an antibody against ZCCHC11. In this study, confocal laser scanning microscopy was used to visualise cells unless stated otherwise. The specificity of the antibody signal was confirmed by a knock down of ZCCHC11, which resulted in a decrease in the antibody signal proportional to the amplitude of the knockdown (Figure 5.1).

In normal condition, ZCCHC11 localises mainly in the cytoplasm with a variable degree of presence in the nucleus in different cell lines (Fig. 5.2). Indeed, there seems to be more ZCCHC11 present in the nucleus of A204 and MDA-MB-231 cells compared to HeLa and HEK293T cells. The localisation of ZCCHC11 was further investigated using GFP-tagged ZCCHC11 (eGFP_ZCCHC11). A plasmid encoding GFP (eGFP only) was used as a control. Looking at the endogenous protein or the tagged version gave similar results, with ZCCHC11 localising predominantly to the cytoplasm under normal conditions (Fig. 5.3).

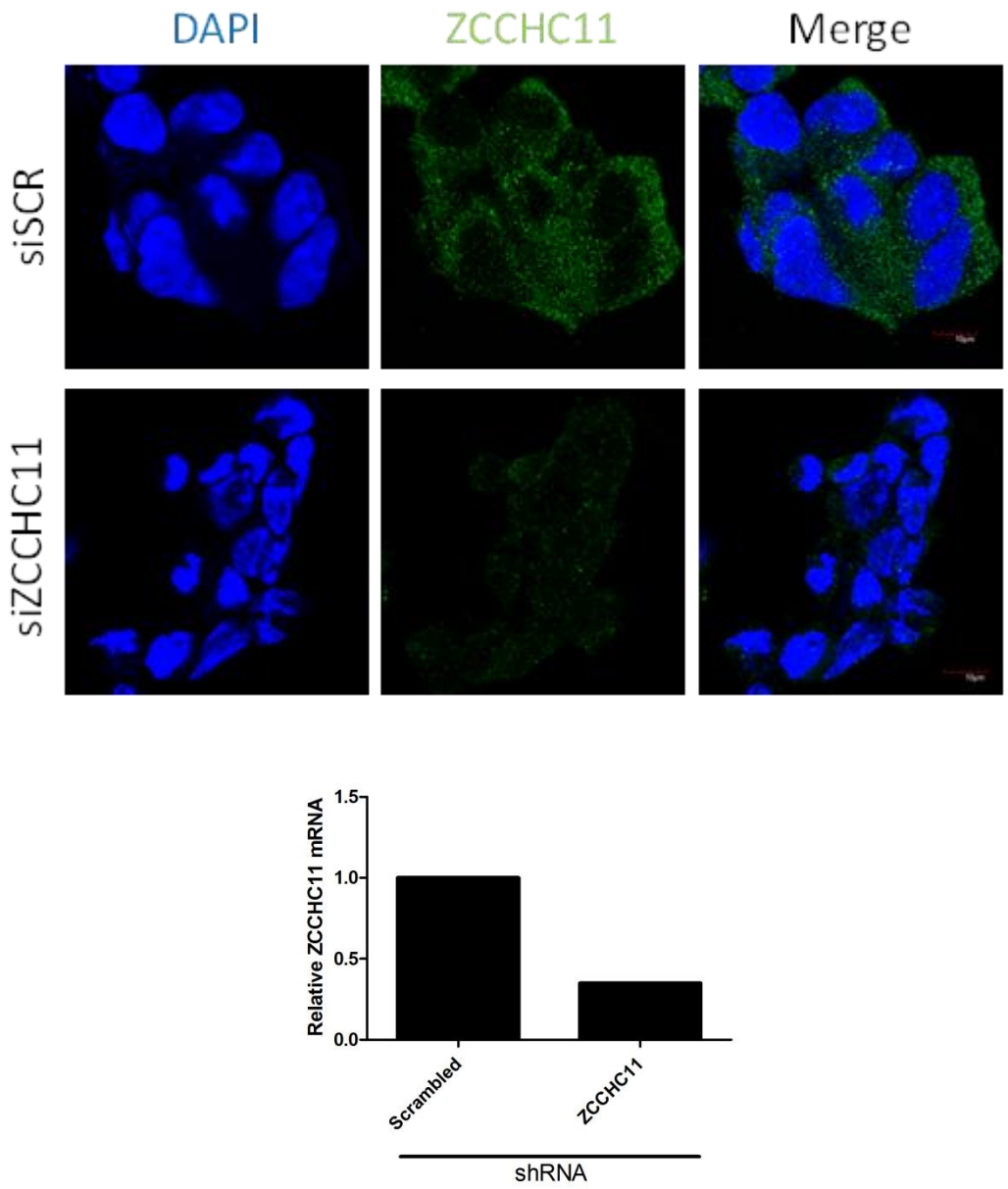


FIGURE 5.1. ZCCHC11 knockdown and immunostaining with anti-ZCCHC11.
HEK293T cells were transfected with plasmids encoding either scrambled shRNA or shRNA targeting the ZCCHC11 mRNA. They were immunostained with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). Lower panel is the qRT-PCR graph showing the knockdown efficiency of ZCCHC11 at the mRNA level.

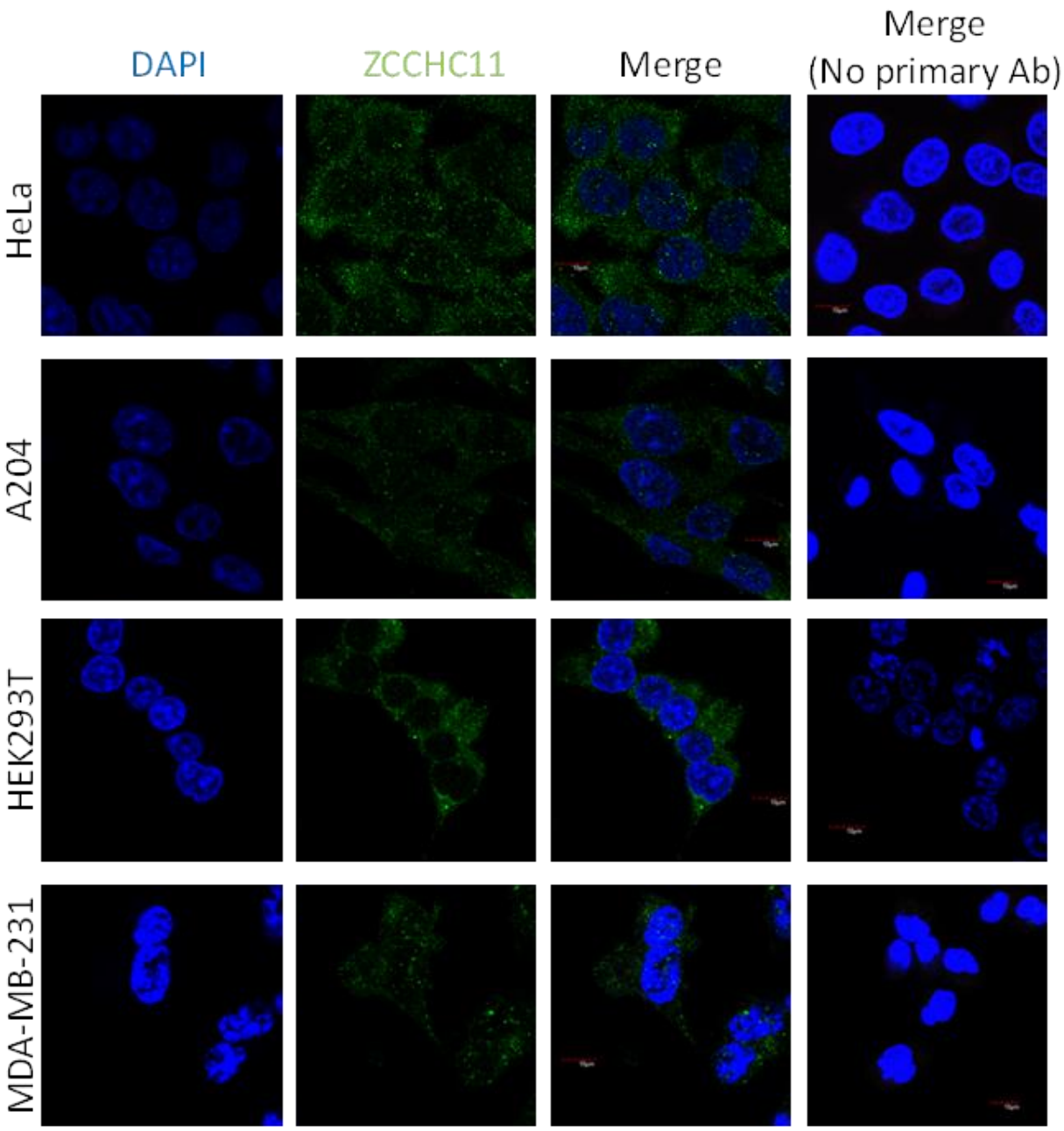


FIGURE 5.2. Subcellular localisation of ZCCHC11 in different cell lines.
HeLa, HEK293T, A204, MDA-MB-231 cells were used in this study. They were immunostained with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). Control groups, which were only stained with secondary antibody, were included for each cell line on the right hand side of the figure.

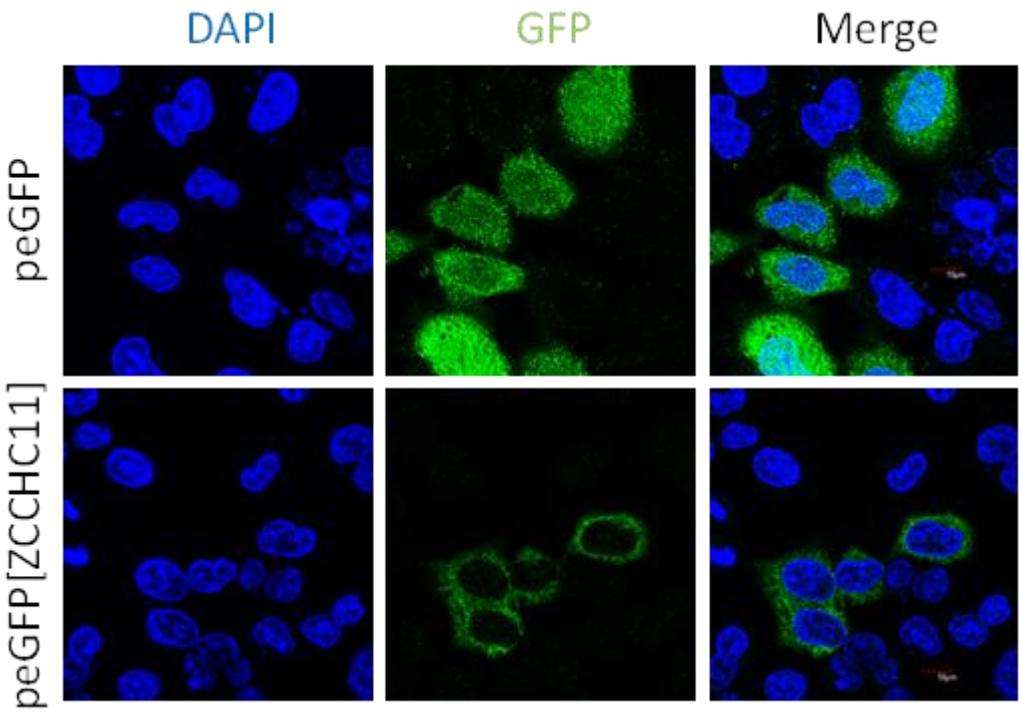
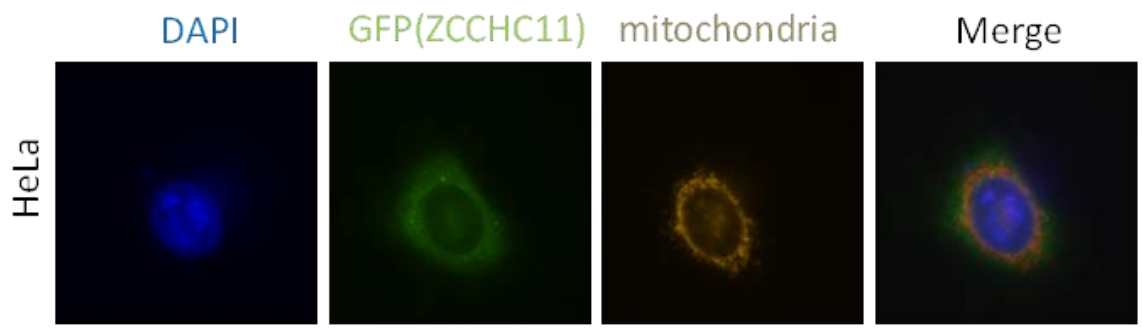


FIGURE 5.3. Subcellular localisation of eGFP-tagged ZCCHC11.
HeLa cells were transfected either with peGFP or peGFP[ZCCHC11]. They were immunostained with anti-GFP antibody (green). Nuclei were stained with DAPI (blue).

5.3.2. ZCCHC11 does not localise to mitochondria or vesicles

The cytoplasmic ZCCHC11 staining was largely diffuse, but also included numerous distinct foci. I therefore decided to investigate whether these foci correspond to known subcellular compartments, to which ZCCHC11 might be preferentially localised. The terminal uridyl transferase MTPAP is largely restricted to mitochondria. Therefore, I investigated whether ZCCHC11 also localises to mitochondria. In order to test this hypothesis, cells were co-stained with MitoTracker Orange CMTMRos, an orange fluorescent dye that stain mitochondria, and visualised using wide-field fluorescence microscopy. However, ZCCHC11 did not co-localise with mitochondria (Fig 5.4a). Next, I investigated whether these foci are membranous structures such as vesicles. Membranous vesicles frequently interact with the microtubular cytoskeleton. I investigated a possible interaction with microtubules by co-staining the cells with an antibody against α-tubulin. However, the foci did not show any sign of interaction with microtubules (Fig 5.4b). Therefore, it is unlikely that ZCCHC11 preferentially localises in membranous vesicles, at least under normal conditions.

a.



b.

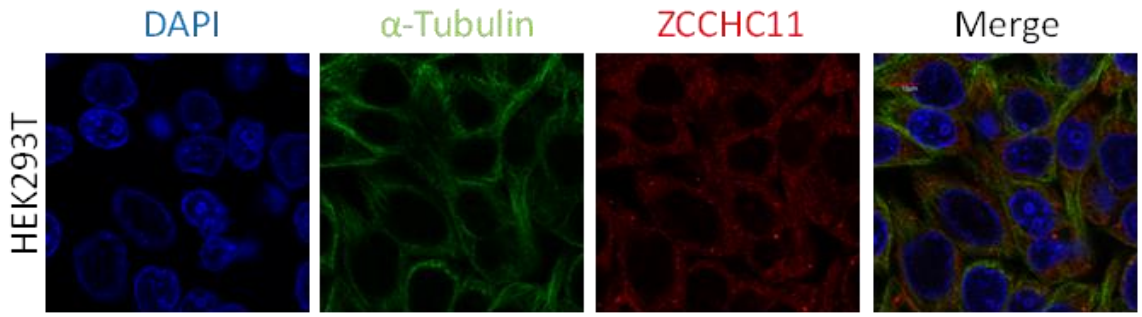


FIGURE 5.4. Investigation of the potential subcellular localisations of ZCCHC11 under normal conditions.

a. HeLa cells were transfected with peGFP[ZCCHC11], and immunostained with anti-GFP antibody (green). Mitochondrial staining was performed using MitoTracker Orange CMTMRos. Visualisation was performed using wide-field fluorescence microscopy. **b**. HEK293T cells were immunostained with anti-ZCCHC11 antibody (red) and with anti-alpha tubulin antibody (green). Nuclei were stained with DAPI (blue).

5.3.3. ZCCHC11 localises to stress granules under oxidative stress

Many proteins involved in RNA metabolism have been found associated with stress granules. To my knowledge, there is currently no study suggesting the association of human terminal uridyl transferases with stress granules. Nevertheless, two observations led me to investigate a potential interaction of ZCCHC11 with stress granules. First, ZCCHC11 inhibits histone biogenesis following genotoxic stress. Second, Lin28, which recruits ZCCHC11 to its pre-*let-7* substrate, is localised to stress granules under stress conditions. Therefore, I investigated whether ZCCHC11 is also localised to stress granules. An ImageJ macro (written by Dr Richard Wheeler) was used in this study for the analysis of colocalisation (Fig. 2.3.). Briefly, the histograms show the proportion of green signal: endogenous or GFP tagged ZCCHC11 staining (y-axis) relative to red signal (x-axis), which corresponds to stress granule or p-body markers. A shift to the right means that a higher proportion of the endogenous or GFP tagged ZCCHC11 co-localises with brighter stained pixels of the markers of these structures.

Stress granules were induced by oxidative stress through sodium arsenite treatment, and were detected with an antibody against the stress granule marker TIA-1. ZCCHC11 colocalised with TIA-1 under oxidative stress (Fig. 5.5), while no colocalisation was observed under normal conditions. Similar results were obtained using the A204 cell line (Fig 5.6.). Another stress granule marker, eIF3B (Buchan et al. 2013; Li et al. 2010), was also used to visualise stress granules in HeLa cells. Similarly, ZCCHC11 colocalised with eIF3B under oxidative stress (Fig 5.7).

Next, I used GFP-tagged ZCCHC11 to investigate its interaction with TIA-1. A plasmid encoding GFP was used as a control in this study. Cells were either left untreated or treated with sodium arsenite before being analysed for TIA-1 and eGFP immunofluorescence. Similarly, GFP-tagged ZCCHC11 localised to stress granules only under stress conditions in HeLa cells (Fig. 5.8-9) and HEK293T cells (Figure 5.10.). These results suggested that ZCCHC11 localises to stress granules under oxidative stress.

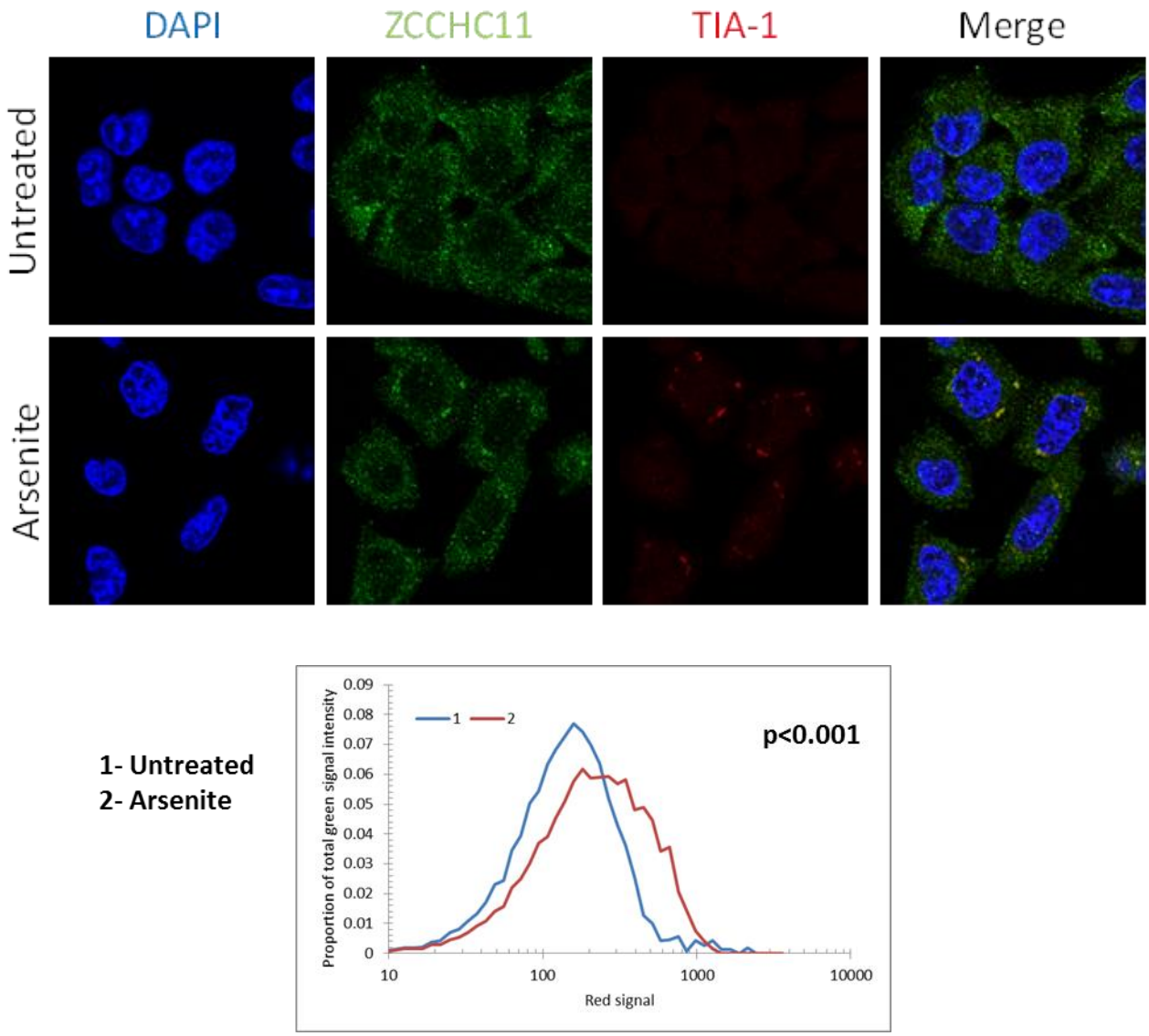


FIGURE 5.5. Colocalisation of ZCCHC11 with stress granule marker TIA-1 in HeLa cells under oxidative stress.

HeLa cells were treated with 0.4 mM sodium arsenite for 45 mins before fixation, and were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). The lower panel is a representative histogram showing the proportion of anti-ZCCHC11 staining (y-axis) relative to anti-TIA-1 staining (x-axis). The Kolmogrov-Smirnov test was used for statistical analysis.

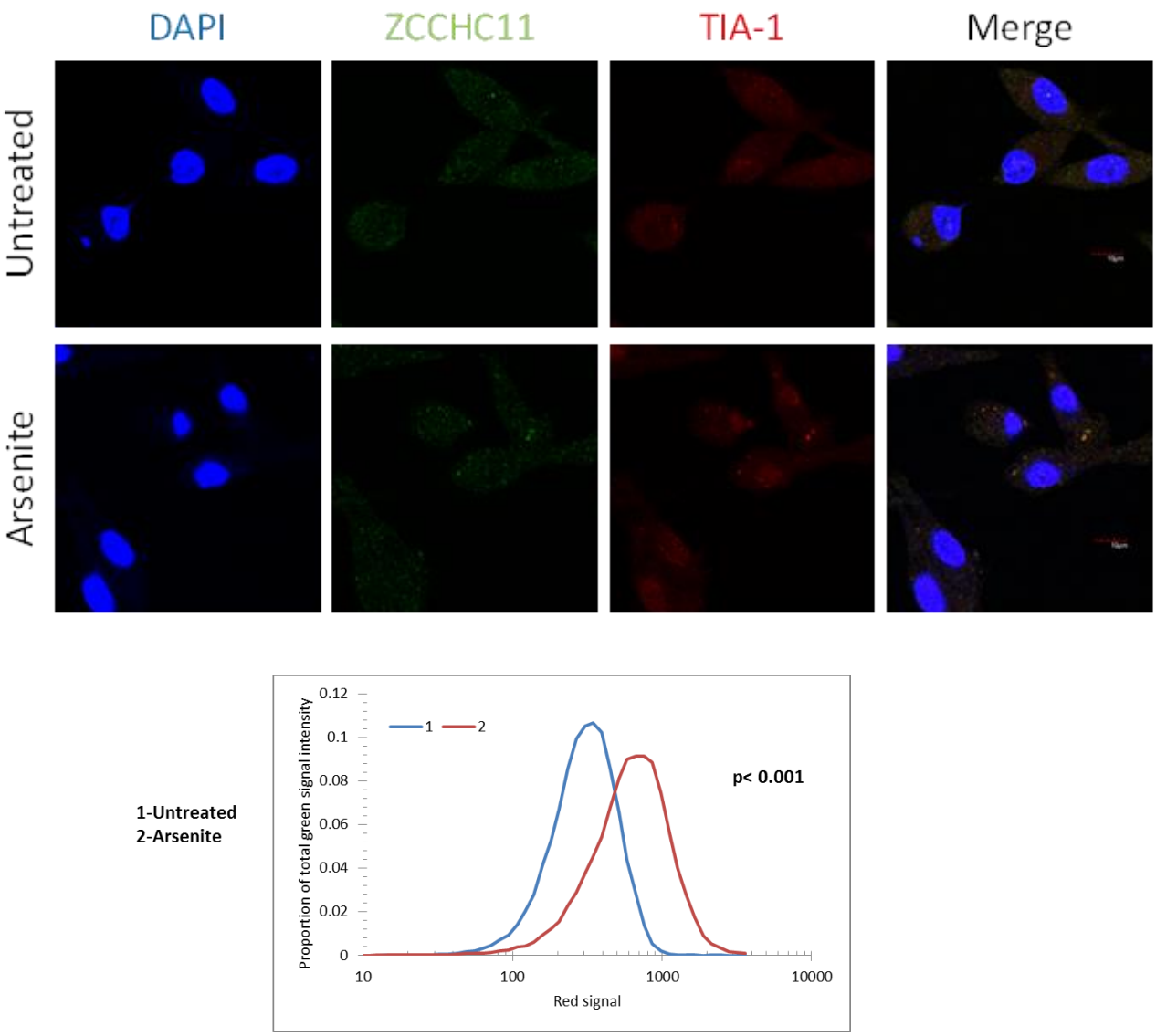


FIGURE 5.6. Colocalisation of ZCCHC11 with stress granule marker TIA-1 in A204 cells under oxidative stress.

A204 cells were treated with 0.4 mM sodium arsenite for 45 mins before fixation, and were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). The lower panel is a representative histogram showing the proportion of anti-ZCCHC11 staining (y-axis) relative to anti-TIA-1 staining (x-axis). The Kolmogrov-Smirnov test was used for statistical analysis.

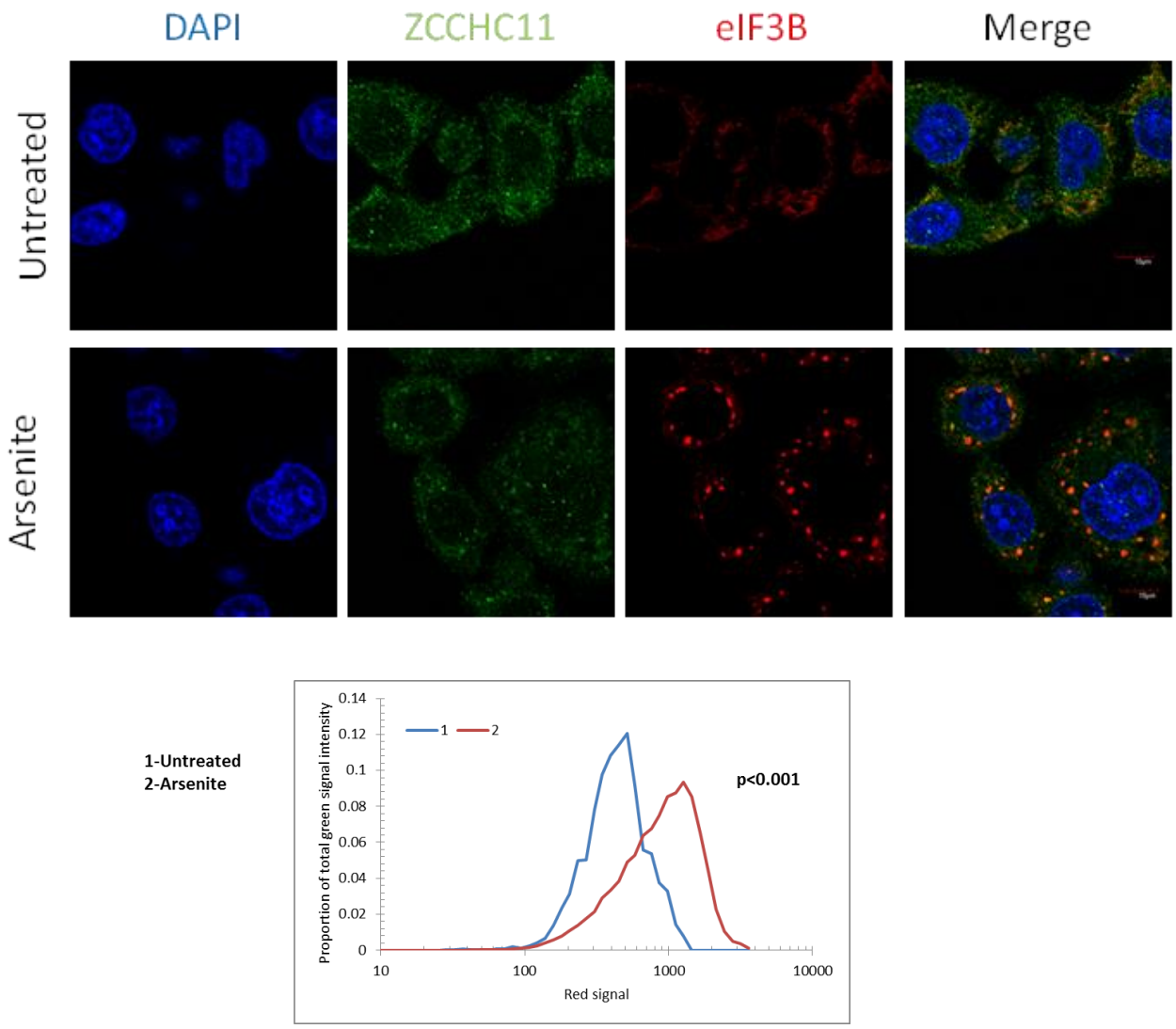


FIGURE 5.7. Colocalisation of ZCCHC11 with stress granule marker eIF3B in HeLa cells under oxidative stress**.**

HeLa cells were treated with 0.4 mM sodium arsenite for 45 mins before fixation, and were immunostained with anti-eIF3B antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). The lower panel is a representative histogram showing the proportion of anti-ZCCHC11 staining (y-axis) relative to anti-eIF3B staining (x-axis). The Kolmogrov-Smirnov test was used for statistical analysis.

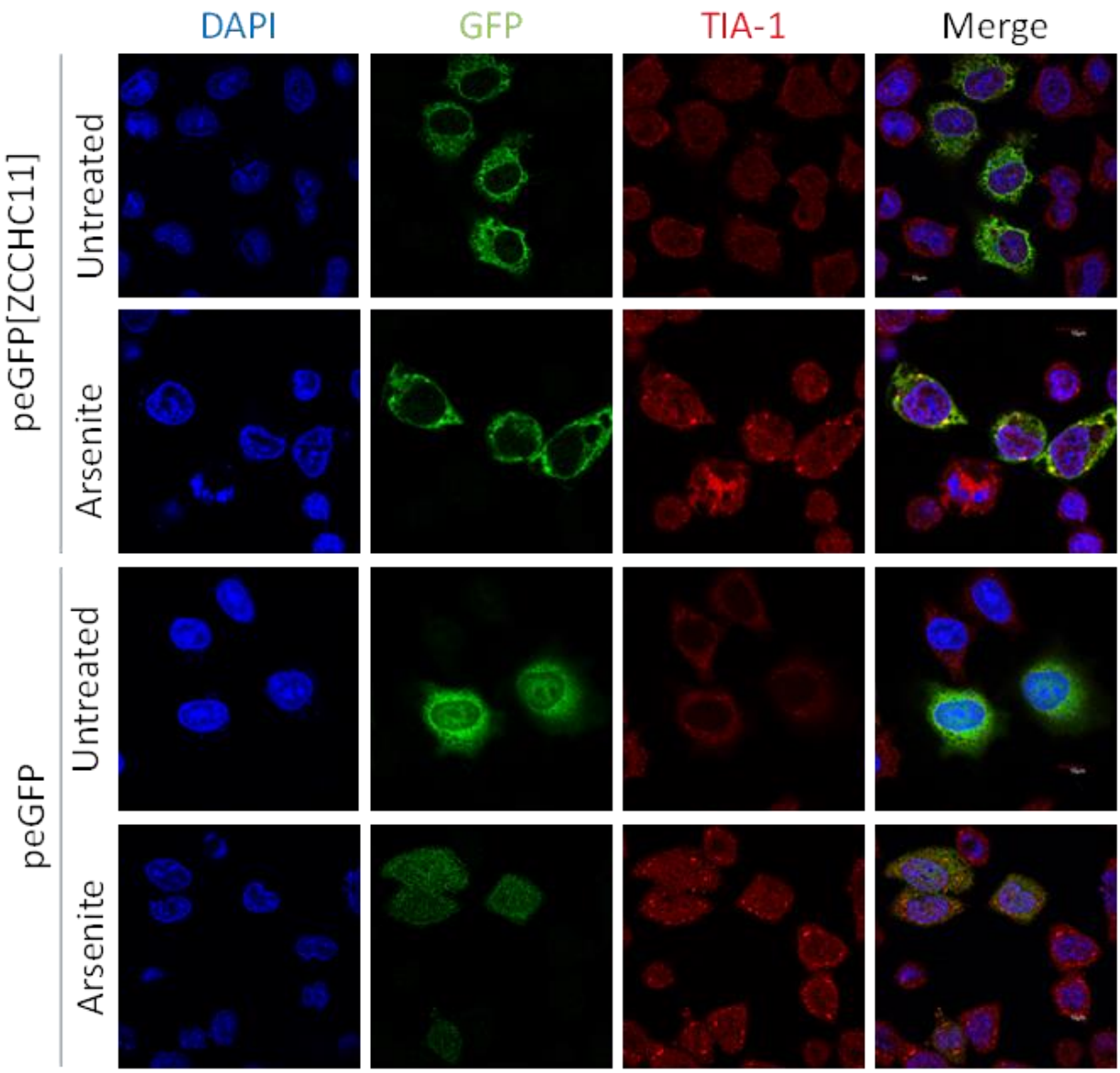


FIGURE 5.8. Colocalisation of GFP tagged ZCCHC11 with stress granule marker TIA-1 in HeLa cells.

HeLa cells were transfected with peGFP or peGFP[ZCCHC11]. They were either left untreated or treated with 0.4 mM sodium arsenite for 45 mins before fixation. They were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-GFP antibody (green). Nuclei were stained with DAPI (blue). Figure 5.9 shows the statistical analysis of this experiment.

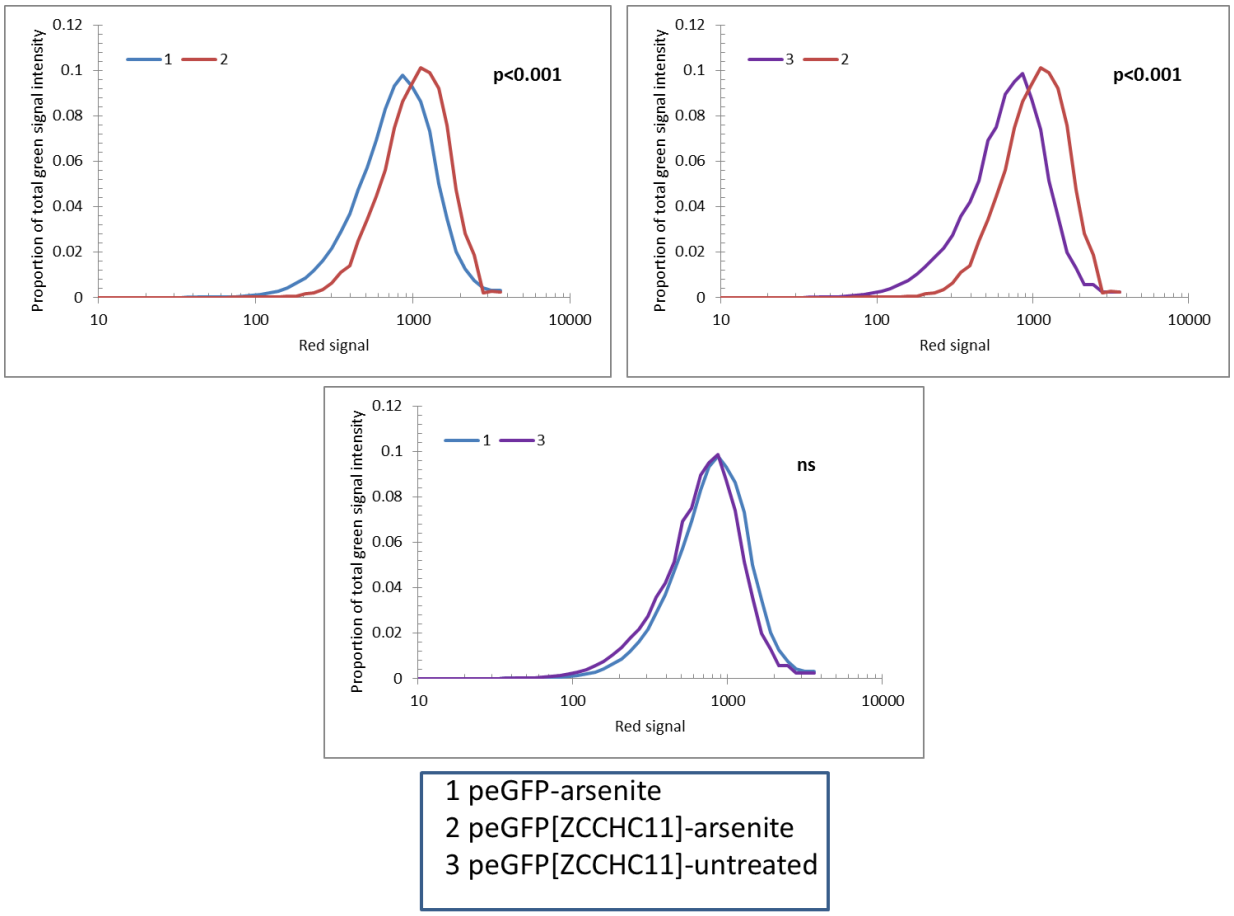


FIGURE 5.9: Statistical analysis of Figure 5.8.

Panels are the demonstrative histograms showing the proportion of anti-GFP staining (y-axis) relative to anti-TIA-1 staining (x-axis) in plasmid-transfected cells. Kolmogrov-Smirnov test was used for statistical analysis.

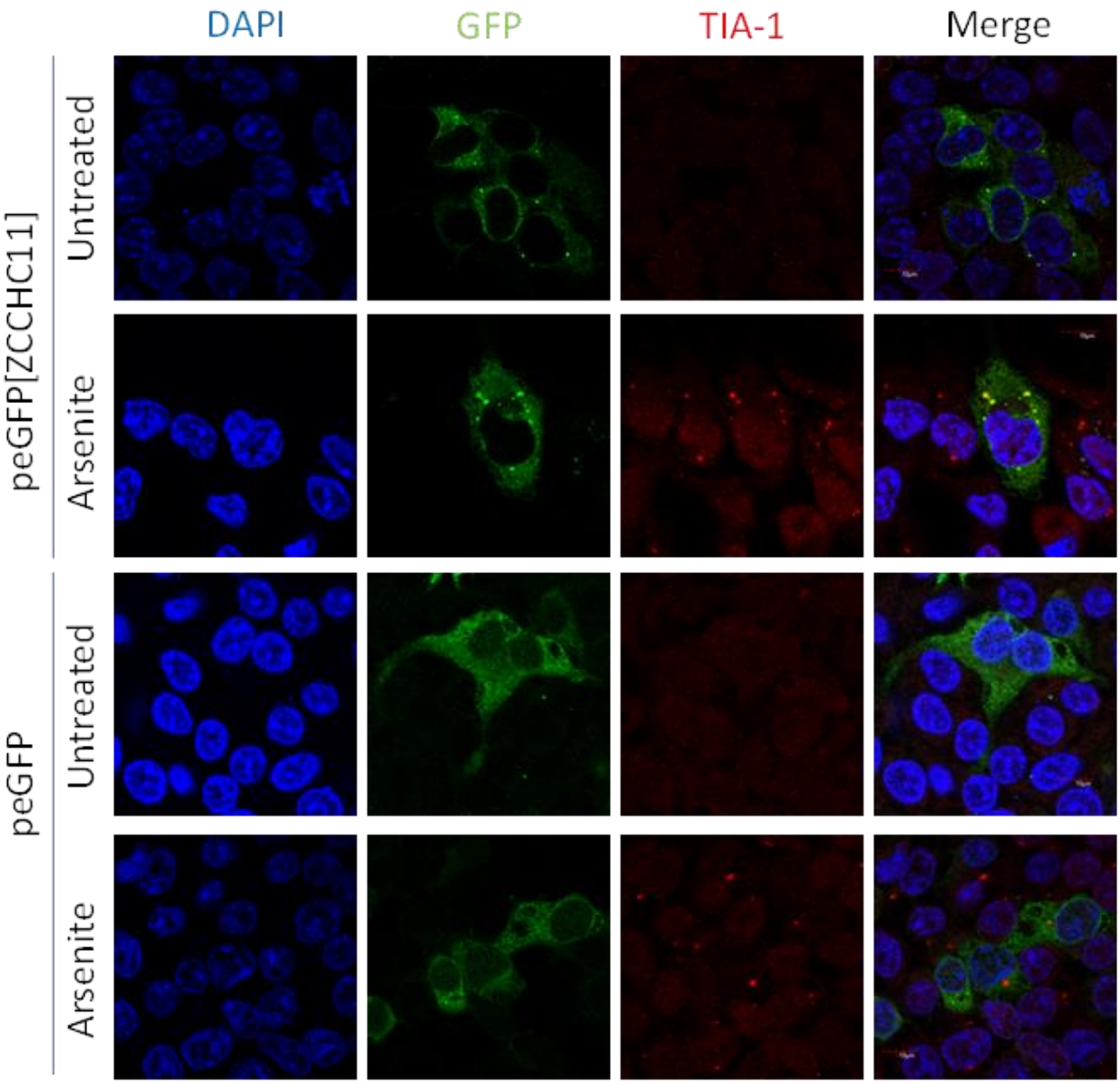


FIGURE 5.10. Colocalisation of GFP-tagged ZCCHC11 with stress granule marker TIA-1 in HEK293T cells.

HEK293T cells were transfected with peGFP or peGFP[ZCCHC11], and were either left untreated or treated with 0.4 mM sodium arsenite for 45 mins before fixation. Immunostaining was performed as described in Figure 5.8.

5.3.4. ZCCHC11 localises to stress granules following heat shock and hyperosmolar stress

Next, I aimed to understand whether ZCCHC11 also localises to stress granules under other cellular stress conditions. In order to test this, HeLa cells were stressed by exposing them to heat shock. When the cells were exposed to heat shock at 45°C for 30 min, localisation of ZCCHC11 to stress granules was observed (Fig 5.11). Similarly, ZCCHC11 localised to cytoplasmic stress granules as visualised by using stress granule marker TIA-1 when the cells were exposed to hyperosmolar stress as described (Hatayama, Fujimoto, and Sakai 1997)(Figure 5.12). In addition, TIA-1 relocalised to a compartment in the nucleus where ZCCHC11 is already present (Fig 5.13). Therefore, it was concluded that the localisation of ZCCHC11 to stress granules was not only induced under oxidative stress, but is a more general phenomenon observed under other stress conditions.

5.3.5. Investigating the effect of hydroxyurea treatment on colocalisation of ZCCHC11 with TIA-1

Next, it was investigated whether genotoxic stress induced by hydroxyurea treatment results in co-localisation of ZCCHC11 with stress granule marker TIA-1. To my knowledge, there are no reports regarding stress granule formation under hydroxyurea stress. In parallel with the literature, my findings also showed no stress granule formation following 30 minutes of hydroxyurea treatment. In addition, ZCCHC11 did not colocalise with TIA-1 following hydroxyurea treatment. Therefore, it can be suggested that

ZCCHC11 does not colocalise with TIA-1 under stress conditions that do not result in stress granule formation (Fig 5.14).

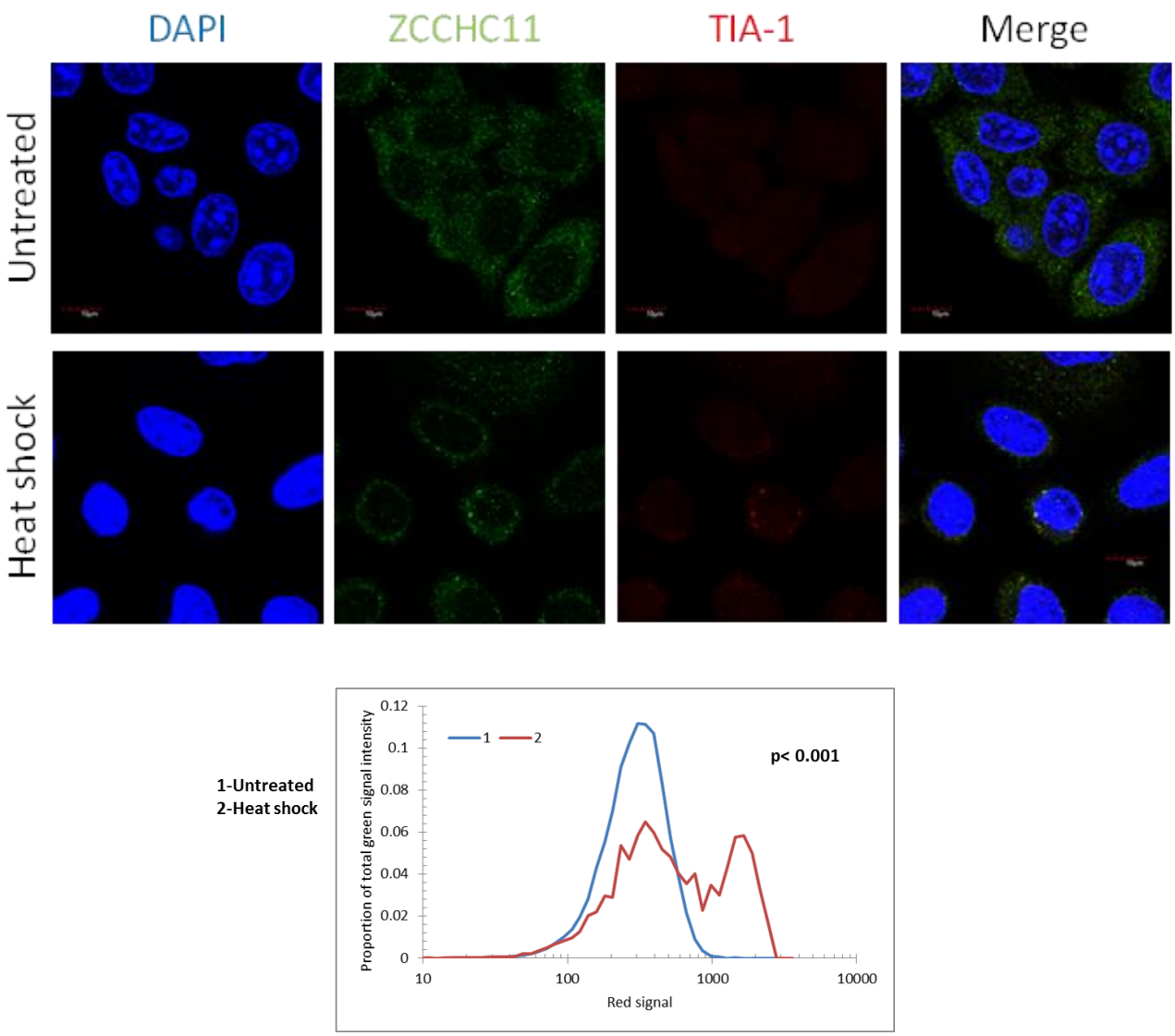


FIGURE 5.11. Colocalisation of ZCCHC11 with stress granule marker TIA-1 in HeLa cells under heat shock stress.

HeLa cells were incubated at 45°C for 30 mins before fixation. They were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). Lower panel is the demonstrative histogram showing the proportion of anti-ZCCHC11 staining (y-axis) relative to anti-TIA-1 staining (x-axis). Kolmogrov-Smirnov test was used for statistical analysis.

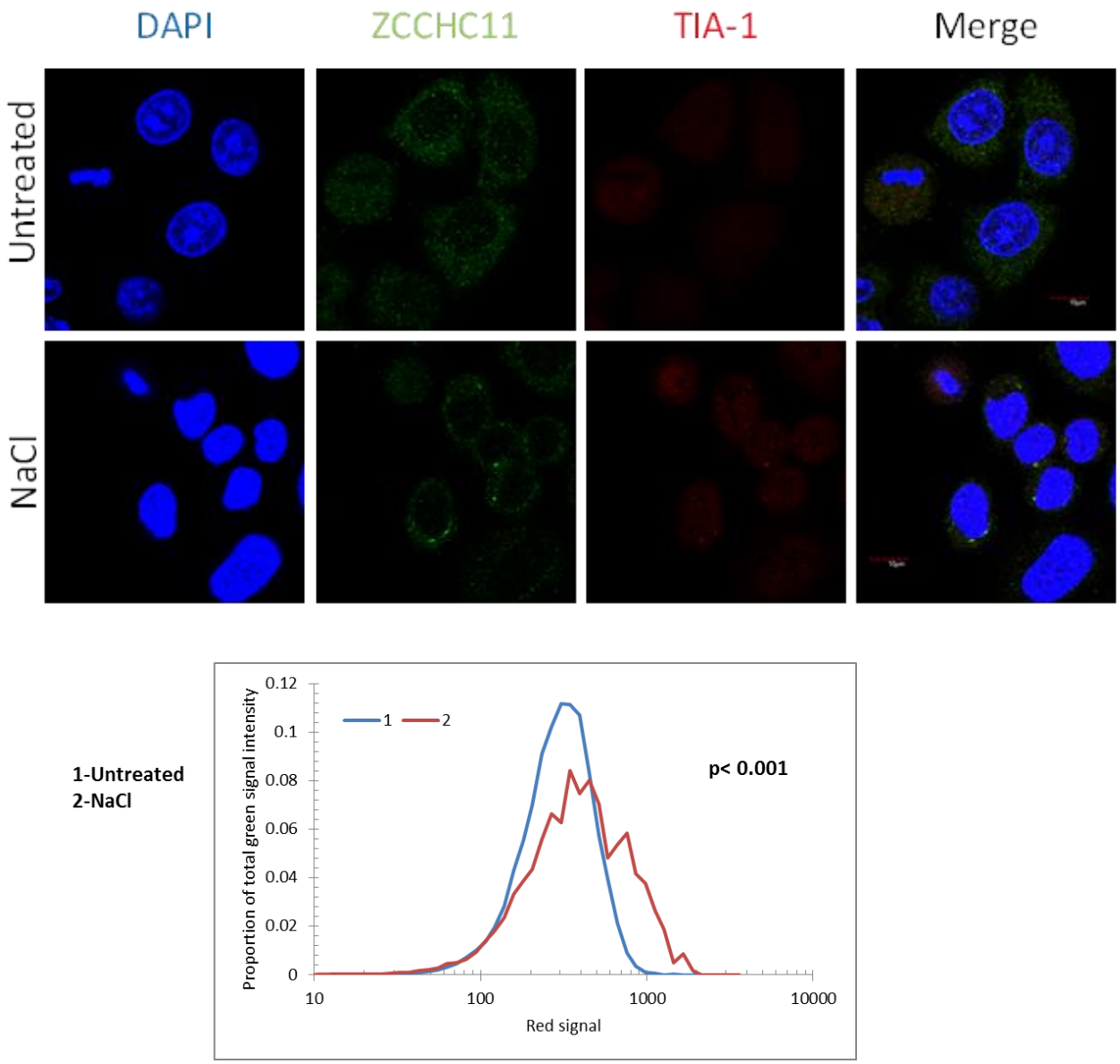


FIGURE 5.12. Colocalisation of ZCCHC11 with stress granule marker TIA-1 in HeLa cells under hyperosmolar stress.

HeLa cells were treated with 150 mM NaCl overnight before fixation. They were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). Lower panel is the demonstrative histogram showing the proportion of anti-ZCCHC11 staining (y-axis) relative to anti-TIA-1 (x-axis) staining. Kolmogrov-Smirnov test was used for statistical analysis.

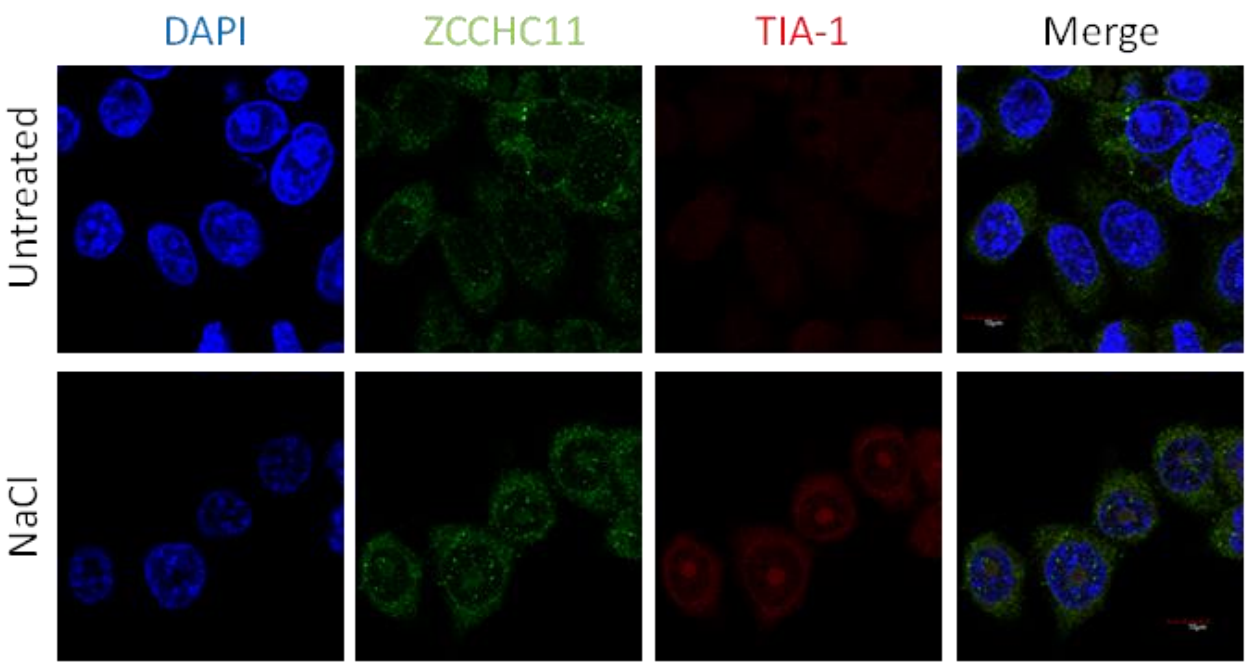


FIGURE 5.13. Colocalisation of ZCCHC11 with stress granule marker TIA-1 in the nucleus of HeLa cells under hyperosmolar stress.
HeLa cells were treated with 150 mM NaCl overnight before fixation. They were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue).

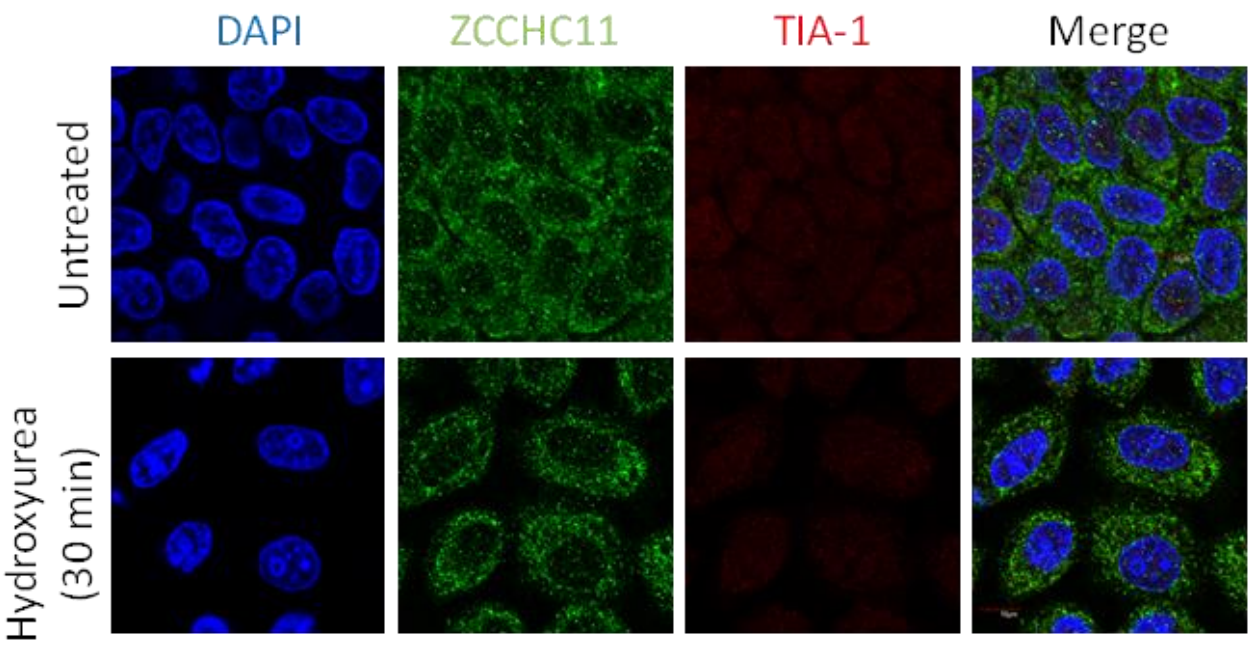


FIGURE 5.14. Investigation of colocalisation of ZCCHC11 with stress granule marker TIA-1 following 30 min hydroxyurea treatment.

HeLa cells were treated with 5 mM hydroxyurea 30 minutes before fixation. They were immunostained with anti-TIA-1 antibody (red) as a stress granule marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue).

5.3.6. Motifs required for localisation of ZCCHC11 to stress granules

My results showed that ZCCHC11 localises to stress granules under cellular stress. However, the underlying molecular mechanism and potential involvement of interactions with other proteins are not known. ZCCHC11 contains three CCHC zinc knuckles and one C2H2 zinc finger (Figure 1.1.). The C2H2 zinc finger of ZCCHC11 was shown to be responsible for functional interaction with Lin28 (Thornton et al. 2012). In the same study, the second most distal zinc knuckle was shown to be important for the uridylation activity of ZCCHC11. Besides, several other studies attributed C2H2 zinc fingers and CCHC zinc knuckles roles in protein-protein/protein-RNA interactions (Matthews et al. 2000; Iuchi 2001; Curtis et al. 1997). In addition, a serine (S104) has been identified as a site of phosphorylation by Cyclin-dependent kinases (CDKs)/ Mitogen-activated protein kinases (MAPK) in public-domain proteomics databases (http://www.phosphosite.org). Therefore, I investigated whether these sites, which have potential roles in the regulation of ZCCHC11 or its interaction with other structures, are important in its localisation to stress granules. Cells were transfected with either peGFP[ZCCHC11] or its mutants lacking individual sites mentioned above. A plasmid encoding GFP (peGFP) was transfected in parallel as a control. All the cells were then treated with arsenite. Mutating the C2H2 motif or S104 by replacing histidine or serine with alanine respectively did not decrease co-localisation rate with stress granule marker TIA-1 in comparison with the peGFP[ZCCHC11]-transfected group. However, mutating the second most distal CCHC knuckle by replacing histidine with alanine significantly decreased the level co-localisation with TIA-1 under stress conditions (Fig.

5.15-16). Nevertheless, the level of colocalisation of peGFP[ZCCHC11_CCHC2_mut] with TIA-1 was slightly higher than peGFP under oxidative stress, suggesting the potential involvement of other motifs in the localisation to stress granules.

Attempts to generate a mutant with more than one CCHC mutated were unsuccessful. However, the second most distal CCHC zinc knuckle plays potentially the most important role in the differential localisation of ZCCHC11 to stress granules following cellular stress, although it is not solely responsible.

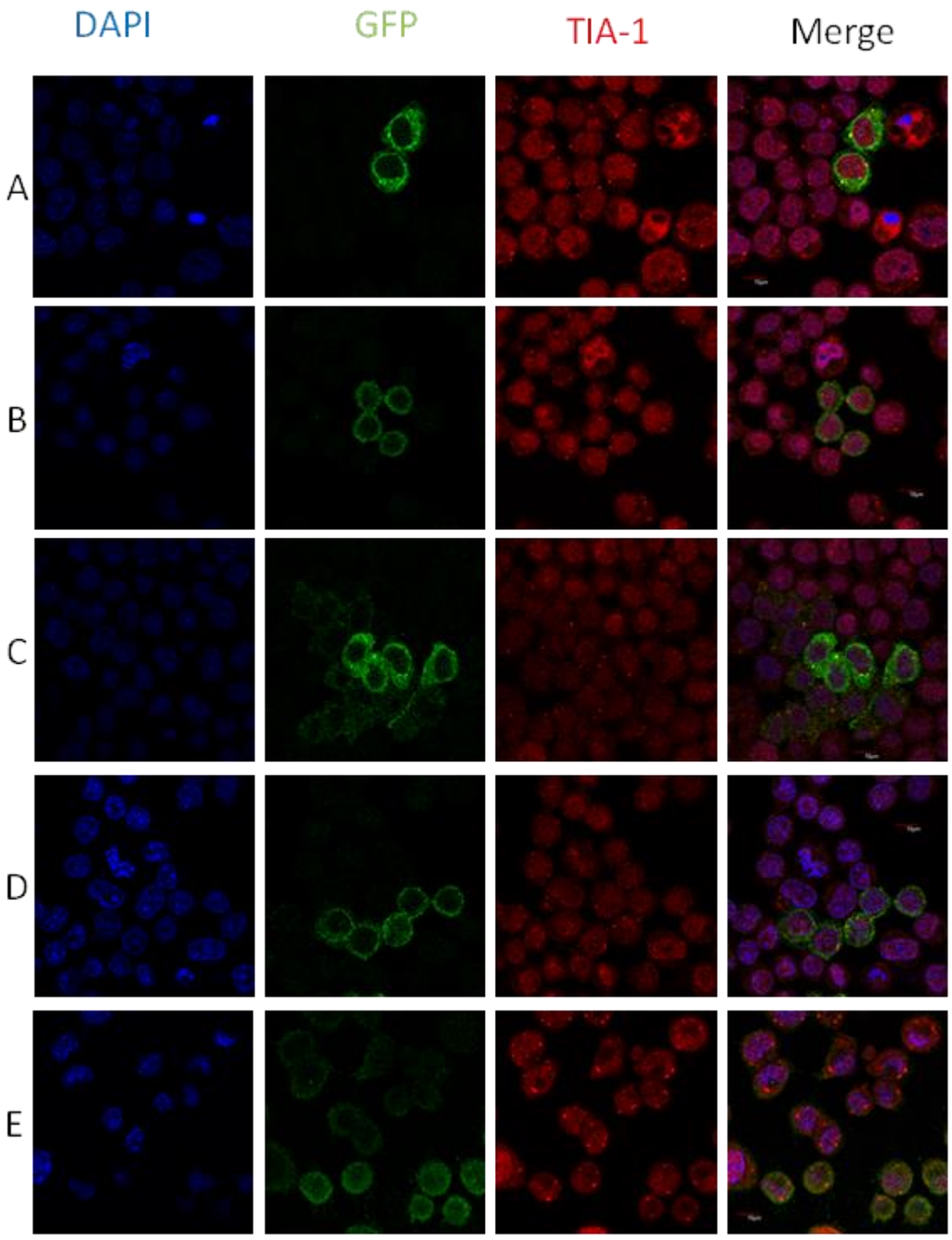


FIGURE 5.15. Investigating the motifs required for the localisation of ZCCHC11 in stress granules.

HeLa cells were transfected with peGFP[ZCCHC11](A), peGFP[ZCCHC11_CCHC2_mut](B), peGFP[ZCCHC11_C2H2_mut](C), peGFP[ZCCHC11_s104a](D), peGFP(E). All of the samples were treated with 0.4 mM sodium arsenite for 45 min before fixation. Immunostaining was performed as described in Figure 5.8. Figure 5.16 shows the statistical analysis of this experiment.

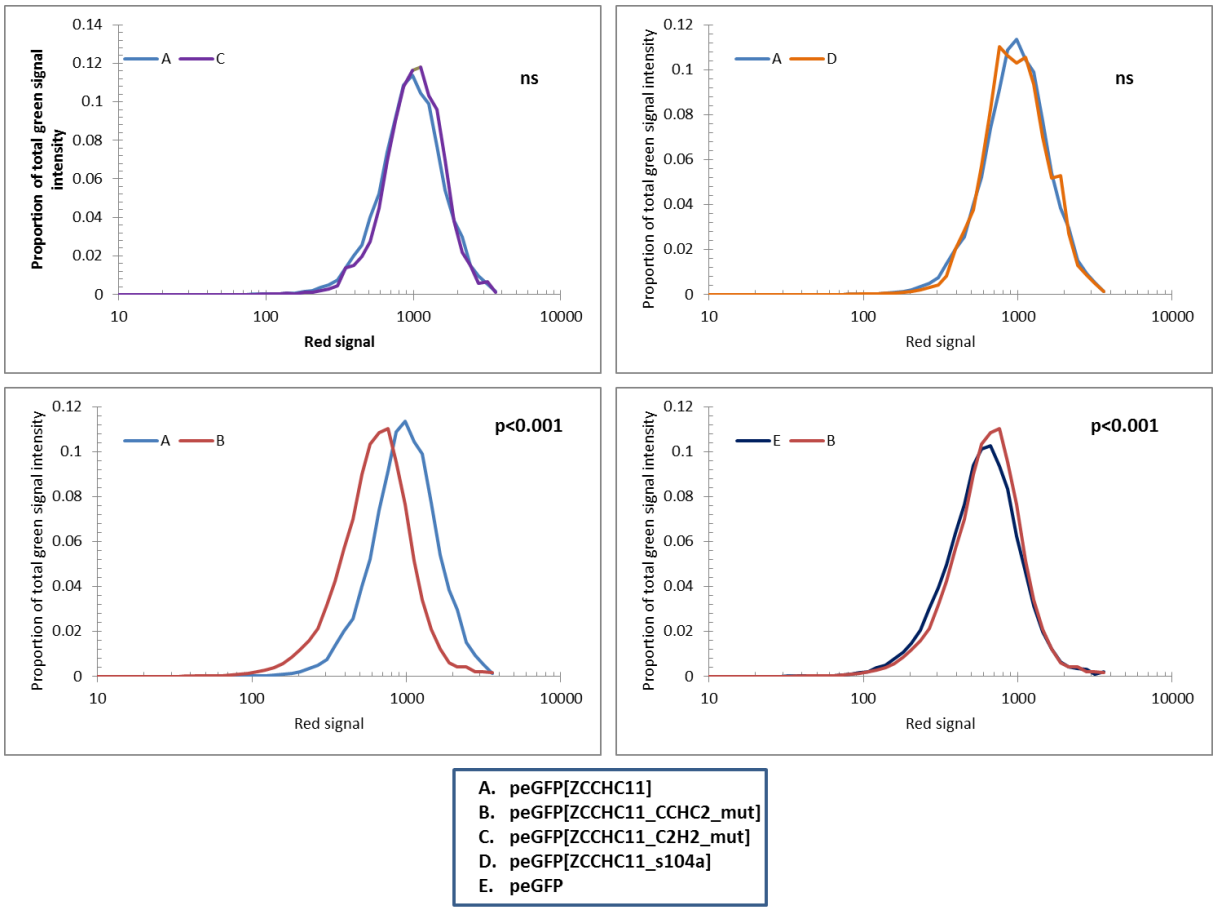


FIGURE 5.16: Statistical analysis of Figure 5.15.

Panels show representative histograms indicating the proportion of anti-GFP staining (y-axis) relative to anti-TIA-1 staining (x-axis) in plasmid-transfected cells. The Kolmogrov-Smirnov test was used for statistical analysis.

5.3.7. Cellular stress results in increased ZCCHC11 levels

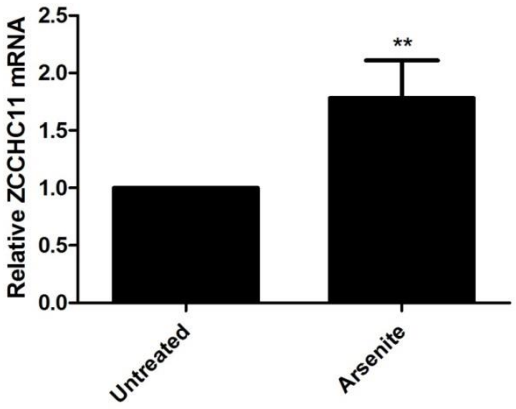
The localisation of ZCCHC11 to stress granules under cellular stress suggested a potential role for ZCCHC11 in cellular survival under stress conditions. Therefore, I hypothesised that ZCCHC11 expression might be regulated following cellular stress. Cells were treated with sodium arsenite for 24 hours before collection. Multiple concentrations of sodium arsenite were tested to determine the concentration at which less than 50% of the cells died. ZCCHC11 increased both in mRNA (Fig. 5.17a) and protein levels following arsenite treatment (Fig 5.17b). These results showed that cellular stress not only induced the localisation of ZCCHC11 to stress granules but also increased its expression.

5.3.8. The ZCCHC11 UTRs take part in up-regulation of ZCCHC11 under oxidative stress

Cells respond to stress conditions by favouring the up-regulation of proteins that promote cellular survival. My results showed that ZCCHC11 localises to stress granules, and its expression increases at both mRNA and protein levels under stress. This may indicate that ZCCHC11 is an important element in the cell survival under stress conditions. Regulation through both UTRs has been shown to function in favouring selective gene expression under these abnormal conditions (Lee, Cevallos, and Jan 2009; von Roretz et al. 2011)). In addition, as ZCCHC11 increases at both mRNA and protein levels under stress conditions, it was hypothesised that a post-transcriptional and/or a translational response involving the UTRs of ZCCHC11 might play a role in this up-regulation. In order to investigate this hypothesis, cells were transfected with peGFP[ZCCHC11_5'UTR_short], peGFP[ZCCHC11_5'UTR_long], peGFP[ZCCHC11_3'UTR]

and peGFP. 24 hours after transfection cells were either treated with sodium arsenite or left untreated. 48 hours after transfection cells were collected and eGFP expression was quantified by flow cytometry. After normalisation of the results to peGFP-transfected cells, it was observed that the 3'UTR and both isoforms of the ZCCHC11 5'UTR favoured gene expression in response to sodium arsenite treatment. The 3'UTR and the shorter 5'UTR isoform of ZCCHC11 mRNA relieved the inhibition of gene expression after the induction of oxidative stress, while the longer 5'UTR isoform of ZCCHC11 increased gene expression substantially (Fig. 5.18). Given that 5'ORFs have been shown to play roles in stress-induced up-regulation of genes, the uAUGs in the 5'UTR of ZCCHC11 were mutated and a similar experimental design was followed. The triple mutation of the uAUGs in the 5'UTR resulted in the complete reversal of the up-regulation conferred by the 5'UTR under stress conditions (Fig 5.18). These results suggest that the UTRs of ZCCHC11 function in maintaining and increasing its expression following cellular stress, and the uAUGs are key to the regulation through the 5'UTR.

a.



b.

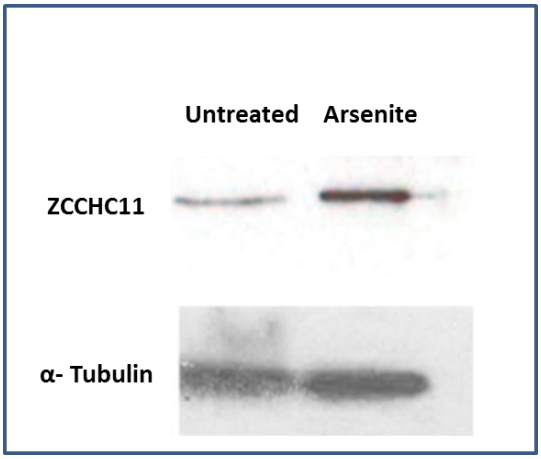


FIGURE 5.17. Effect of cellular stress on ZCCHC11 expression.
Oxidative stress was induced using 15 µM sodium arsenite for 24 hours. **a.** The graph shows the effect of oxidative stress on ZCCHC11 mRNA levels. Columns represent the mean of the qRT-PCR results of at least three independent experiments ±SD. The result of sodium arsenite-treated group was compared with untreated cells whose ZCCHC11 mRNA expression value is considered as one. Data were normalised against β-Actin mRNA levels. Student's t-test was applied and p values are determined using the peGFP control as a reference (** $p<0.01$). **b.** Immunoblot showing the effect of sodium arsenite treatment on ZCCHC11 expression. α-Tubulin was used as a loading control. The immunoblot is representative of at least three independent experiments.

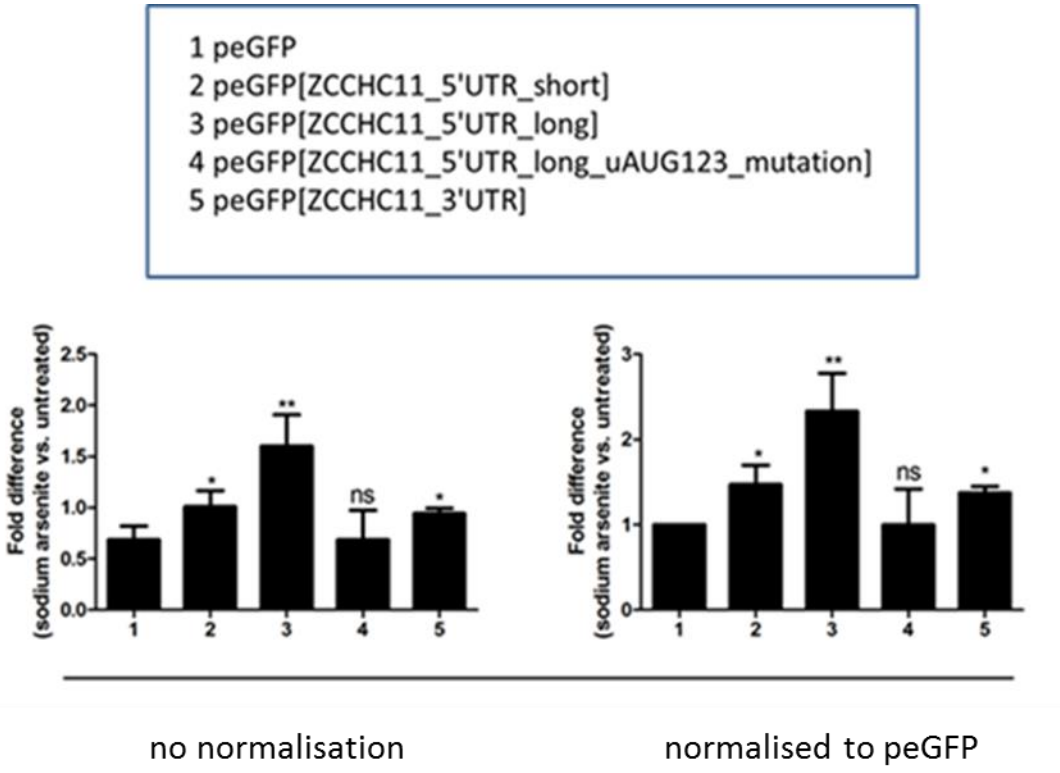


FIGURE 5.18. The effect of the UTRs of ZCCHC11 on its regulation under oxidative stress.
Cells were transfected with peGFP, peGFP[ZCCHC11_3'UTR], peGFP[ZCCHC11_5'UTR_short], peGFP[ZCCHC11_5'UTR_long] or peGFP ligated to the long 5'UTR of ZCCHC11 whose uAUGs are mutated in combination (peGFP[ZCCHC11_5'UTR_long_uAUG123]). 24 hours after transfection cells were seeded into different wells and either left untreated or treated with 15 µM sodium arsenite. Upper panel shows the transfected plasmids. Lower panel demonstrates eGFP expression estimated by flow cytometry either before (left) or after (right) normalisation to peGFP treated group. Student's t-test was applied and p values were determined using peGFP in each figure as reference (ns= nonsignificant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

5.3.9. ZCCHC11 localises to p-bodies under cellular stress

The results discussed in this chapter suggest that ZCCHC11 localises to the cytoplasm where it forms foci. The preferential localisation of ZCCHC11 to certain cytoplasmic compartments did not overlap with mitochondria or vesicular structures. Although ZCCHC11 localises to stress granules, this does not explain foci formation under normal circumstances, as localisation of ZCCHC11 to stress granules occurred only under stress conditions. p-bodies are cellular structures that comprise a variety of proteins and they are present in normal conditions. Given that Lin28A was found to localise in both stress granules and p-bodies, I investigated whether ZCCHC11 also localises to p-bodies. GW182, a marker for p-bodies, was used to visualise p-bodies in this study. Under normal conditions, ZCCHC11 did not localise to p-bodies. Given that p-bodies can be induced by cellular stress (Souquere et al. 2009; Iwaki and Izawa 2012), one group of cells was treated with sodium arsenite. Interestingly ZCCHC11 localised to p-bodies following cellular stress (Fig. 5.19). These results suggest that ZCCHC11 localises to both stress granules and p-bodies under cellular stress.

5.3.10. Does ZCCHC11 localise to the nucleus in S-phase?

The studies in this chapter suggested that ZCCHC11 is predominantly cytoplasmic. However, the presence of ZCCHC11 in the nucleus, albeit at a lower level, led me to speculate that ZCCHC11 activity may be regulated by nucleo-cytoplasmic shuttling. A previous study from our group showed that ZCCHC11 level decreases in mid-S phase. (Schmidt, West, and Norbury 2011). This downregulation may be particularly important when the inhibitory effect of ZCCHC11 on histone mRNA, and the requirement of cells to synthesize histones during DNA replication are taken into consideration. The results

discussed in Chapter 3 suggested that downregulation of ZCCHC11 might be conferred by its 3'UTR, at least in part. One other way of regulating the biological activity of a protein is to alter its subcellular localisation during certain phases of the cell cycle. For example, the activity of the transcription factor E2F-4 is regulated by a change in subcellular localisation in a cell cycle-dependent manner (Lindeman et al. 1997; Verona et al. 1997). I therefore aimed to understand whether analogous regulation is exerted on ZCCHC11 to decrease its cytoplasmic presence in S-phase by a cell cycle-dependent relocalisation to the nucleus. In order to investigate this, cells were treated for 24 hours with 2 mM hydroxyurea to arrest them in S phase as described in Chapter 3. However, there was no significant change in the nuclear immunostaining of ZCCHC11, suggesting that nucleo-cytoplasmic shuttling is not a means of regulation of ZCCHC11 activity during S phase (Fig 5.20.).

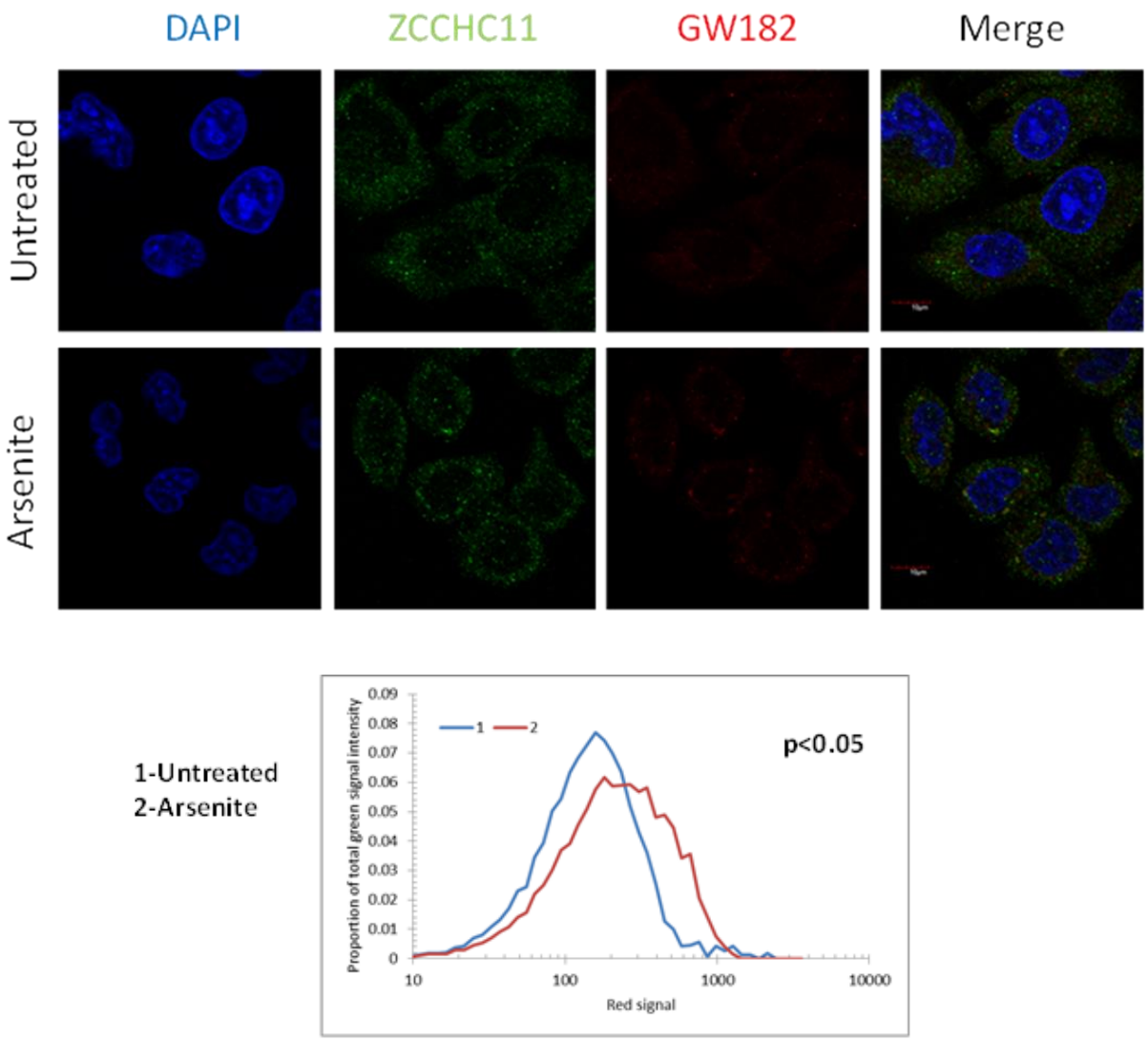


FIGURE 5.19. ZCCHC11 colocalises with p-body marker GW182 under oxidative stress in HeLa cells.

HeLa cells were treated with 0.4 mM sodium arsenite for 45 mins before fixation. They were immunostained with anti-GW182 antibody (red) as a p-body marker and with anti-ZCCHC11 antibody (green). Nuclei were stained with DAPI (blue). Lower panel is the demonstrative histogram showing the proportion of anti-ZCCHC11 (y-axis) staining relative to anti-GW182 (x-axis) staining. Kolmogrov-Smirnov test was used for statistical analysis.

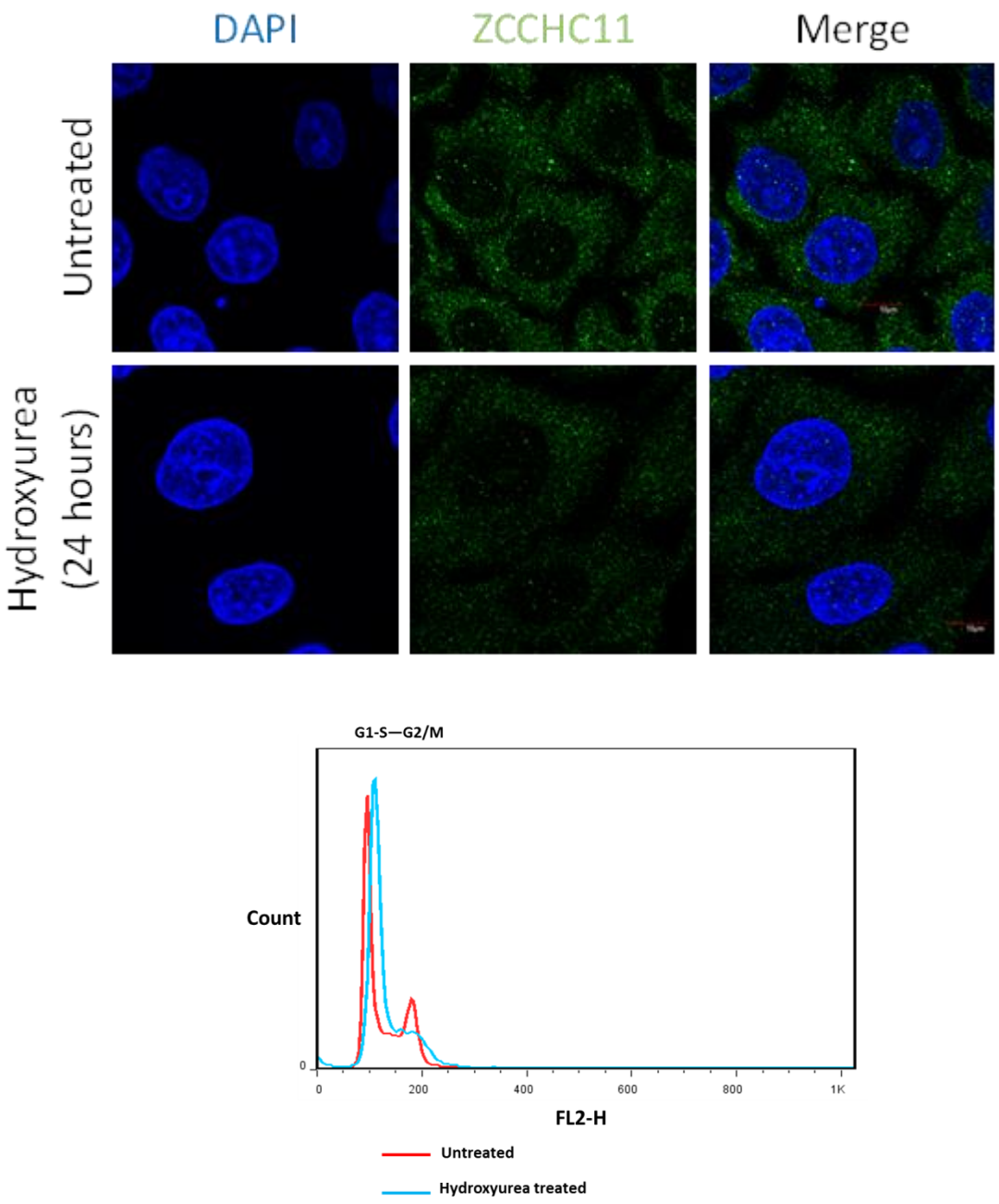


FIGURE 5.20. Investigating the effect of S-phase arrest on the subcellular localisation of ZCCHC11.

HeLa cells were treated with 2 mM hydroxyurea for 24 hours before fixation. They were immunostained with anti-ZCCHC11 antibody (green). Nuclear staining is performed using DAPI (blue). The lower panel shows PI staining (x-axis) in flow cytometry to confirm the S-phase arrest.

5.4. Discussion

In the previous chapters, post-transcriptional regulation of ZCCHC11 through its UTRs was discussed. In order to have a better understanding of the regulation of ZCCHC11, its subcellular localisation must be studied in detail. Given that cells regulate the activity of certain proteins through alteration of their subcellular localisation, especially in response to external stimuli, one other basis for the regulation of ZCCHC11 might be a change in its subcellular localisation.

Previous studies in the literature suggested different sub-cellular localisations for ZCCHC11. First, it was suggested that ZCCHC11 localises to the nucleus and translocates to the cytoplasm following LPS treatment in RAW 264.7, a macrophage cell line (Minoda et al. 2006). However, a previous study from our group suggested a cytoplasmic localisation for ZCCHC11 in HEK293T cells (Schmidt, West, and Norbury 2011). The results in this chapter show that ZCCHC11 localises predominantly to the cytoplasm and weakly to the nucleus in HeLa, HEK293T, A204 and MDA-MB-231 cell lines. The degree of nuclear localisation varied between cell lines. However, the pattern of foci observed for both the endogenous ZCCHC11 and the GFP- tagged ZCCHC11 led me to investigate potential sub-cytoplasmic compartments to which it could localise. ZCCHC11 did not localise to the mitochondria, as shown using a mitochondrial staining reagent, MitoTracker Orange CMXRos. Given that vesicular structures often interact with the microtubular cytoskeleton, I also investigated whether these foci are membranous vesicles using an antibody against α-tubulin. However, no co-localisation was seen, decreasing the possibility that the cytoplasmic ZCCHC11 foci are vesicles.

In this study, the regulation of the expression and subcellular localisation of ZCCHC11 under stress conditions were studied. The rationale behind this investigation can be explained in two ways. First, ZCCHC11 was shown by our group to promote histone mRNA degradation following genotoxic stress. Second, although none of the human terminal uridyl transferases were previously shown to localise either to p-bodies or to stress granules, Lin28A, a key interacting partner of ZCCHC11, was shown to localise to both (Balzer and Moss 2007). One possible mechanism of ZCCHC11 regulation would involve its translocation to certain sub-cellular structures following stress. Stress granule assembly is induced by several stress conditions such as oxidative stress, hyperosmolar stress and heat shock. On the other hand, p-bodies assemble under non-stress conditions. However, several studies suggested that the formation of p-bodies might occur following cellular stress (Kilchert et al. 2010; Souquere et al. 2009; Iwaki and Izawa 2012). My results show that ZCCHC11 localises to stress granules under oxidative stress. Although the stress granule formation after heat shock or hyperosmolar stress occurred at a reduced frequency in comparison with oxidative stress, ZCCHC11 also localised to stress granules under these stress conditions. Interestingly, ZCCHC11 did not localise to p-bodies under normal circumstances, but did so only after stress induction. This is in line with reports that show either the localisation of several proteins to p-bodies after stress induction or the increase in their presence in p-bodies under stress conditions (Quaresma et al. 2009; Vidalino et al. 2012). Despite the significant localisation of ZCCHC11 to stress granules and p-bodies under stress conditions, there was not a complete overlap between the foci of ZCCHC11 and foci of stress granule marker TIA-1 or p-body marker GW182. This can be explained by the fact that p-bodies and stress granules lie in close proximity to each other and ZCCHC11 localises to both structures.

Given that only one of these structures was visualised together with ZCCHC11 at a time, non-overlapping parts of ZCCHC11 could in fact be a part of the other structure. On the other hand, both structures studied here are dynamic and have a variety of components. The composition of these granules can change from granule to granule, therefore a proportion of the stress granules or p-bodies may not contain ZCCHC11. Nevertheless, my findings showing translocation of ZCCHC11 to stress granules and p-bodies under stress revealed two things. First, ZCCHC11, an important protein in RNA decay, localises in these two structures that are important in RNA metabolism. Second, ZCCHC11 might play a role in cellular stress responses. Hypothetically, ZCCHC11, which increases upon cellular stress, would be recruited to stress granules and p-bodies upon stress induction by an RNA-binding protein, which is a component of these structures, and binds specifically to RNA targets of ZCCHC11. Upon recruitment of ZCCHC11 to these structures, it may uridylate and promote the degradation of these RNAs.

ZCCHC11 contains numerous structural features, including a C2H2 zinc finger, three CCHC zinc knuckle motifs and a serine residue (S104), which is known to be phosphorylated, potentially by CDK/MAPK. These motifs might be potentially important for molecular interactions or for signals responsible for the re-localisation to stress granules. However, site-directed mutagenesis experiments revealed that the C2H2 finger and S104 are not required for this phenotype. Mutating the second most distal CCHC motif, on the other hand, caused significantly reduced co-localisation with stress granule marker TIA-1. It is possible that this mutation might potentially disrupt the interaction of ZCCHC11 with the molecule that recruits it to the stress granules. In order to understand the stress-related regulation of ZCCHC11 in more detail, the expression of ZCCHC11

following oxidative stress was investigated. Interestingly, ZCCHC11 increased at both mRNA and protein levels when cells were stressed with sodium arsenite. Both UTRs function in mediating this regulation. The up-regulation conferred by the 5'UTR of ZCCHC11 under sodium arsenite stress requires the uORFs, as the mutation of the uAUGs resulted in complete reversal of this effect. Although uORFs are well known for their function in decreasing gene expression, they can also promote gene expression under cellular stress. In this study, it was found that the uORFs in the 5'UTRs of ZCCHC11 mRNAs down-regulate expression mainly by decreasing ZCCHC11 translation. However, it was also found that the uORFs up-regulate ZCCHC11 expression following sodium arsenite treatment, and this effect was more potent in the case of the longer 5'UTR isoform. Therefore, it can be concluded that the 5'UTR of ZCCHC11 has a complicated regulatory function through its uORFs; down-regulatory under normal circumstances and up-regulatory under stress conditions.

My results showed stress-induced up-regulation of ZCCHC11 expression as well as translocation of ZCCHC11 to stress granules and p-bodies under stress. This suggests that ZCCHC11 plays a role in cellular stress responses. On the other hand, the pattern of foci formed by ZCCHC11 in normal circumstances could not be explained by any of the sub-cellular structures that were investigated in this study. It can be speculated that ZCCHC11 forms novel foci with several other enzymes important in RNA metabolism.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

Chapter 6: CONCLUSIONS AND FUTURE DIRECTIONS

This chapter discusses the results presented in Chapters 3, 4 and 5. It also includes suggested future directions in the light of current literature and the findings presented in this thesis regarding the post-transcriptional regulation of ZCCHC11 and its stress-dependent regulation.

Increasing evidence from the literature suggests that ZCCHC11 is an important element in many diseases, especially in cancer, but very little is known about its regulation. This thesis aimed to investigate the regulation of the expression of ZCCHC11, especially at the post-transcriptional level. In particular, a role of the ZCCHC11 3'UTR in restricting its expression was identified. The data presented suggest that ZCCHC11 regulation through its 3'UTR involves complex mechanisms such as different *trans*-acting factors that influence gene expression in opposite directions, the impact of alternative polyadenylation and regulation by the orthologous enzyme ZCCHC6. The AU-rich sequences of the 3'UTR of ZCCHC11 were strong candidates for regulatory elements. The findings in this thesis strongly support such a regulation, and show that the AU-rich binding protein HuR regulates ZCCHC11 expression. The proximal part of the 3'UTR is responsible for the overall repression of expression, whereas the distal part of the 3'UTR functions in up-regulation of gene expression. A similar dichotomy was observed in relation to the *trans*-acting factors involved. HuR increased ZCCHC11 expression while TTP down-regulated it. The effect observed following TTP knockdown only partially rescued the 3'UTR-mediated down-regulation, suggesting either that there may be other factor(s) involved in down-regulation via the 3'UTR, or that the extent of TTP knock-down was insufficient to eliminate its activity completely.

In the case of HuR, which affects the expression of ZCCHC11 through both halves of its 3'UTR, it can be speculated that there is a competition between HuR and AUBPs that negatively regulate ZCCHC11 gene expression.

The results presented in Chapter 3 showed the influence of HuR on ZCCHC11 regulation through its 3'UTR. This effect may be direct or indirect. AUBPs recognize and bind AU-rich elements; nevertheless, they can have biological effects through indirect mediators such as non-coding RNAs (Kundu et al. 2012; Yoon et al. 2012b). In order to find out whether this regulation requires an interaction between the 3'UTR and the AUBPS, an RNA-IP approach could be followed. Either endogenous HuR or an epitope-tagged version could be immunoprecipitated, and the presence of ZCCHC11 mRNA investigated using RT-PCR. High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) would also make it possible to determine whether this interaction is direct.

Understanding all the factors responsible for the regulation of ZCCHC11 through its 3'UTR requires further studies. The approach of knocking down candidate AUBPs has been followed in this study and this revealed HuR and TTP as key factors in this regulation. An RNP affinity purification method followed by mass spectrometric analysis could provide further details regarding the proteins binding to the 3'UTR.

ZCCHC6, the other orthologue of Cid1 in human cells, was found to down-regulate ZCCHC11 expression through a mechanism involving its 3'UTR. This study also revealed HuR as a common positive regulator of both ZCCHC6 and ZCCHC11. The complicated functional interplay between these two terminal uridyl transferases may relate to their partially redundant functions in regulating *let-7* biogenesis (Thornton et al. 2012). The

substrate recognition by terminal uridyl transferases is poorly understood, but may require some element near the 3' end of the RNA target. ZCCHC6, an enzyme that uridylates its substrates, also down-regulates ZCCHC11. Nevertheless, the nature of this regulation is currently not understood. This study showed that the 3'UTR of ZCCHC11 is important in the down-regulation by ZCCHC6. Whether this involves the 3' uridylation of ZCCHC11 mRNA by ZCCHC6 has yet to be determined.

Characterisation of the 3' end of the message revealed different ZCCHC11 mRNA isoforms that differ in their 3' end sequence, suggesting potentially regulatory alternative polyadenylation. The mutation of the potential alternative polyadenylation site in experiments using an eGFP reporter system increased the proportion of the longer isoform to shorter isoform and decreased eGFP reporter construct expression. Alternative polyadenylation of genes participates in gene regulation by exclusion of *cis*-acting elements such as miRNA binding sites in the shorter isoform and the inclusion of them only in the longer isoform. In the case of ZCCHC11 a potential miRNA binding site distal to the alternative polyadenylation site is a potential regulatory element. However, contrary to a previous study, in which the effect of *miR-26* binding to ZCCHC11 was reported to be down-regulatory, such a role was not supported under normal conditions by the site-directed mutagenesis analysis of the potential miRNA binding site performed here.

The 5'UTRs of mRNAs are also important in the regulation of the gene expression. This study characterised the 5' end of the ZCCHC11 mRNA in detail. The 5'UTR seems to limit ZCCHC11 expression under normal circumstances through the involvement of uORFs. This effect is solely translational and does not involve the mRNA decay machinery.

The results presented in Chapter 3 mainly address regulation of ZCCHC11 under normal conditions. A previous study from our group described a decrease of ZCCHC11 expression during S phase; the data presented here implicate the ZCCHC11 3'UTR in this S phase-dependent regulation. The dynamics of post-transcriptional regulation are complex, involving competition and redundancy between multiple *trans*-acting factors. My findings show that the post-transcriptional regulation of ZCCHC11 is also complex. Conditions such as cellular stress might alter the regulation through the ZCCHC11 UTRs by favouring the effect of a particular *trans*-acting factor while inhibiting others. Interestingly, this study revealed that both UTRs of ZCCHC11 favoured its expression under cellular stress. The *trans*-acting factors involved in this regulation remain to be identified. Nevertheless, my results showed that uORFs in the 5'UTR take part in up-regulation of ZCCHC11 under stress. This is particularly interesting when their down-regulatory role in normal circumstances is taken into consideration. The stress-dependent regulation of ZCCHC11 was also reflected at the protein level. Cells shut down bulk protein synthesis as part of a general response to stress, while maintaining or increasing the expression of selected proteins with stress survival functions. Therefore, these results indicate a likely requirement of cells for ZCCHC11 under stress conditions.

This thesis also demonstrated that ZCCHC11 localises predominantly to the cytoplasm and, to a varying degree, the nucleus. The foci-forming pattern of its cytoplasmic localisation under normal conditions was investigated, but none of the sub-cellular structures tested in this study, including mitochondria, vesicles and p-bodies, were found to overlap with these foci under normal circumstances. These novel foci require

further investigation, and the identification of proteins interacting with ZCCHC11 might help to clarify their formation.

One mode of biological regulation is to change the localisation of proteins, thus changing their activity. This thesis revealed that ZCCHC11 localises to stress granules under various stress conditions. This was confirmed using two different protein markers to visualise the stress granules. The potential importance of ZCCHC11 in stress responses is further supported by its relocalisation to stress granules. Interestingly, under stress conditions ZCCHC11 also localised to p-bodies. These results suggest that ZCCHC11 is a component of both p-bodies and stress granules, which are structures of RNA metabolism, under cellular stress. However, the mechanism of the recruitment of ZCCHC11 to these granules remains unknown. ZCCHC11 is recruited to pre-*let-7* by Lin28, which is also a component of stress granules and p-bodies. ZCCHC11 might be recruited to these structures by certain proteins that recognize *cis*-acting elements in the RNAs targeted by ZCCHC11 under stress. The second most distal CCHC motif of ZCCHC11, which was previously shown to be important in the uridylation of pre-*let-7*, was shown to be important in the localisation of ZCCHC11 to stress granules. Further experiments, such as mutating all of the CCHC zinc knuckles or generating truncation mutants, could be performed to investigate the motifs required for this localisation.

There is increasing evidence that ZCCHC11 promotes tumour growth, and its knock-down results in decreased cell number or tumour volume in cancers (Blahna et al. 2011; Piskounova et al. 2011). The ability of cancer cells to survive, especially under stress conditions, may be related to the activity of ZCCHC11. Cancer cells might benefit from

the activity of ZCCHC11 during survival from chemotherapeutic or radiotherapeutic stress.

In summary, under normal conditions, ZCCHC11 is down-regulated post-transcriptionally through its UTRs and translationally through its 5'UTR. The regulation through its 3'UTR requires interactions with AUBPs. In addition, the 3'UTR decreases expression even further in S phase-arrested cells and favours gene expression during cellular stress. The 5'UTR also plays an important role in the increased ZCCHC11 expression observed under stress. Finally, ZCCHC11 was found to localise to stress granules and p-bodies under stress, which further suggest a potential role of ZCCHC11 in cellular stress responses.

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Appendix A: Sequences of the 3'UTR of ZCCHC11 or its fragments and subfragments

AU-rich elements are displayed with colour codes. (ATTTA: red, ATTTTA: yellow, ATTTTTA: blue, NNUUNNUUU: green, *miR-132/212* potential binding site: grey, *let-7* potential binding site: brown, *miR-26* potential binding site: pink)

3'UTR of peGFP[ZCCHC11_3'UTR]

TGCGAGTCCATTTTCTTTCAGCTGGTCTACCGATGCACAGCAACCAGCCAAT
CCTGCTGTCTCAAGGGTATCCGTACCTCAATGTCAGTTACATTCAGCAGAAA
AAGTGACATTTAATGGAAAGCAAAACAAATCAGGTCCTGATTTAAAATTTTAA
CACGTTTAGATAGCTTATTTAAATTCTAAGACTGTTTTTACAGACTGTATTATT
TGTATATAAATATGAACTATAGTTTTTACTAGTATCACCAAATTGAGTGATACA
AATTTCATCTATTTTATTACCCTATTTTTAAGGGAATTTTATGTTTTGTTTAACC
TTGATGAATTGTATAAAGAGAGAATTTAAAAAATTACATTCTTTATTTTCTATT
AGCTGTATTCATACTTTGGTTGCTTCAGACTTTATATATTGTTCTTAAAAGTTA
GGAAAGAATTTCATGTGTTTTAAATTGTCACTTCTATTCTTTACAGAACCTTGT
AGTGCCAAAAACTTTTTAAAAGGTAAAAAAATAAAATAAAATTACAACATGAA
GATTCAGATTTTTTTTCTTTTCCATACTTGTATATTGAACATTCAAACTTGAAT
GGAAAGTTGATTTTGTCTGTATTTGAAGTACTTCTATTTTAAGTT

3'UTR of peGFP[ZCCHC11_3'UTR_Fr1]

TGCGAGTCCATTTTCTTTCAGCTGGTCTACCGATGCACAGCAACCAGCCAAT
CCTGCTGTCTCAAGGGTATCCGTACCTCAATGTCAGTTACATTCAGCAGAAA
AAGTGACATTTAATGGAAAGCAAAACAAATCAGGTCCTGATTTAAAATTTTAA
CACGTTTAGATAGCTTATTTAAATTCTAAGACTGTTTTTACAGACTGTATTATT
TGTATATAAATATGAACTATAGTTTTTACTAGTATCACCAAATTGAGTGATACA
AATTTCATCTATTTTATTACCCTATTTTTAAGGGAATTTTATGTTTTGTTTAACC
TT

3'UTR of peGFP[ZCCHC11_3'UTR_Fr2]

CCCTATTTTTAAGGGAATTTTATGTTTTGTTTAACCTTGATGAATTGTATAAAG
AGAGAATTTAAAAAATTACATTCTTTATTTTCTATTAGCTGTATTCATACTTTG
GTTGCTTCAGACTTTATATATTGTTCTTAAAAGTTAGGAAAGAATTTCATGTG
TTTTAAATTGTCA

3'UTR of peGFP[ZCCHC11_3'UTR_Fr3]

CATTCTTTATTTTCTATTAGCTGTATTCATACTTTGGTTGCTTCAGACTTTATA
TATTGTTCTTAAAAGTTAGGAAAGAATTTCATGTGTTTTAAATTGTCACTTCTA
TTCTTTACAGAACCTTGTAGTGCCAAAAACTTTTTAAAAGGTAAAAAAATAAA
ATAAAATTACAACATGAAGATTCAGATTTTTTTTCTTTTCC

3'UTR of peGFP[ZCCHC11_3'UTR_Fr4]

AGAACCTTGTAGTGCCAAAAACTTTTTAAAAGGTAAAAAAATAAAATAAAATT
ACAACATGAAGATTCAGATTTTTTTTCTTTTCCATACTTGTATATTGAACATTC
AAACTTGAATGGAAAGTTGATTTTGTCTGTATTTGAAGTACTTCTATTTTAAGT
T

3'UTR of peGFP[ZCCHC11_3'UTR_sf1a]

TGCGAGTCCATTTTCTTTCAGCTGGTCTACCGATGCACAGCAACCAGCCAAT
CCTGCTGTCTCAAGGGTATCCGTACCTCAATGTCAGTTACATTCAGCAGAAA
AAGTGACATTTAATGGAAAGCAAAACAAATCAGG

3'UTR of peGFP[ZCCHC11_3'UTR_sf1b]

TACATTCAGCAGAAAAAGTGACATTTAATGGAAAGCAAAACAAATCAGGTCC
TGATTTAAAATTTTAACACGTTTAGATAGCTTATTTAAATTCTAAGACTGTTTT
TACAGACTGTATTATTTGTATATAAATATGAACTATA

3'UTR of peGFP[ZCCHC11_3'UTR_sf1c]

CTAAGACTGTTTTTACAGACTGTATTATTTGTATATAAATATGAACTATAGTTT
TTACTAGTATCACCAAATTGAGTGATACAAATTTCATCTATTTTATTACCCTAT
TTTTAAGGGAATTTTATGTTTTGTTTAACCTT

3'UTR of peGFP[ZCCHC11_3'UTR_Fr23]

CCCTATTTTTAAGGGAATTTTATGTTTTGTTTAACCTTGATGAATTGTATAAAG
AGAGAATTTAAAAATTACATTCTTTATTTTCTATTAGCTGTATTCATACTTTG
GTTGCTTCAGACTTTATATATTGTTCTTAAAAGTTAGGAAAGAATTTCATGTG
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AAAAGGTAAAAAAATAAAATAAAATTACAACATGAAGATTCAGATTTTTTTCT
TTTCC

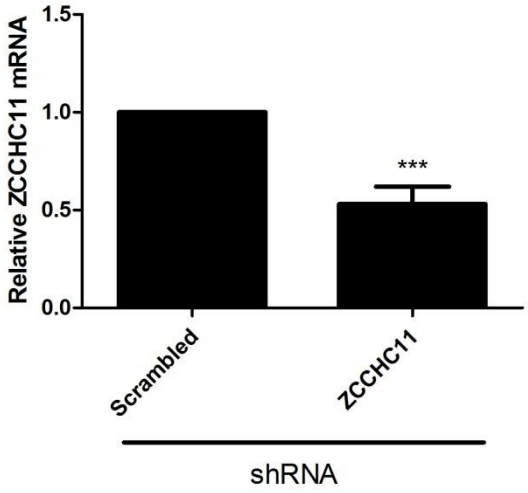
3'UTR of peGFP[ZCCHC11_3'UTR_Fr34]

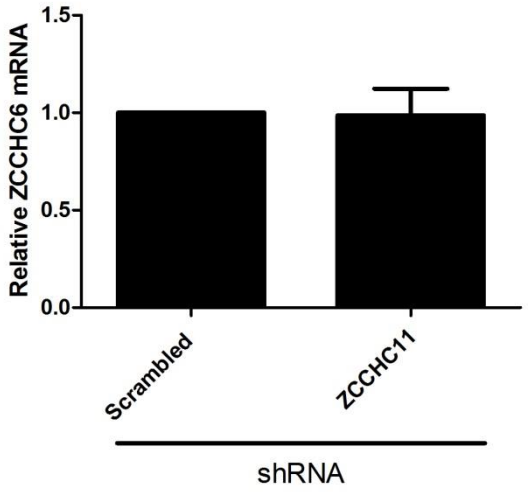
CATTCTTTATTTTCTATTAGCTGTATTCATACTTTGGTTGCTTCAGACTTTATA
TATTGTTCTTAAAAGTTAGGAAAGAATTTCATGTGTTTTAAATTGTCACTTCTA
TTCTTTACAGAACCTTGTAGTGCCAAAAACTTTTTAAAAGGTAAAAAAATAAA
ATAAAATTACAACATGAAGATTCAGATTTTTTTTCTTTTCCATACTTGTATATT
GAACATTCAAACTTGAATGGAAAGTTGATTTTGTCTGTATTTGAAGTACTTCT
ATTTTAAGTT

3'UTR of peGFP[ZCCHC11_3'UTR_Fr234]

CCCTATTTTTAAGGGAATTTTATGTTTTGTTTAACCTTGATGAATTGTATAAAG
AGAGAATTTAAAAAATTACATTCTTTATTTTCTATTAGCTGTATTCATACTTTG
GTTGCTTCAGACTTTATATATTGTTCTTAAAAGTTAGGAAAGAATTTCATGTG
TTTTAAATTGTCACTTCTATTCTTTACAGAACCTTGTAGTGCCAAAAACTTTTT
AAAAGGTAAAAAAATAAAATAAAATTACAACATGAAGATTCAGATTTTTTTCT
TTTCCATACTTGTATATTGAACATTCAAACTTGAATGGAAAGTTGATTTTGTCT
GTATTTGAAGTACTTCTATTTTAAGTT

Appendix B: Investigation of ZCCHC6 levels following ZCCHC11 knockdown





HeLa cells were either transfected with plasmids encoding shRNA against ZCCHC11, or with a scrambled control shRNA. The results were normalised to the group transfected with scrambled control shRNA, the value of which is to one (n=3). Upper panel shows the quantification of knock down efficiency, which was performed using qRT-PCR. Lower panel shows the investigation of the effect of the knockdown of ZCCHC11 on ZCCHC6 expression at the mRNA level.

Appendix C: List of primers used in this study

Primers used in site directed mutagenesis

Mu1_forward	gaattctgcgagtccGCCCCctttcagctggtctaccga
Mu1_reverse	tcggtagaccagctgaaagGGGGCggactcgcagaattc
Mu2_forward	tcagcagaaaaagtgacGCCCGatggaaagcaaaacaaa
Mu2_reverse	tttgttttgctttccatCGGGCgtcacttttctgctga
Mu3_forward	aaacaaatcaggtcctgGCCCGaaattttaacacgttta
Mu3_reverse	taaacgtgttaaaatttCGGGCcaggacctgatttgttt
Mu4_forward	caggtcctgatttaaaGCCCCGacacgtttagatagct
Mu4_reverse	agctatctaaacgtgtCGGGGCtttaaatcaggacctg
Mu5_forward	ttaacacgtttagatagcttGCCCGaattctaagactgtttttac
Mu5_reverse	gtaaaaacagtcttagaattCGGGCaagctatctaaacgtgttaa
Mu6_forward	gtgatacaaatttcatctGCCCCGttaccctatttttaaggga
Mu6_reverse	tcccttaaaaatagggtaaCGGGGCagatgaaatttgtatcac
APAS_mut_F	tttaaaaggtaaaaaaatCGCGtaaaattacaacatgaaga
APAS_mut_R	tcttcatgttgtaattttaCGCGatttttttaccttttaaa
*mir26*_mut_F	gtatattgaacattcaaaTCCAaatggaaagttgattttgtctgta
*mir26*_mut_R	tacagacaaaatcaactttccattTGGAtttgaatgttcaatatac

APPENDIX

let7_mut_F	gtctcaagggtatccgtaTTCTGatgtcagttacattcagcagaaaa
let7_mut_R	ttttctgctgaatgtaactgacatCAGAAtacggatacccttgagac
uORF_1_mut_F	cggcgcggcggacatAgcggtggagtcctga
uORF_1_mut_R	tcaggactccaccgcTatgtccgccgcgccg
uORF_2_mut_F	caggagccactgagatAaggaggcgtcgcgc
uORF_2_mut_R	gcgcgacgcctcctTatctcagtggctcctg
uORF_3_mut_F	aacttaagcttcacaatAcagttcgcggcgcggcg
uORF_3_mut_R	cgccgcgccgcgaactgTattgtgaagcttaagtt
HC11_s104a_F	agcaaaaaaatttcctaatGCaccggtgaaagccgaaaag
HC11_s104a_R	cttttcggctttcaccggtGCattaggaaatttttttgct
HC11_C2H2_mut_F	atataccaattgtcggtatctaGCCaaactttgcttaattcacattg
HC11_C2H2_mut_R	caatgtgaattaagcaaagtttGGCtagataccgacaattggtatat
HC11_CCHC2_mut_F	gtgtgtggaaaaataggcGCTtacatgaaagactgcccta
HC11_CCHC2_mut_R	tagggcagtctttcatgtaAGCgcctatttttccacacac

Primers used in cloning

3'UTR_HC11_EcoRI	ggggGAATTCtgcgagtccattttctttc
3'UTR_HC11_XbaI	gggggTCTAGAaactcttgtcaaaaagatttattaac
eGFP-BamHi (3'UTR exp.)	ggggGGATCCatggtgagcaagggcga
eGFP-EcoRI (3'UTR exp.)	ggggGAATTCttacttgtacagctcgtcca
3'UTR_HC11_fr1_EcorI	ggggGAATTCtgcgagtccattttctt
3'UTR_HC11_fr1XbaI	gggggTCTAGAaaggttaaacaaaacataaaatt
3'UTR_HC11_fr2_EcorI	ggggGAATTCccctatttttaagggaattttatg
3'UTR_HC11_fr2_XbaI	ggggTCTAGAtgacaatttaaaacacatgaaatt
3'UTR_HC11_fr3_EcorI	ggggGAATTCcattctttattttctattagctgtat
3'UTR_ HC11_fr3_XbaI	ggggTCTAGAggaaaagaaaaaaaatctgaatc
3'UTR_ HC11_fr4_EcorI	ggggGAATTCagaaccttgtagtgcca
3'UTR_ HC11_fr4_XbaI	gggggTCTAGAaacttaaaatagaagtacttcaaatac
3'UTR_ HC11_fr1a_EcorI	ggggGAATTCtgcgagtccattttctt
3'UTR_ HC11_fr1a_Xba1	gggggTCTAGAcctgatttgttttgctttc
3'UTR _ HC11_fr1b_ECoRI	ggggGAATTCtacattcagcagaaaaagt
3'UTR_ HC11_fr1b_Xba1	gggcggTCTAGAtatagttcatatttatatacaaa
3'UTR_ HC11_fr1c_ECorI	ggggGAATTCctaagactgtttttacaga

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3'UTR_ HC11_fr1c_XbaI	gggggTCTAGAaacttaaaatagaagtacttcaaatac
3'UTR_ HC11_Fr1only_Xba1	gggggTCTAGAtaataaaatagatgaaatttgtatcac
5'UTR_long_HC11_HindIII	aaaAAGCTTCACAATGCAGTTCGCG
5'UTR_short_HC11_HindIII	aaaAAGCTTTTCGCGGCGCGGCGGACATG
5'UTR_HC11_BamHI	aacGGATCCtatttgaaaatctgtttctttcc
5'UTR_secondhalf_forward	TTTGCTTGCCTAAACTGGAC
5'UTR_firsthalf_reverse	CTGCGAGGCCCGCGAGCTA
eGFP_HindIII	ggggAAGCTTatggtgagcaagggcga
eGFP_BamHI	aacGGATCCttacttgtacagctcgtcca
eGFP_NotI	aattaGCGGCCGCatggtgagcaagggcga
eGFP_XbaI	gggggTCTAGAttacttgtacagctcgtcca

Primers used in cRACE

Erdem cRACE 5' inner	agtttaaattgcttcaagtcc
Erdem cRACE 5' outer	ctttggttcatgattttcact
Erdem cRACE 3' outer	tttattaccctatttttaaggga
Erdem cRACE 3' inner	acattctttattttctattagctg

Primers used in qRT-PCR

BRF1_F		gcctgtaagtacggggacaa
BRF1_R		ctcttcagcgttgtggatga
cMYC_F		aatgaaaaggcccccaaggtagttatcc
cMYC_R		gtcgtttccgcaacaagtcctcttc
KSRP_F		tctcgcctgttctgtgtctc
KSRP_R		aagttctgctttgctctttgtg
eGFP_F	(proximal pair)	actacctgagcacccagtcc
eGFP_R	(proximal pair)	cttgtacagctcgtccatgc
HuR_F		cgcagagattcaggttctcc
HuR_R		ccaaacccttgcacttgtt
ZCCHC11_F		tcaacaggtggctggttcagctcag

ZCCHC11_R	tagcagctgactgggaagagttctg
ZCCHC6_F	atctttggttcagggcatct
ZCCHC6_R	gatgttgtcgaatttgtggaa
3'UTR distal pair_F	gaaccttgtagtgccaaaaactt
3'UTR distal pair_R	tttccattcaagtttgaatgttc
TTP_F	tttaagggaggcaatgaacc
TTP_R	caggagacactggaacctca
TAP_F	atgctcaggcgccgaaagta
TAP_R	gaaggcgtttcgttgttcttcgt
GAPDH_F	atgggtgtgaaccatgagaa
GAPDH_R	gtgctaagcagttggtggtg

Appendix D: shRNAs and TTP siRNA used in this study

AUF1 (This study)

AUF1_shRNA_F

gatccccaagattgacgccagtaagattcaagagatcttactggcgtcaatctttttttc

AUF1_shRNA_R

tcgagaaaaaaagattgacgccagtaagatctcttgaatcttactggcgtcaatcttggg

KSRP (Trabucchi et al. 2009)

KSRP_shRNA_F

gatccccgatcaaccggagagcaagattcaagagatcttgctctccggttgatctttttc

KSRP_shRNA_R

tcgagaaaaagatcaaccggagagcaagatctcttgaatcttgctctccggttgatcggg

BRF1 (Lee et al. 2005)

BRF1_shRNA_F

gatcccccaagatgctcaactatagtttcaagagaactatagttgagcatcttgtttttc

BRF1_shRNA_R

tcgagaaaaacaagatgctcaactatagttctcttgaaactatagttgagcatcttgggg

HuR (Tiedje et al. 2012)

HuR_shRNA_F

gatcccccagaaacatttgagcattgttcaagagacaatgctcaaatgtttctgttttttc

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HuR_shRNA_R

tcgagaaaaacagaaacatttgagcattgtctcttgaacaatgctcaaatgtttctgggg

ZCCHC11 (Schmidt, West, and Norbury 2011)

ZCCHC11_shRNA_F

gatccccggttgcttcagactttatattcaagagatataaagtctgaagcaacctttttc

ZCCHC11_shRNA_R

tgcagaaaaaggttgcttcagactttatatctcttgaatataaagtctgaagcaaccgggg

Scrambled control (This Study)

Scrambled_shRNA_F

gatccccgtgacctcgatttattagtttcaagagaactaataaatcgaggtcacttttc

Scrambled_shRNA_R

tgcagaaaaagtgacctcgatttattagttctcttgaaactaataaatcgaggtcacggg

Tristetraprolin (Jin et al. 2012)

TTP siRNA cgacgauauaauuauuaua

References of Appendix

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