

**The anti-proliferative activity of BTG/TOB
proteins is mediated via the Caf1a
(CNOT7)/Caf1b (CNOT8) deadenylase
enzymes**

Rachel Louise Doidge, BSc (Hons)

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Abstract

The human BTG/TOB protein family comprises six members (BTG1, BTG2/PC3/Tis21, BTG3/Ana, BTG4/PC3B, TOB1/Tob, and TOB2) that display anti-proliferative activity in a number of cell types. They are characterised by a conserved N-terminal BTG domain that mediates interactions with the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylases. It was unclear whether the anti-proliferative activity of the BTG/TOB proteins was mediated through interactions with Caf1a (CNOT7) and Caf1b (CNOT8). To address this we further characterised the amino acid residues located along the BTG2 and TOB1 interaction surface with Caf1a (CNOT7)/Caf1b (CNOT8) to identify residues required for the interaction. We then analysed the role of BTG2 and TOB1 in the regulation of cell proliferation, translation and mRNA abundance using a mutant that is no longer able to interact with Caf1a (CNOT7)/Caf1b (CNOT8). We conclude that the anti-proliferative activity of BTG/TOB proteins is mediated through interactions with the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylase enzymes. We also demonstrate that recruitment of BTG2 and TOB1 to mRNA leads to reduced protein levels and mRNA degradation. Furthermore, we show that the regulation of mRNA abundance and protein levels is dependent on Caf1a (CNOT7)/Caf1b (CNOT8), but does not appear to require other Ccr4-Not components, including the Ccr4a (CNOT6)/Ccr4b (CNOT6L) deadenylases, or the non-catalytic subunits CNOT1 or CNOT3.

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Abbreviations List

2'PDE	2' phosphodiesterase
AD	Activation domain
Ago	Argonaute
ARE	AU-rich element
ATP	Adenosine triphosphate
β-Gal	β-Galactosidase
BMP	Bone morphogenic protein
BrdU	Bromo-deoxyuridine
BREd	Downstream BRE element
BREu	Upstream BRE element
BSA	Bovine serum albumin
BTG	B cell translocation gene
CDK	Cyclin dependant kinase
CFIm	Pre-Messenger RNA Cleavage Factor I
CPEB3	Cytoplasmic polyadenylation element binding protein 3
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage stimulation factor
DBD	DNA binding domain
DCP2	Decapping enzyme 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EEP	Endonuclease-phosphate
eIF	Elongation initiation factor
eIF4	Elongation initiation factor 4
ErbB2	Erythroblastic leukaemia viral oncogene homolog 2
ERα	Estrogen receptor α
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
ERBB2	ErbB2 tyrosine kinase receptor
Inr	Initiator motif
IP	Immuno-precipitation
IP6	Inositol hexakisphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
LB	Luria-bertani
m7G	7-methyl-guanosine cap
miRISC	MicroRNA induced silencing complex
miRNA	MicroRNA
mRNA	Messenger RNA
mRNP	Ribonucleoprotein
NEC	Nuclear export complex

NELF	Negative elongation factor
NMD	Nonsense-mediated decay
ORF	Open reading frame
P bodies	Processing bodies
PABP	Poly A binding protein
PARN	Poly(A) specific ribonuclease
PAS	Polyadenylation signal
PCR	Polymerase chain reaction
PDB	Protein data bank
PIC	Pre-initiation complex
Poly(A)	Poly adenosine tail
PRMT1	Protein arginine methyl-transferase 1
PTC	Premature termination codons
P-TEFb	Positive transcription elongation factor b
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAPI	RNA polymerase I
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
RNAPIV	RNA polymerase IV
RNAPV	RNA polymerase V
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Short interfering RNA

snRNA	Small nuclear RNA
SLBP	Stem loop binding protein
TBP	TATA box protein
TFII(A,B,D,F,H,R)	Transcription factor for RNA pol II
Tob	Transducer of ErbB2
TPA	12-O-tetradecanoylphorbol-13-acetate
TREX	Transcription-export complex
tRNA	Transfer RNA
TTP	Tristetraprolin
UTR	Untranslated region

Chapter 1

Introduction

1. Introduction

1.1 Eukaryotic transcription and nuclear RNA processing

1.1.1 Eukaryotic Transcription

The survival and development of multi-cellular organisms is directly linked to the rigorous control of gene expression. Any loss of this control can lead to disease, cancer and cell death. Transcription is the first step in gene expression and one of the most tightly regulated. It is the process by which the DNA sequence of a gene is copied into a complementary messenger RNA strand. Genomic DNA is organised into chromatin where the DNA strand is wrapped around histone proteins, which in turn are packaged into fibres. Chromatin acts to protect DNA from damage, reduce its volume and functions to control gene expression by altering the availability of DNA to transcription factors and RNA polymerases. A gene's complementary mRNA strand, produced by transcription, is mobile and also less structured than genomic DNA, making it a preferred template for protein synthesis. In eukaryotes, transcription can be divided into three major steps: initiation – the recruitment of RNA polymerase to the DNA; elongation – the extension of the mRNA molecule; and termination – the removal of the RNA polymerase enzyme and release of the nascent mRNA.

There are five types of RNA polymerase enzymes present in eukaryotic cells and they all transcribe different sub-sets of genes. All protein coding genes are transcribed by RNA polymerase II (RNAPII) (along with most micro RNAs) making it the most studied of the RNA polymerase enzymes. RNAPII is unable to initiate transcription alone as it cannot directly recognise DNA binding motifs, it therefore requires the presence of transcription factors and other protein co-factors to localise it to DNA (Juven-Gershon et al., 2008). To this end, genes are flanked by a series of regulatory elements that denote the start site, end point and regulate the level and timing of the transcription. For transcription initiation to begin a transcription complex must be formed on

the promoter of the target gene. Promoter regions are a complex series of regulatory DNA sequences that can be located close to, or some distance away from, an ATG start codon. The region to which RNAPII is recruited is called the core promoter and is normally located close to the transcriptional start site (Juven-Gershon and Kadonaga, 2010). Core promoters are not formed from a generic sequence but display wide diversity in terms of structure and function.

There are two main types of core promoter in eukaryotes: focused and dispersed. In focused promoter initiation, transcription begins within a narrow region of nucleotides which usually contains just one transcriptional start site (Juven-Gershon et al., 2008). This type of promoter is commonly found on tightly regulated genes allowing greater accuracy of gene expression levels. The second type of initiation is dispersed; here promoters contain multiple weak start sites over a broad region of 50 to 100 nucleotides. Dispersed initiation is more common in complex organisms like vertebrates where around two thirds of all genes are regulated by diverse promoters (Juven-Gershon et al., 2008). These promoters are also found to control more constitutively expressed genes that do not require tight regulation of gene expression (Juven-Gershon and Kadonaga, 2010). It is because of the biological significance of tightly regulated genes that focused promoters have been most highly studied and shown to contain a combination of conserved sequence motifs. These motifs bind a number of different transcription factors that in combination recruit RNAPII. They are called the basal transcription factors and include TFIIA, TFIIB, TFIID, TFIIR, TFIIF and TFIIH (transcription factor for RNA polymerase II). TFIID is the key basal transcription factor and able to bind to several different promoter elements including the TATA box, the initiator and the downstream promoter elements (DPE). It is a multi-subunit complex comprising of the TATA box binding protein (TBP) and several association factors (Figure 1.1) (Thomas and Chiang, 2006).

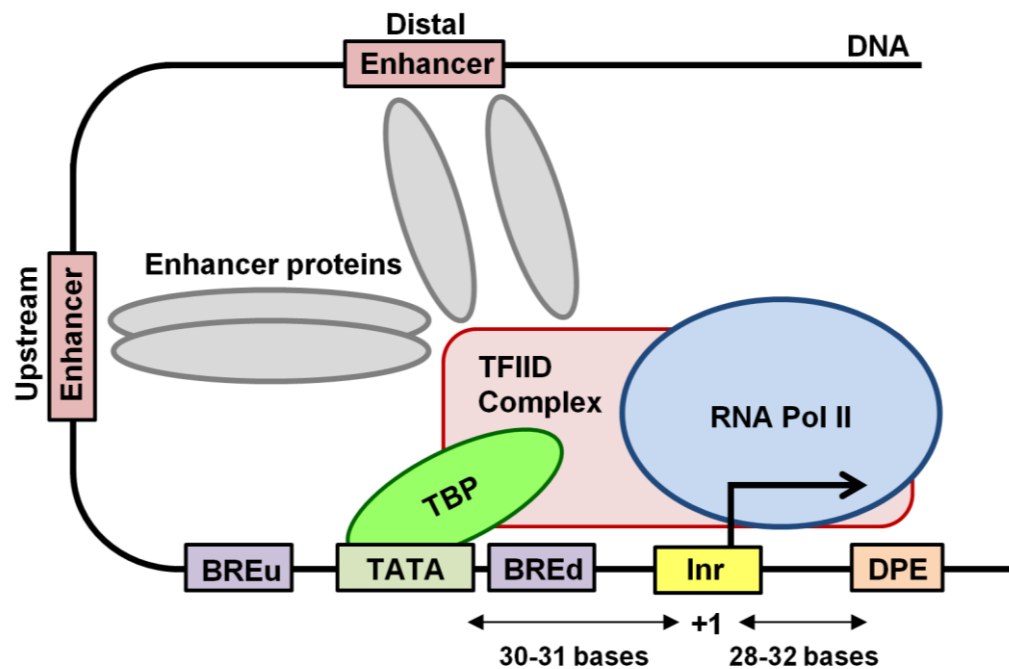


Figure 1.1 Schematic representation of a eukaryotic core promoter. Typical mammalian genes are regulated by sequence elements located up and down stream of the transcription initiation site (Inr). The down stream promoter element (DPE) work in conjunction with the Inr sequence to recruit the TFIID complex and RNA Pol II to the start codon. The distance between the Inr and DPE is highly regulated at 28-32 nucleotides. The TATA box promoter sequence is bound by the TATA box binding protein (TBP) which forms part of the TFIID complex and is flanked by B recognition element (BRE elements) located with upstream or downstream of the TATA box, which recruit further proteins that can promote or inhibit transcription initiation.

Before elongation can begin the Negative Elongation Factor (NELF) must be released from RNAPII. This is driven by the phosphorylation of the C-terminal domain of RNAPII, causing a mass exchange of protein factors and the release of the polymerase from the promoter (Buratowski, 2009; Kostrewa et al., 2009). This frees the polymerase to progress along the DNA, synthesising the mRNA. This newly formed mRNA is unstable and by the time its 5' end has left the RNAPII enzyme it is modified and given a 7-methyl-guanosine cap (m⁷G). Along with stabilisation the cap is also responsible for nuclear transport, mRNA processing and translation. This early elongation is subject to pauses and stalling during synthesis of the first 50 nucleotides until the elongation complex has undergone the necessary conformational changes (Nechaev and Adelman, 2011). The recruitment of the Positive Transcription Elongation Factor b (P-TEFb) drives the phosphorylation of RNAPII from primarily Ser-5 to Ser-2, which encourages elongation and termination factor binding to promote mRNA extension (Peterlin and Price, 2006).

1.1.2 mRNA processing and termination of transcription

The mRNA molecule produced by RNAPII is known as the pre-mRNA and contains protein coding exons and non-coding introns. The pre-mRNA undergoes three primary modifications to convert it to a mature mRNA: the addition of a 5' 7-methyl-guanosine cap (m⁷G), intron removal and polyadenylation. The m⁷G cap is added to the mRNA during transcription elongation as the newly formed mRNA exits the nucleus. A guanylyl transferase forms a 5' to 5' triphosphate bridge attaching a guanidine residue to the 5' end of the mRNA. This guanidine residue is then methylated at position seven by a methyltransferase to form the 5' cap that stabilises the mRNA. The cap structure is required for efficient nuclear export of the mRNA, translation initiation and to prevent mRNA degradation via exonucleases.

Introns are removed during splicing to leave the protein coding exon sequence. The process of alternative splicing is unique to eukaryotes and involves connecting the exons of a gene in multiple different ways to produce alternative mRNA transcripts (Johnson et al., 2003; Johnson and Vilardeell, 2012). This process increases the number of proteins that can be coded for in one mRNA molecule. The splicing reaction is catalysed by the spliceosome, a multi-subunit complex of proteins and small nuclear RNA (snRNA) molecules (McManus and Graveley, 2011).

The final step to produce a mature mRNA is the addition of a 3' polyadenosine tail. This step signals the end of transcription and stimulates the release of the mature mRNA. The poly(A) tail is the key element responsible for mRNA half-life and stability. It has been widely published that mRNA molecules with longer poly(A) tails are more stable with increased half-lives. In fact, mRNA molecules that do not undergo 3' processing are often confined to the nucleus and quickly degraded (Colgan and Manley, 1997; Eckmann et al., 2011; Mandel et al., 2008; Proudfoot, 2012). The polyadenylation site is defined by three conserved primary sequence elements and regulated by two auxiliary sequence elements (Mandel et al., 2008; Millevoi and Vagner, 2010), each of which associate with a complex of proteins forming the 3' end processing machinery (Figure 1.2). This complex includes: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I (CFIm), and cleavage factor II (Mandel et al., 2006). The polyadenylation signal (PAS) was the first identified sequence element thought to play a role in 3' mRNA processing. This sequence is an AAUAAA hexamer located upstream of the pre-mRNA cleavage site and is recognised by the CPSF complex (Mandel et al., 2008; Proudfoot, 2012). Point mutations within the PAS sequence significantly reduce the levels of polyadenylation and can lead to misregulation of gene expression and disease.

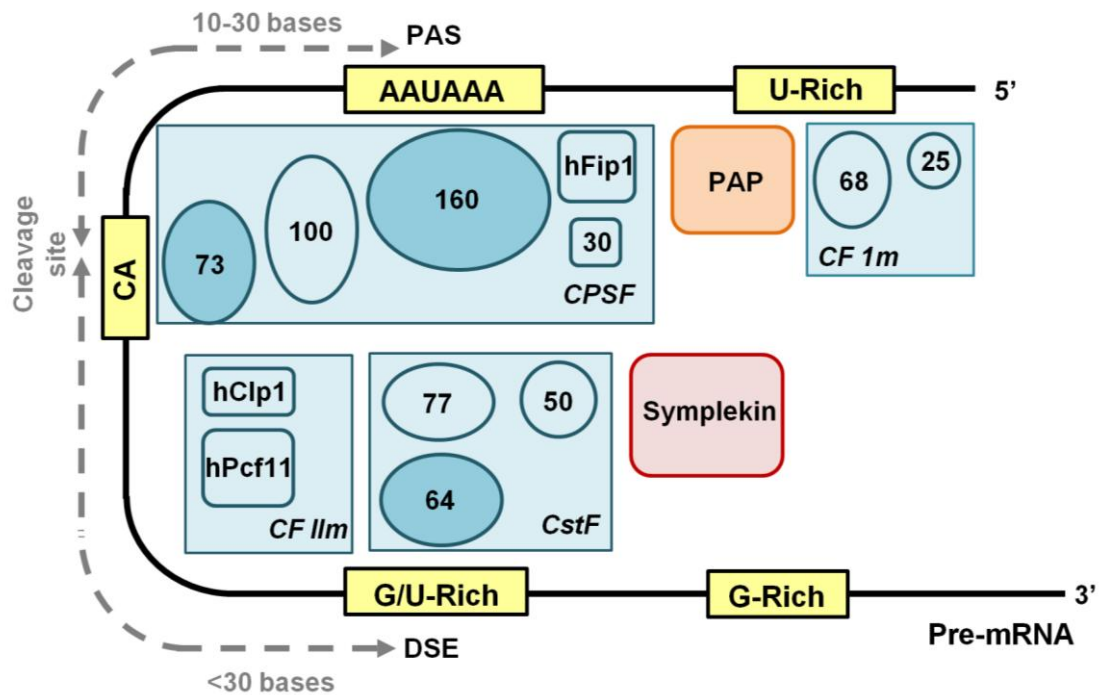


Figure: 1.2 Mammalian pre-mRNA 3' end processing machinery. Schematic of the pre-mRNA 3' end processing machinery complex. Cleavage and polyadenylation specificity factor (CPSF) 160 recognises the polyadenylation signal (PAS). Cleavage stimulation factor (CstF) 64 recognises the downstream element (DSE) (Mandel et al., 2008).

The PAS signal is recognised by an RNA binding protein, CPSF-160, the largest component of the CPSF complex and the most well studied. CPSF-160 has also been shown to interact with TFIID a member of the basal transcription factor machinery indicating it may play a role in transcription rates and termination (Proudfoot, 2004). The CPSF complex also binds directly to the pre-mRNA cleavage site via the CPSF-73 subunit – a suspected zinc dependant nuclease (Mandel et al., 2008). The optimal cleavage site is generally a CA sequence with cleavage taking place after the adenosine residue (Mandel et al., 2006). Located downstream of the cleavage site is the U/GU-rich region that is recognised by the CstF protein complex, in particular CstF-64 (Takagaki and Manley, 1998; Takagaki et al., 1996). Eventual termination of transcription occurs through the accumulation of 3' end processing factors, PAP and termination factors onto the nascent mRNA. Evidence indicates that poly(A) site cleavage can occur while RNAPII is still bound to the mRNA with factors in the elongation complex affecting termination. The release of RNAPII is encouraged through exonuclease degradation downstream of the PAS signal by XRN2 (West et al., 2008).

1.1.6 Nuclear–cytoplasmic mRNA transport

The three primary modifications required to form a mature mRNA (5' capping, splicing and polyadenylation) impact on mRNA nuclear export. Each processing step acts to recruit protein factors that build the nuclear export complex (NEC), without which nuclear export is severely inhibited. Studies in *Xenopus* oocytes demonstrated that uncapped mRNA was poorly exported or not exported to the cytoplasm because it was no longer able to bind the cap binding complex (Cheng et al., 2006). During splicing the exon junction complex works in conjunction with the 5' cap to recruit the transcription-export complex (TREX) (Cheng et al., 2006; Masuda et al., 2005). This conserved complex consists of the THO complex along with numerous export

factors including, ALY and a DEAD-box RNA helicase called UAP56 (Table 1.1) (Carmody and Wente, 2009).

Most mRNA is exported from the nucleus via a non-karyopherin mechanism involving a heterodimer of Nxf1 (metazoan; also known as TAP; Mex67 in *S. cerevisiae*) and Nxt1 (metazoan; also known as p15; Mtr2 in *S. cerevisiae*). The binding of the Nxf1-Nxt1 dimer requires a series of recruitment steps to build up the NEC on the mRNA. Firstly the DEAD box RNA helicase UAP56, in an ATP bound state, recruits ALY; hydrolysis of the ATP transfers the mRNA to ALY and releases UAP56 (Taniguchi and Ohno, 2008). ALY is then able to recruit Nxf1-Nxt1 before being released resulting in a messenger ribonuclearprotein (mRNP) bound to export receptors (Hautbergue et al., 2008). Not all mRNAs are exported via this mechanism. There are a small subset that use a karyopherin receptor mechanism. The karyopherin Crm1 (Xpo1 in *S. cerevisiae*) can mediate the export of unspliced, partially spliced and some viral mRNAs. Crm1 cannot bind directly to mRNA but requires adaptor proteins which are often mRNA specific (Cullen, 2003).

Both karyopherin and non-karyopherin mechanisms for nuclear export require the mRNP to dock with FG-Nups proteins that are found on the nucleoplasmic face of the nuclear pore complex (NPC). It is thought that the mRNP passes through the pore by a means of facilitated diffusion before entering into the cytoplasm (Weis, 2007). For Crm1 transported mRNAs this is achieved through the hydrolysis of RanGTP. For the majority of mRNAs transported via the Nxf1-Nxt1 pathway, release is driven by two export factors Dbp5 and Gle1 and further requires inositol hexakisphosphate (IP₆) (Cole and Scarcelli, 2006). Dbp5 is an RNA-dependent ATPase of the DEAD-box protein family, and binds to the NPC cytoplasmic face (Schmitt et al., 1999; Tseng et al., 1998). Gle1 specifically binds to IP₆ and docks to a neighbouring NPC protein hCG1 (Alcazar-Roman et al., 2006; Strahm et al., 1999). IP₆ bound Gle1 stimulates the ATPase activity of Dbp5 inducing a

conformational change in Dbp5 triggering the directional release of the mRNA to the cytoplasm (Carmody and Wentz, 2009; Tran et al., 2007).

Protien	Function
ALY	Member of TREX complex, recrutes Nxf1-Nxft1 to mRNP
CBC	Nuclear 5' cap binding complex
Crm1	Karyopherin protein export recptor
Dbp5	DEAD-box protein, RNA-dependant ATPase that dock at the NPC
EJC	Exon-junction complex, recruits THO complex
Gle1	Docks at NPC, binds IP ₆ and stimulates Dbp5 ATPase activity
hCG1	Cytoplasmic nucleoporin protein
hnRNPs	Hetergenousous nuclear ribonucleoprotein
IP ₆	Inositol hexakisphosphate
Nup214	Cytoplasmic nucleoporin protein
Nup358	Cytoplasmic nucleoporin protein
Nxf1	mRNA export receptor
Nxt1	Forms heterodimer with Nxf1
THO complex	Protein complex involved in transcription elongation comprising of: Tho2, Hpr1, Mft1 and Thp2
TREX complex	Transcription export complex comprising of THO complex, UAP56 and ALY
UAP56	DEAD-box protein, recruits ALY to TREX complex

Table 1.1 Factors involved in mRNA export (table adapted from Carmody and Wentz, 2009)

1.2 Regulation of translation

Translation is as highly regulated as transcription and it has as great an effect on overall gene expression. The rate of translation, the numbers of ribosomes present on an mRNA and the stability of mRNA all impact on the physical amount of protein being produced from a gene. Furthermore, features within an mRNA can intrinsically alter its translational efficiency.

The key driving force in translation is the ribosome, a complex of RNAs and proteins with peptidyl transferase activity. The ribosome is divided into two subunits: the 40S which positions the mRNA codons in place to be read by the 60S subunit which recruits tRNA and catalyses peptide bond formation. To prime the 40S ribosome subunit for attachment to mRNA it forms a complex with several elongation initiation factors including eIF1, eIF1A, eIF2, eIF3 and eIF5. Subsequently the mRNA is activated with the addition of eIF4F and eIF4B or eIF4H that unwind the 5' m⁷G cap structure, opening it up to ribosome binding (Gingras et al., 1999; Jackson et al., 2010; Sonenberg and Dever, 2003).

The initiation of translation can be regulated by two distinct mechanisms, sequence-specific regulation involving RNA binding proteins or microRNA (miRNA) and more general regulation through eIF protein modifications. The best-characterised mechanism for eIF regulated translation is via the phosphorylation status of eIF2. In mammals there are four protein kinases capable of phosphorylating eIF2 at Ser-51 (Jackson et al., 2010). These kinases are all activated through forms of cellular stress such as amino acid starvation, misfolded proteins or the presence of double stranded RNA, possibly due to viral infection. eIF2 is essential for protein synthesis and forms a ternary complex with Met-tRNA and GTP. The protein holds intrinsic GTPase activity and upon codon binding between Met-tRNA and the mRNA, the GTP is hydrolysed through the recruitment of eIF5, releasing eIF2. In order for eIF2 to be recycled for the next round of translation its GDP must be replaced by fresh GTP with the help of eIF2B, a guanine nucleotide exchange

factor. When phosphorylated eIF2 sequesters eIF2B, the strong bond between the two proteins disrupts its activity thus preventing GTP recharging. This causes a global reduction in active eIF2 and consequently a general reduction in translation (Jackson et al., 2010; Jiang and Wek, 2005).

There are many cases linking translation activation to the phosphorylation of several of the eIF factors including eIF1, eIF2 β , eIF2B ϵ , several eIF3 subunits, eIF4G, eIF4B, eIF4H, eIF5 and eIF5B (Jackson et al., 2010). For example, increased levels of eIF4E phosphorylation have been linked to growth factor stimulation, mitogens and changes in hormone levels (Scheper and Proud, 2002). The MAP-kinases Mnk1 and Mnk2 phosphorylate eIF4E and appear to increase its ability to form the cap binding complex (Scheper and Proud, 2002). Conversely the dephosphorylation of eIF4E causes a decrease in translation but its physiological importance is still unresolved (Goodfellow and Roberts, 2008).

The poly(A) tail of mRNA plays a key role in promoting translation. Upon arrival into the cytoplasm the poly(A) tail is bound by poly A binding protein (PABP) which stabilises the mRNA and facilitates translation (Gorgoni and Gray, 2004). PABP has far reaching roles in mRNA biogenesis, translation efficiency and mRNA stability. It can bind directly to the 5' cap structure via eIF4G, circularising the mRNA and bringing it into the optimal 'closed loop' configuration for translation (Jackson et al., 2010). Studies in *Xenopus* oocytes revealed that disrupting the interaction between PABP and eIF4G dramatically reduces the translation rates of polyadenylated mRNAs (Wakiyama et al., 2000). Currently there are three suggested mechanisms to explain how this interaction stimulates translation: promoting ribosome recycling, stimulating the recruitment to the 60S ribosome and increasing the affinity of eIF4F for the cap (Kahvejian et al., 2005). However, it is likely that stimulation of translation by PABP is caused by a combination of these factors and may vary depending upon the specific mRNA.

1.3 Cytoplasmic messenger RNA decay

The abundance of messenger RNA in the cytoplasm is highly regulated and acts as an alternative method to transcription for regulating gene expression. The rate of mRNA degradation often changes in response to stimuli and provides cells with a rapid method of altering or fine-tuning gene expression. Secondly mRNA quality control can also target specific mRNA for degradation due to the presence of premature termination codons (PTC), lack of a termination codon or due to errors in RNA biogenesis (Garneau et al., 2007; Goldstrohm and Wickens, 2008; Morozov and Caddick, 2012; Wu and Brewer, 2012). In most cases the precursor to cytoplasmic mRNA degradation is the removal of the poly(A) tail by deadenylase enzymes. Furthermore, deadenylation is often the rate limiting step for mRNA decay and transcriptional silencing, making it an ideal control point to regulate protein production. To stimulate mRNA degradation the poly(A) tail must be reduced to 15-25 nucleotides in length by one of a combination of deadenylase enzymes, after which the mRNA is exposed to ribonucleases (Couttet et al., 1997; Decker and Parker, 1993; Muhlrads et al., 1994). There are three major classes of RNA-degrading enzymes: endonucleases that cut RNA internally, 5' exonucleases that hydrolyse the RNA from the 5' end and 3' exonucleases that degrade RNA from the 3' end (Houseley and Tollervey, 2009). Along with these enzymes there are further RNA degradation cofactors such as 5' decapping enzymes, RNA helicases, RNA binding proteins and recruitment factors that work together to degrade the mRNA (Table 1.2) (Houseley and Tollervey, 2009). After deadenylation Lsm proteins (1-7) bind in a ring-like structure to the 3' end of the mRNA and aid the decapping enzymes DCP1 and DCP2 to remove the 5' m⁷G cap. This exposes the 5' end of the mRNA to degradation via the exoribonuclease XRN1 (Houseley and Tollervey, 2009; Wu and Brewer, 2012). Conversely, deadenylation exposes the 3' end to degradation via the exosome, a multi-subunit complex with RNase activity, RNA binding and helicase properties (Figure 1.3) (Houseley et al., 2006).

Complex	Human names	Function
Decapping factors	DCP1A, DCP1B	Member of DCP decapping complex
	DCP2	Catalytic subunit of decapping complex
	Lsm 1-7	Lsm RNA binding complex containing Lsm subunits 1 to 7
	Pat1	Required for mRNA decapping
Exoribonuclease enzymes	XRN1	Cytoplasmic 5' exoribonuclease
	Exosome	Multi subunit exonuclease containing two enzymatic subunits:
	Rrp44	Exosome 3' hydrolytic exonuclease
	EXOSC10	Exosome 3' hydrolytic exonuclease
Deadenylase enzymes	CNOT6 (Ccr4a)	EEP-type deadenylases of the Ccr4-Not complex
	CNOT6L (Ccr4b)	EEP-type deadenylases of the Ccr4-Not complex
	CCR4L (Nocturnin)	EEP-type deadenylases
	Angel (Ccr4d)	EEP-type deadenylases
	Angel2 (Ccr4e)	EEP-type deadenylases
	CNOT7 (Caf1a)	DEDD-type deadenylases of the Ccr4-Not complex
	CNOT8 (Caf1b)	DEDD-type deadenylases of the Ccr4-Not complex
	TOE1 (Caftz)	DEDD-type deadenylases
	PARN	DEDD-type deadenylases stimulated by cap binding
	PAN2	DEDD-type deadenylases that forms a dimeric complex with PAN3
Helicase enzymes	SUPV3L1, SKIV2L	Cytoplasmic helicases, members of the exosome cofactors Ski complex. Involved in nonstop-mediated decay
	UPF1	RNA-dependent helicase and ATPase involved in nonsense-mediated decay

Table 1.2 Factors involved in cytoplasmic mRNA degradation

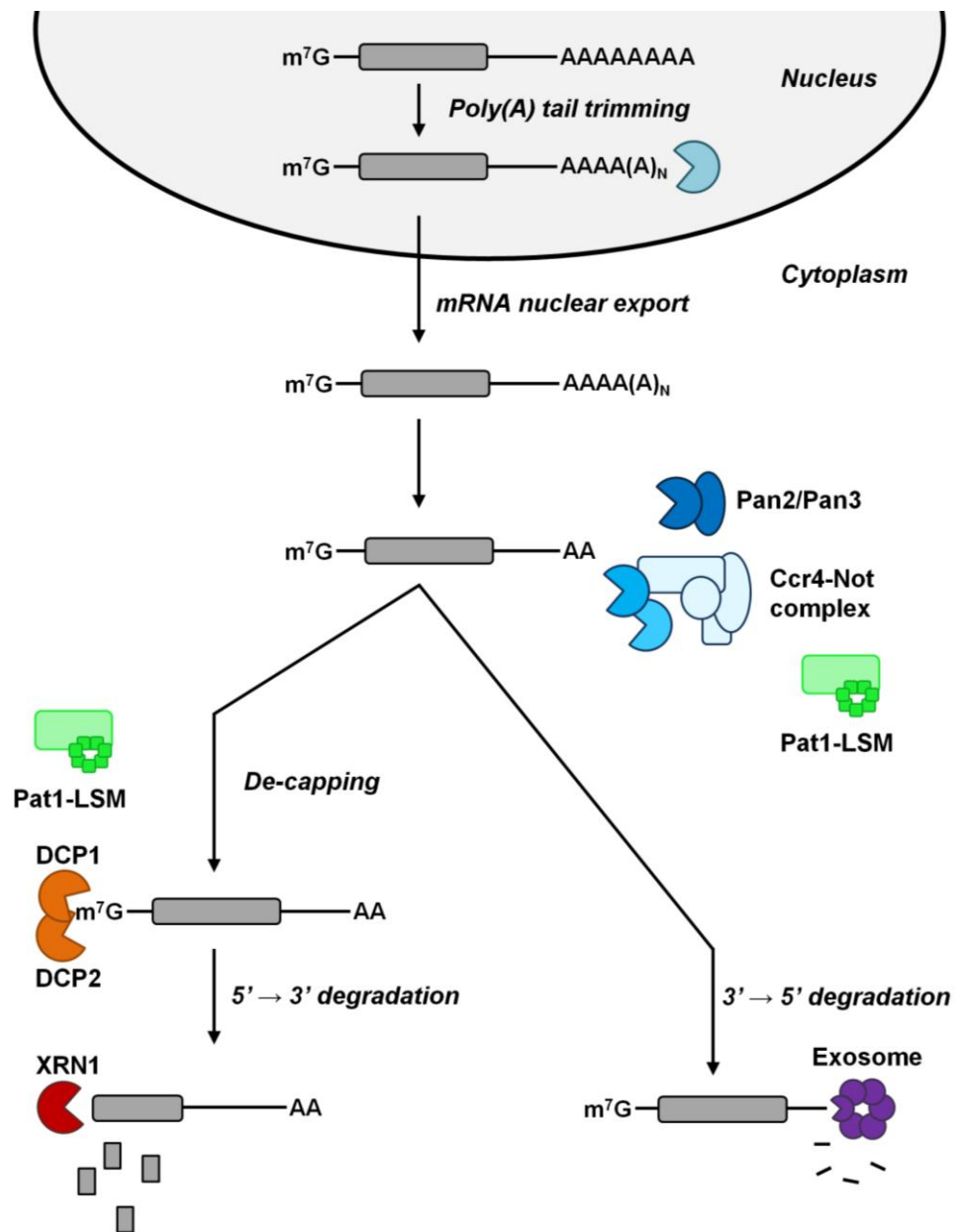


Figure 1.3 mRNA degradation pathways. In the nucleus deadenylases trim the poly(A) tail. In the cytoplasm deadenylases enzymes shorten the poly(A) tail. The Pat1-LSM complex can interact with the poly(A) tail to recruit the Ccr4-Not complex and DCP1-DCP2. Removal of the poly(A) tails stimulates mRNA degradation via two pathways, mRNA decapping by DCP1/DCP2 followed by 5' → 3' degradation by XRN1, or 3' → 5' degradation via the exosome complex.

1.3.1 Diversity of deadenylase enzymes

There are a wide range of deadenylases including ten different enzymes in humans. All deadenylases are magnesium-dependant exoribonucleases that hydrolyse RNA in a 3' to 5' direction. Comparisons between the known deadenylases demonstrated that they belong to one of two groups: the DEDD type or the exonuclease-endonuclease-phosphate (EEP) family. The classifications are based on the conserved catalytic domains. The DEDD-type nucleases contain Asp and Glu residues that co-ordinate Mg^{2+} ions across three exonuclease motifs (Zuo and Deutscher, 2001). The EPP-type deadenylases have conserved Asp and His residues in their nuclease domain (Dlakic, 2000). In humans the DEDD deadenylase include Caf1a (CNOT7), Caf1b (CNOT8), TOE1, Poly(A)-specific ribonuclease (PARN) and the PAN family (Table 1.2). The EEP-type nucleases use Asp and His residues to co-ordinate Mg^{2+} ions. This group includes Ccr4a (CNOT6), Ccr4b (CNOT6L), ANGLE, Nocturnin and the 2' phosphodiesterase family (2'PDE). Many of the enzymes exist in multiple forms in several species possibly due to genome duplication events, for example Caf1a (CNOT7) and Caf1b (CNOT8) in humans share 89% similar sequence identity. The advantage of having so many different deadenylases remains unclear. Do the different enzymes target different mRNAs, are they expressed under specific conditions or do they overlap to provide genetic security? The *Drosophila* Nocturnin enzyme is necessary during a narrow time window at late pupal stage for proper wing morphogenesis in adult flies. Knockdown of this gene leads to the well-known curled wing phenotype (Gronke et al., 2009). This enzyme is widely expressed during the lifetime of the fly however strong transcript enrichment occurs in specific regions like the salivary glands and the ring gland during development. This deadenylase appears to regulate a specific gene and only in specific locations in *Drosophila*. The expression levels of Nocturnin are also modulated by the circadian clock in several species including *Xenopus* and mice where levels peak at night and are thought to regulate metabolism and

lipid trafficking (Douris et al., 2011). Together, this provides evidence that this deadenylase may regulate defined sets of genes.

PDE12 is a human mitochondrial deadenylase for the phosphodiesterase family. It forms part of an antiviral defence mechanisms through its ability to hydrolyse 2'-5' linked oligoadenylates (Kubota et al., 2004).

The Pan deadenylases were first identified in yeast as DEDD type nucleases required to trim poly(A) tails to their required length of ~80 nucleotide in the nucleus. They do not play a role in deadenylation once the mRNA has entered the cytoplasm hence appear specific to nuclear RNA processing (Brown et al., 1996). In humans PAN2 conducts initial poly(A) tail shortening of β -globin reporter constructs (Yamashita et al., 2005). Like many deadenylases PAN2 cannot bind directly to mRNA but must form a complex with PAN3 which in turn associates with PABP (Uchida et al., 2004). Recently the Pan complex has also been linked to miRNA-mediated gene silencing through interactions with AIN1 a protein capable of binding to Ago the structural backbone of the miRNA RISC complex. Whether the function here is to initiate poly(A) shortening as demonstrated in previous published works remains undetermined but it does demonstrate a role for this deadenylase in gene silencing (Kuzuoglu-Ozturk et al., 2012).

PARN is a unique deadenylase in that its activity is enhanced in the presence of the 5' cap. PARN is a dimeric enzyme where each subunit is composed of three DEDD structural motifs. It is believed that PARN is recruited through AU-rich elements in an mRNA's 3'UTR. Once bound it interferes with the eIF4E, eIF4G, and PABP interaction disrupting translation and initiating deadenylation (Gao et al., 2000). Conversely, mRNA stability factors like HuR proteins bind to the AU-rich element and prevent PARN binding to increase mRNA stability (Gao et al., 2001). The presence of PABP on the poly(A) tail inhibits the activity of PARN, the exact reason for this is unknown but it is suspected that it may simply prevent access of the deadenylase to its poly(A) substrate (Gao et al., 2000).

The Caf1a (CNOT7), Caf1b (CNOT8) DEDD-type and Ccr4a (CNOT6), Ccr4b (CNOT6L) EEP-type enzymes form part of a multi-subunit complex called the Ccr4-Not complex which is involved both in transcription and translational regulation. They were first identified as the predominant cytoplasmic deadenylases in yeast (Caf1 and Ccr4), where they regulate global poly(A) tail lengths and mRNA turnover of a wide variety of genes (Tucker et al., 2001). The enzymes form heterodimers conferring high levels of sequence similarity with Caf1a (CNOT7)/Caf1b (CNOT8) sharing 89% sequence similarity and Ccr4a (CNOT6)/ Ccr4b (CNOT6L) 88%. Caf1a (CNOT7) and Caf1b (CNOT8) bind directly to the Ccr4-Not complex but only one of these DEDD-type nucleases can be bound at any one time (Lau et al., 2009). These deadenylases are recruited to mRNA through a variety of different cofactors. In addition to their catalytic role, Caf1a (CNOT7)/Caf1b (CNOT8) also interact with the leucine-rich repeat domains of Ccr4a (CNOT6)/Ccr4b (CNOT6L), thereby associating them with the complex (Basquin et al., 2012; Clark et al., 2004). Like most deadenylases, Ccr4a (CNOT6) and Ccr4b (CNOT6L) do not contain RNA binding domains but require a variety of interaction partners to target them to specific mRNAs.

1.3.2 ARE-mediated mRNA decay

Bioinformatic analysis indicated that between 5-8% of the transcriptome contains AREs (Halees et al., 2008). These are AU-rich elements predominantly found in the 3' UTR of short-lived mRNAs and act to modulate mRNA translation, stability and degradation (Wu and Brewer, 2012). ARE sequences are diverse but can be broadly grouped into three classifications (Chen and Shyu, 1995; Wilusz et al., 2001). Class I AREs contain 1-3 interspersed copies of AUUUA pentamers surrounded by U-rich regions and are found in mRNAs for FOS and MYC. Class II AREs contain multiple overlapping copies of the AUUUA motif and are found in Tumour Necrosis Factor α (*TNF α*). Finally, class III AREs contain U-rich sequences but lack the AUUUA motif present in class I and class II (Wu and Brewer, 2012). These different classes of AREs confer binding specificity for RNA binding proteins that often act as cofactors to recruit degradation and deadenylase enzymes. For example PABP can directly recruit the deadenylase enzymes Pan2-Pan3 to stimulate deadenylation (Garneau et al., 2007). Furthermore, the anti-proliferative protein TOB1 can function as a binding intermediate between PABP and the Ccr4-Not complex deadenylase Caf1a (CNOT7) (Ezzeddine et al., 2007). However RNA binding protein recruitment is not limited to deadenylases; many RNA binding proteins gather together a number of different mRNA processing factors to further increase the rate of degradation. For example the human protein Tristetraprolin (TTP) binds to AU-rich elements in mRNA 3' UTR regions and functions as a scaffold protein to recruit the decapping enzymes DCP1 and DCP2, the Ccr4-Not deadenylase Ccr4a (CNOT6) and the exoribonuclease XRN1 (Lykke-Andersen and Wagner, 2005).

1.3.3 Nonsense-mediated mRNA decay (NMD)

The NMD pathway is a surveillance system designed to detect premature termination codons (PTC) in mRNA and prevent them from being translated into truncated proteins. There are two proposed pathways for identifying PTC-containing mRNA over normal mRNA. This first hypothesis suggests that the exon-exon junction complex (EJC) plays a key role in PTC identification. During the pioneering round of translation the EJC is displaced. However if the ribosome encounters a PTC upstream of the EJC, the ribosome stalls, the SURF complex (containing SMG1, UPF1 and eRFs) is recruited and the mRNA is targeted for NMD (Wen and Brogna, 2010; Wu and Brewer, 2012). Key components of NMD are the UPF genes (UPF1, UPF2 and UPF3) that form a trimeric complex with a prematurely terminating ribosome and the SMG phosphatidylinositol 3-kinase-related kinase proteins (Conti and Izaurralde, 2005; Wu and Brewer, 2012). Phosphorylation of UPF1 by SMG1 increases its affinity for mRNA decay factors thereby recruiting decapping factors (DCP1-DCP2), deadenylases, exoribonucleases (XRN1) and in some cases the exosome to stimulate mRNA degradation (Figure 1.4) (Brogna and Wen, 2009; Eulalio et al., 2007; Wu and Brewer, 2012).

A second hypothesis suggests the main regulator of NMD is the distance between the stop codon and the poly(A) tail of an mRNA. In this case an inability of PABPC1 to interact with the terminating ribosome or the EJC may signal for NMD, however debate still exists between the two models (Silva and Romao, 2009; Wen and Brogna, 2010; Wu and Brewer, 2012).

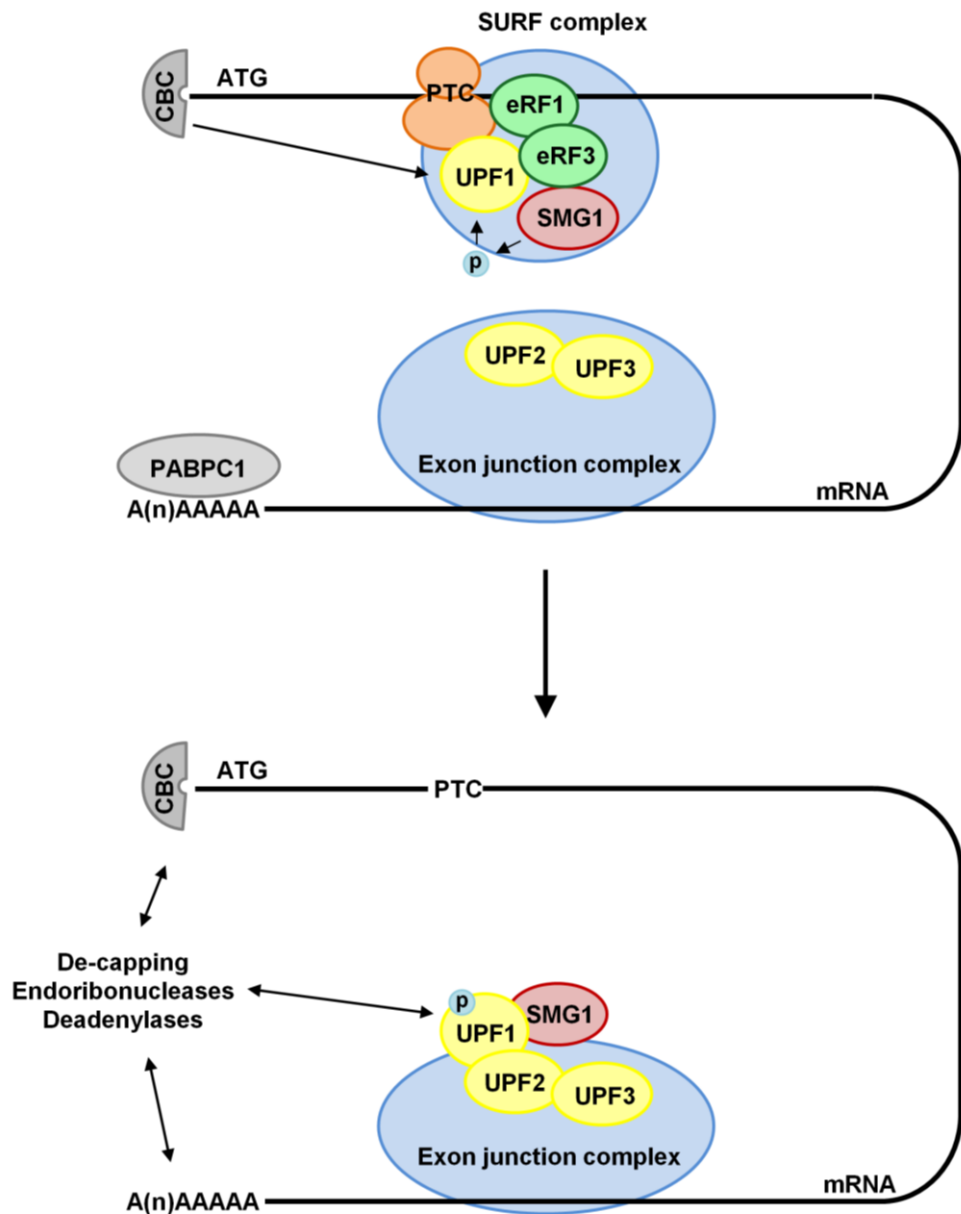


Figure 1.4 Nonsense-mediated decay (NMD). During pre-mRNA splicing the exon junction complex (EJC) is deposited 20-24nt upstream of an exon-exon junction. Upon export to the cytoplasm the cap-binding complex interacts with UPF1 during the pioneer round of translation. If the ribosome encounters a premature termination stop codon (PTC), CBC-UPF1 promotes SMG1-UPF1 formation and binding to eRF1-eRF3 to form the SURF complex. SMG1-UPF1 bind to UPF2-UPF3 in the EJC before SMG1 induces the phosphorylation of UPF1 and subsequent recruitment of decapping, deadenylation and endoribonuclease enzymes to degrade the mRNA (modified from Wu and Brewer 2012).

1.3.4 Nonstop-mediated mRNA decay (NSD)

Messenger RNAs lacking a termination codon are unstable and subject to degradation (Frischmeyer et al., 2002; van Hoof et al., 2002; Wu and Brewer, 2012). During translation of an mRNA lacking a stop codon the ribosome continues scanning beyond the protein coding region into the mRNA 3' UTR and poly(A) tail. When the ribosome reaches the end of the mRNA its unoccupied A-site is recognised and bound by the C-terminal domain of Ski7. Ski7 then forms a complex with Ski2, Ski3 and Ski8 which together recruit the exosome to initiate 3' – 5' mRNA decay (van Hoof et al., 2002; Wu and Brewer, 2012). Furthermore the mRNA is subject to decapping via DCP1 and DCP2 followed by 5' – 3' degradation by XRN1, however this process is noticeably less efficient than exosome degradation (Figure 1.5) (Frischmeyer et al., 2002).

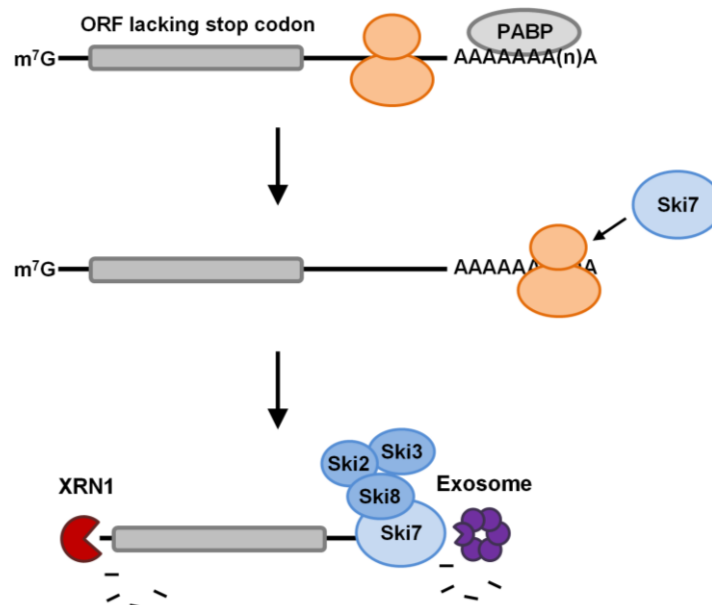


Figure 1.5 Nonstop-mediated decay (NSD). Upon translation of mRNAs lacking a stop codon, the ribosome is allowed to transverse the 3' UTR and stall in the poly(A) tail. PABP dissociates from the mRNA before binding of Ski7 onto the stalled ribosome to release it from the transcript. Ski7 then forms a complex with Ski2, Ski3 and Ski8 before recruiting the exosome to stimulate 3' - 5' mRNA degradation. Furthermore, decapping via DCP1 and DCP2 and 5' – 3' XNR1 degradation occurs at the 5' end of the mRNA (modified from Wu and Brewer 2012).

1.3.5 MicroRNA-mediated decay

Micro-RNAs (miRNAs) are short (~21 nucleotides) non coding RNA molecules capable of altering protein expression through direct binding to a specific mRNA. Hundreds of miRNAs have been identified and bioinformatic studies indicate they may each regulate numerous genes covering a large proportion of the transcriptome (Bushati and Cohen, 2007). Most miRNAs repress transcription through imperfectly base pairing to a target mRNA, often within the mRNA 3' UTR. This interaction leads to inhibition of translation and/or deadenylation and mRNA decay (Huntzinger and Izaurralde, 2011). The repression of gene expression by miRNAs is thought to take place by one of four methods: (1) co-translational protein degradation, (2) inhibition of translation initiation, (3) inhibition of translation elongation and (4) premature termination of translation (Eulalio et al., 2007). Translation may also be silenced by sequestering mRNA away to Processing bodies (P-bodies) where the mRNA can remain in an idle state before being released back into the cytoplasm for translation at a later point. During miRNA biogenesis the newly created single stranded miRNA incorporates into microRNA ribonucleoprotein (miRNP) known as the RNA induced silencing complex (miRISC) (Huntzinger and Izaurralde, 2011; Wu and Brewer, 2012). Ago proteins (of which there are four in humans) form the core of the miRISC complex and recruit an assortment of accessory factors including the RNA helicase Rck and the GW182 proteins (Eulalio et al., 2008b; Hutvagner and Zamore, 2002; Jackson et al., 2010; Wu and Brewer, 2012). The miRISC complex facilitates translational silencing and mRNA degradation via two pathways. Ago2 possesses "slicer" activity and can directly catalyse endoribonucleic cleavage if there is near-perfect complementarity between the mRNA and miRNA (Figure 1.6A). This generates 5' and 3' mRNA cleavage products which are degraded by XRN1 and the exosome (Huntzinger and Izaurralde, 2011; Wu and Brewer, 2012).

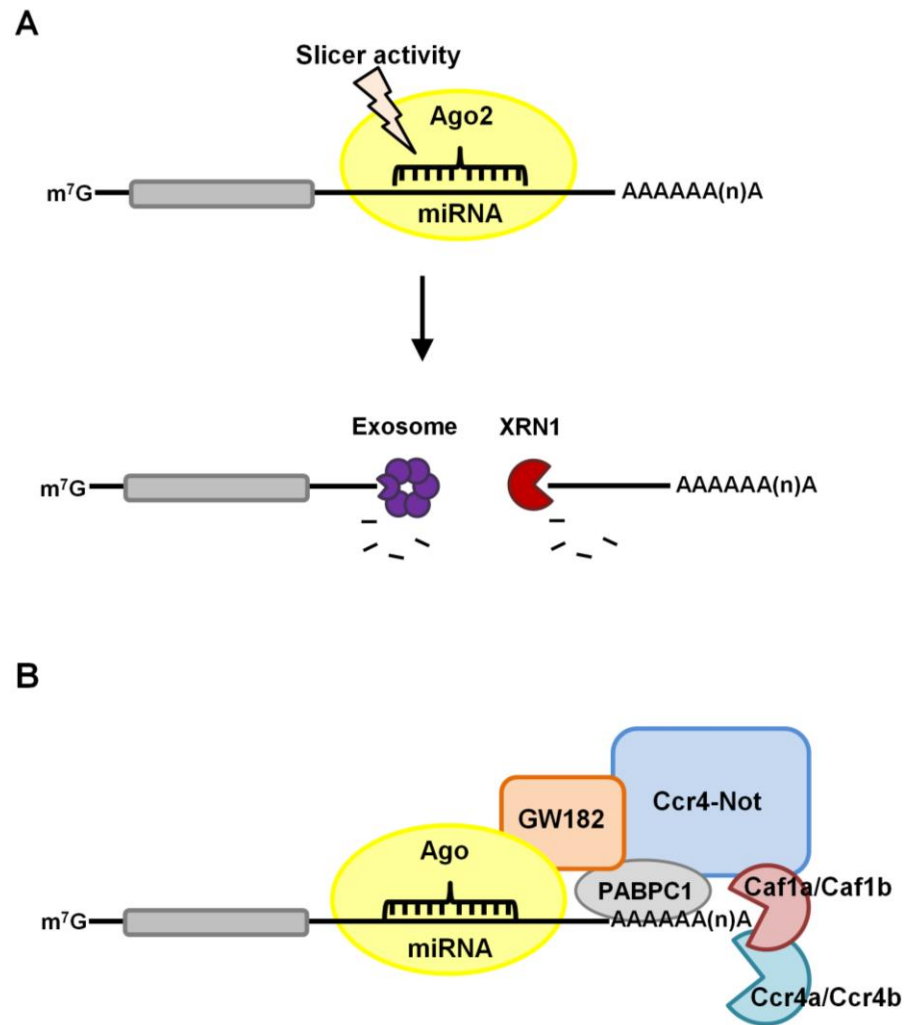


Figure 1.6 MicroRNA-mediated mRNA decay. (A) Ago2 catalysed endoribonuclease degradation. Ago2 possesses 'slicer' activity to catalyse endoribonuclease activity when there is near perfect complementarity between the miRNA and target mRNA. The endoribonuclease generates 5' and 3' cleavage products which are targeted by XRN1 for degradation. **(B) miRNA-mediated recruitment of the Ccr4-Not complex to mRNA.** miRNA bound to Ago recruits GW182 through GW repeats in GW182 and in turn interacts with PABP via a PAM motif. GW182 recruits the Ccr4-Not complex through CNOT1. The Ccr4-Not complex contains two deadenylase enzymes, Caf1a (CNOT7) or Caf1b (CNOT8) that bind to CNOT1 and Ccr4a (CNOT6) or Ccr4b (CNOT6L) that bind via Caf1a (CNOT7)/Caf1b (CNOT8). These deadenylase enzymes degrade the poly(A) tail to reduce translational efficiency and stimulate mRNA degradation.

The second mode of mRNA degradation via miRNA binding is the recruitment of decapping and deadenylation enzymes. RNA tethering assays demonstrated that the miRISC component GW182 promotes deadenylation initially via Pan2-Pan3 before complete poly(A) tail removal via the Ccr-Not complex followed by decapping via DCP1-DCP2 (Behm-Ansmant et al., 2006; Chekulaeva et al., 2011; Fabian et al., 2011; Pillai et al., 2004; Zipprich et al., 2009). GW182 contains numerous distinct protein interaction domains including an N-terminal Ago binding domain and a C-terminal protein binding domain that interacts with CNOT1 of the Ccr4-Not complex (Figure 1.6B) (Behm-Ansmant et al., 2006; Chekulaeva et al., 2011; Eulalio et al., 2008b; Fabian et al., 2011; Wu and Brewer, 2012)

1.3.3 Degradation of mRNAs without a poly(A) tail

Not all mRNAs possess a poly(A) tail. In the case of histone mRNAs, their 3' end is stabilised by a series of stem loop motifs that are bound by stem loop binding proteins (SLBP) (Dominski and Marzluff, 2007). Histone mRNA degradation is stimulated by the addition of uridine residues to the mRNA 3' via 3' uridylyl transferase (Mullen and Marzluff, 2008; Schmidt et al., 2011). To date, the extent of pyrimidine tagging of polyadenylated mRNAs in eukaryotes remains unclear, however tagging has been demonstrated in *Aspergillus nidulans* and *Schizosaccharomyces pombe* (Morozov et al., 2010a; Rissland and Norbury, 2009). Furthermore, poly(U) tagging has been shown to target mRNA for degradation by increasing the affinity of the Lsm-Pat1 complex (Morozov and Caddick, 2012; Rissland and Norbury, 2009; Song and Kiledjian, 2007) and via the NMD pathway through interactions with Upf1 (Isken and Maquat, 2008; Morozov and Caddick, 2012).

1.3.7 RNA processing bodies

P-bodies are cytoplasmic foci that contain proteins involved in a diverse range of post-transcriptional mRNA processing. Factors required for deadenylation, degradation, NMD and miRNA-mediated gene silencing co-localise with target mRNAs within dynamic centres termed P-bodies (Kulkarni et al., 2010). Upon arrival in the cytoplasm not all mRNAs are immediately translated. Some enter a silenced state before being transported to a desired location for activation. mRNAs are also subject to quality control and made available to regulatory proteins or miRNAs that alter their translation rates before eventually being degraded. P-bodies function as factories determining an mRNA's fate, bringing together the required proteins and RNAs to improve the efficiency of mRNA processing. The existence of P bodies was first hypothesised in 1997 when the exoribonuclease XRN1 showed punctuated cytoplasmic localisation in a mouse fibroblast cell line (Bashkirov et al., 1997). The significance of this discovery was only realised years later when the decapping enzyme DCP2 and its co-factors also demonstrated the same granular staining pattern in mammalian and yeast cells (Sheth and Parker, 2003; van Dijk et al., 2002). Furthermore P bodies have also been found to contain the Lsm proteins, the decapping enhancer Pat1, RNA helicases like Rck and deadenylases such as Pan2-Pan3, Caf1 (CNOT7/8) and Ccr4 (CNOT6/6L). Interestingly, the exosome is one mRNA processing enzyme not found in P-bodies, suggesting that the two mRNA degradation pathways may occur in different cellular locations (Lebreton et al., 2008; Sheth and Parker, 2003).

Interestingly the inhibition of the deadenylase CCR4 in *S. cerevisiae* causes the disappearance of P-bodies (Sheth and Parker, 2003). This result is replicated in mouse cells where the knockdown of the Caf1 deadenylases - or over expression of catalytically inactive Caf1 - prevents P-body formation (Zheng et al., 2008). Furthermore, disruption of Caf1 activity has been shown to prevent the formation and dynamic response of P-bodies to environmental stimuli in *Aspergillus nidulans* (Morozov et al., 2010b). Noticeably, blocking

the later stages of mRNA decay through XRN1 knockdown or blocking decapping increases P-body numbers, suggesting that P-body formation is dependent upon deadenylation (Sheth and Parker, 2003). This is further confirmed with the presence of deadenylation intermediates within P-bodies and the absence of PABP from P-bodies, inferring that mRNAs localised to a P-body have shorter poly(A) tails (Kedersha et al., 2005). One current model for P-body function is that mRNAs undergoing deadenylation actively move into P-bodies where they become trapped and undergo decapping and 5'-3' degradation. However entering a P-body does not always signal the degradation of that mRNA, as there have been several documented accounts of mRNAs exiting P-bodies and re-entering translation (Kulkarni et al., 2010).

1.4 The Ccr4-Not complex

1.4.1 Enzymatic properties of the Ccr4-Not complex

The Ccr4-Not complex is a heterogeneous multi-subunit complex containing one DEDD-type deadenylase, either Caf1a (CNOT7) or Caf1b (CNOT8), and one EEP-type subunit, either Ccr4a (CNOT6) or Ccr4b (CNOT6L). In addition to the enzymatic subunits, the complex also contains several non-catalytic components including the scaffold protein CNOT1 (Figure 1.7).

The Ccr4-Not complex is a global regulator of gene expression that is conserved from yeast to humans. It has the ability to affect both transcriptional regulation and mRNA turnover and has been shown to localise in both the nucleus and cytoplasm. First identified and most highly studied in yeast, the Ccr4-Not yeast protein complex consists of nine core subunits: CCR4, CAF1, CAF40, CAF130 and five NOT proteins (which exist in two complex formations: one of 1.9 MDa and another at 1.0 MDa in size) (Chen et al., 2001; Collart, 2003).

Two enzymatic roles are associated with the Ccr4-Not complex: deadenylation and ubiquitination. The CNOT4 protein possesses an N-terminal RING domain that can interact with the UbcH5b E2 enzymes and provide ubiquitin protein ligase activity (Albert et al., 2002). This enzyme is a permanent member of the yeast Ccr4-Not complex but has a transitory association in humans (Jeske et al., 2006; Lau et al., 2009). CNOT4 also contains an RRM motif which provides putative RNA-binding activity. Given its association with the Ccr4-Not deadenylase complex, it is tempting to speculate that it will interact with oligo(A) residues, but this remains to be confirmed. The deadenylase activity is conferred by Ccr4 and Caf1 in yeast and its human homologs Ccr4a (CNOT6) and Ccr4b (CNOT6L), Caf1a (CNOT7) and Caf1b (CNOT8) (Collart and Panasenko, 2012; Dupressoir et al., 2001). One Ccr4-Not subunit capable of binding directly to nucleic acids is RQCD-1, a dimeric protein containing six armadillo-repeat like structures

that form the nucleic acid binding cleft. Surprisingly this protein discriminates against poly(A) sequences, preferring poly(G), poly(C) or poly(T), suggesting it may not be involved in mRNA degradation (Garces et al., 2007). There remains insufficient evidence linking direct binding of Ccr4-Not components to mRNA, however a convincing model is emerging which demonstrates that RNA-binding co-factors act to target the Ccr4-Not deadenylase to mRNA (Ezzeddine et al., 2007; Ezzeddine et al., 2012; Funakoshi et al., 2007; Hosoda et al., 2011; Mauxion et al., 2008; Sandler et al., 2011).

Despite the crystallisation of several individual Ccr4-Not members, the overall structure of the complex remains a mystery. Recent electron microscopy data has begun to shed light on the global shape of the complex. Crosslinking, followed by purification of the yeast scaffold protein Not1 (homologue of human CNOT1), produced an L shaped complex with two arms of similar length (Basquin et al., 2012; Nasertorabi et al., 2011). The largest subunit, NOT1, contains HEAT-repeat organisation (rod-like helical structures) with MIF4G related regions reminiscent of other scaffold proteins related to mRNA metabolism (Basquin et al., 2012). Yeast two-hybrid and pull down assays demonstrated that most factors associate with the C-terminal domain of Not1/CNOT1, with Caf1a (CNOT7) or Caf1b (CNOT8) bound into the centre (Basquin et al., 2012; Lau et al., 2009). The current model suggests binding availability of the Not1 N-terminal for regulatory factors and rationalises how cross-talk may occur between the deadenylases and other Not proteins (Basquin et al., 2012; Lau et al., 2009). However the protein composition of the Ccr4-Not complex may shift and alter depending upon cellular factors like localisation, the cell cycle, or through stimulation of signalling molecules. This heterogeneity explains why there is little confirmed structural data available (Collart and Panasenko, 2012).

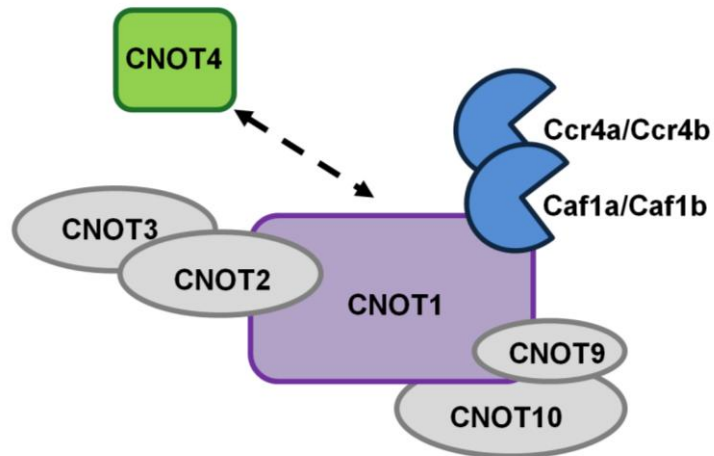


Figure 1.7 Schematic representation of the Ccr4-Not complex. CNOT1 scaffold protein in purple, core components in grey, interchangeable components in blue (deadenylases) and green (E3 ligase). Based on Lau et al., 2009.

1.4.2 The role of the Ccr4-Not complex in mRNA decay

Following decapping and deadenylation, both ends on an mRNA are exposed and subject to exonucleolytic degradation. The exoribonuclease Xrn1 acts in a 5' to 3' direction and is often found in P body-like structures whereas 3' to 5' degradation occurs via the exosome (Parker and Song, 2004). In both cases the event that stimulates mRNA degradation is the removal of the poly(A) tail.

The Ccr4-Not deadenylases show specificity for poly(A) substrates and their catalytic activity *in vitro* is independent of other Ccr4-Not components (Bianchin et al., 2005; Chen et al., 2002). Humans possess four Ccr4-Not deadenylases grouped into two pairs based on their catalytic site: the DEDD type Caf1a (CNOT7) and Caf1b (CNOT8) and the EEP type Ccr4a (CNOT6) and Ccr4b (CNOT6L) (Figure 1.8). A human Ccr4-Not complex can only contain two deadenylase enzymes, one from each sub-family (Lau et al., 2009). Using siRNA-mediated knockdown approaches in combination with genome-wide expression profiling, it is evident that the Caf1a (CNOT7) and Caf1b (CNOT8) regulate largely identical gene sets (Aslam et al., 2009). The same observation was made for Ccr4a (CNOT6) and Ccr4b (CNOT6L), which also regulate the mRNA abundance of identical gene sets (Aslam et al., 2009). Intriguingly upon comparison Caf1a (CNOT7)/Caf1b (CNOT8) and Ccr4a (CNOT6)/Ccr4b (CNOT6L) appear to regulate the expression of distinct groups of mRNA with little overlap (Aslam et al., 2009). Thus, despite the fact that both enzymes have similar substrate specificity and reside in the same protein complex, they appear not to cooperate in regulating the same mRNAs. This is particularly intriguing because Ccr4a (CNOT6)/Ccr4b (CNOT6L) bind to the Ccr4-Not complex via Caf1a (CNOT7)/Caf1b (CNOT8). The mechanism through which the two deadenylase types specifically target different mRNAs, despite spatially being in close proximity, is an interesting question yet to be answered.

Several members of the Ccr4-Not complex are required for effective cell proliferation including CNOT1, CNOT3, Caf1a (CNOT7)/Caf1b (CNOT8) and Ccr4a (CNOT6)/Ccr4b (CNOT6L) but a role in survival and senescence appears to be specific to Ccr4a (CNOT6)/Ccr4b (CNOT6L) (Mittal et al., 2011; Morita et al., 2007). P-body numbers and their constituent components also appear to be affected by the Ccr4-Not deadenylases. The knockdown of Caf1a (CNOT7)/Caf1b (CNOT8) leads to the reduction of P bodies containing RCK, DCP1a and eIF4E suggesting that deadenylation may stimulate P-body formation. The reduction in P-bodies is less pronounced with Ccr4a (CNOT6)/Ccr4b (CNOT6L) knockdown and interestingly foci containing eIF4E actually increases (Mittal et al., 2011). This suggests that Ccr4a (CNOT6)/Ccr4b (CNOT6L) may act at a different stage of the deadenylation process, causing the accumulation of alternate P-body components.

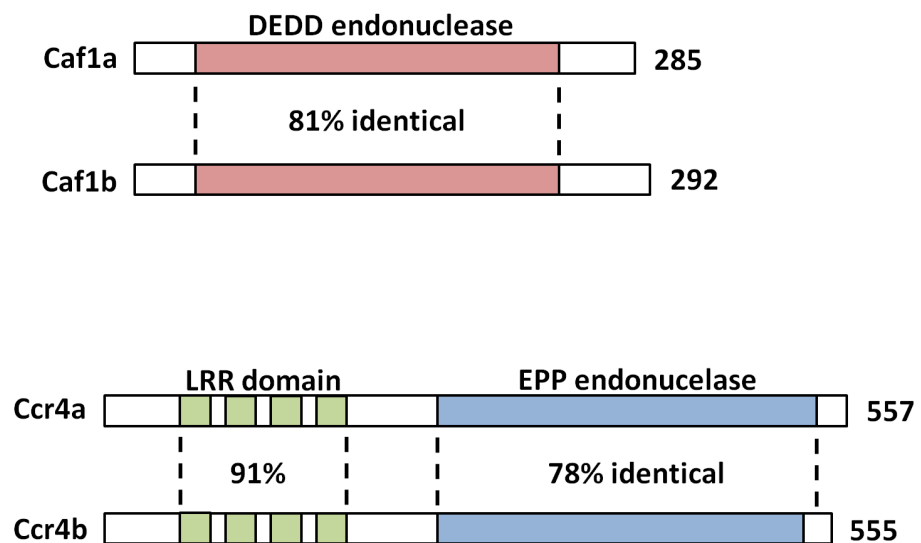


Figure 1.8 Relationship between the Ccr4-Not deadenylases. The schematic representation of the Ccr4-Not deadenylases highlights conserved domains, DEDD endonuclease domain (red), EPP endonuclease domain (blue), LRR domains (green). Also indicated are the size of the proteins.

1.4.3 Regulation and recruitment of the Ccr4-Not complex

As described above, the deadenylase activity of the Ccr4-Not complex does not degrade poly(A) tails indiscriminately. Thus, the Ccr4-Not complex is selectively targeted to specific mRNAs by the action of RNA-binding proteins, as well as components involved in microRNA-mediated repression (Doidge et al., 2012).

1.4.3.1 Interactions with members of the BTG/TOB protein family

The six BTG/TOB proteins (BTG1, BTG2, BTG3, BTG4, TOB1 and TOB2) display anti-proliferative activity and are identified through a conserved N-terminal domain, which can bind to Caf1a (CNOT7)/Caf1b (CNOT8) (Ikematsu et al., 1999; Prevot et al., 2001; Rouault et al., 1998; Yoshida et al., 2001). The BTG/TOB proteins have divergent C-terminal domains, which allow them to mediate further protein-protein interactions. The C-terminal domain of TOB1 contains a PAM2 motif which mediates an interaction with the C-terminal domain of PABP. This interaction suggests a role for TOB1 as an adaptor protein that facilitates the recruitment of the Ccr4-Not complex to specific mRNAs (Figure 1.9A) (Ezzeddine et al., 2007; Funakoshi et al., 2007; Mauxion et al., 2008). By examining the crystal structures of the TOB1–Caf1a (CNOT7) complex and the related DEDD-type deadenylase PARN bound to RNA, it can be predicted that the N-terminal domain of TOB1 binds to Caf1a (CNOT7) away from its active site (Winkler, 2010). This suggests that the binding of TOB1 to Caf1a (CNOT7) does not affect the catalytic activity of Caf1a (CNOT7) further strengthening the hypothesis that the BTG/TOB proteins function as adaptor proteins that recruit Caf1a (CNOT7)/Caf1b (CNOT8) to mRNA.

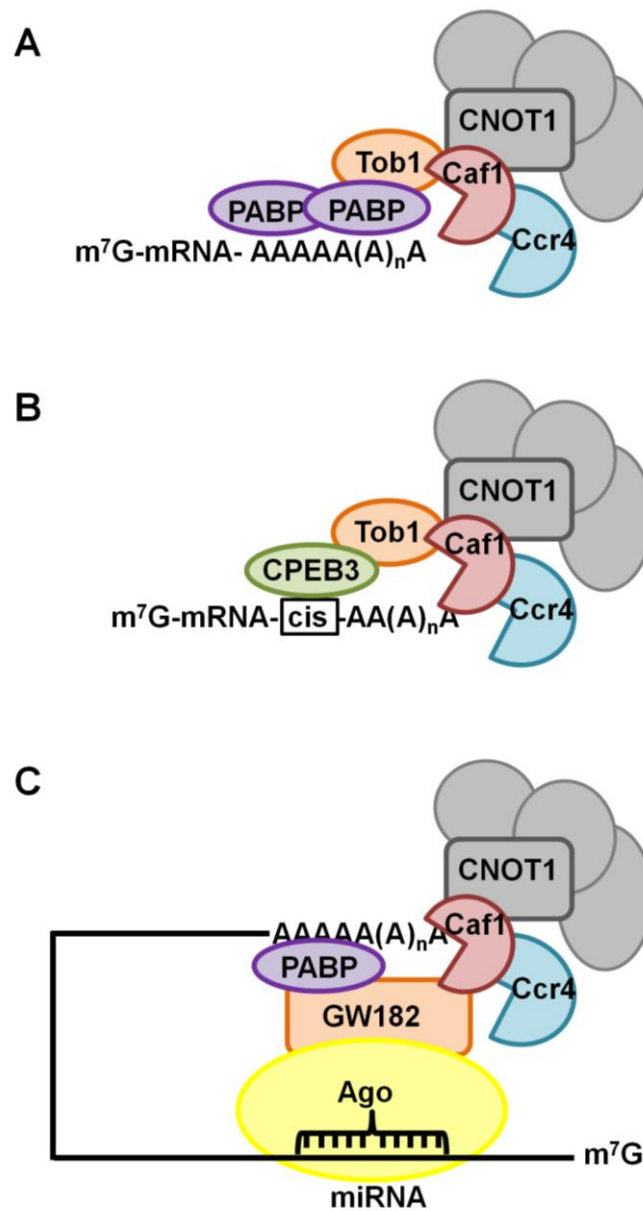


Figure 1.9 Speculative models for the recruitment of the Ccr4-Not deadenylase complex. The Ccr4-Not complex may be recruited via **(A)** the BTG/TOB proteins, **(B)** sequence specific RNA binding proteins involved with PABP interactions, **(C)** factors involved with the miRNA machinery.

1.4.3.2 Role of RNA-binding proteins

The Ccr4-Not complex can be recruited to mRNA through interactions with RNA binding proteins, often mediated via the BTG/TOB proteins. Deadenylation can be stimulated through interaction with eukaryotic release factor eRF1-eRF3 mediated via their PAM2 motifs. Competitive interactions between the PAM2 motifs of eRF3, TOB1 and the deadenylase Pan3 with PABPC1 stimulate the activation of the Pan2-Pan3 and Ccr4-Not deadenylases (Fabian et al., 2011; Uchida et al., 2002). In addition to activating general mRNA degradation, the Ccr4-Not deadenylases and TOB1 can stimulate more targeted sequence-specific mRNA degradation. Cytoplasmic Polyadenylation Element-Binding protein 3 (CPEB3) binds to the C-terminal domain of TOB1 (Hosoda et al., 2011). CPEB3 also binds directly to U-rich RNA sequences through two RRM domains located in the protein's C-terminal (Hosoda et al., 2011). Recruitment of CPEB3 to the 3' UTR of mRNA results in deadenylation and mRNA turnover, which is dependent on both the presence of TOB1 and Caf1a (CNOT7)/Caf1b (CNOT8) (Figure 1.9B). This suggests that Caf1a (CNOT7)/Caf1b (CNOT8) are recruited to specific sequences of mRNA to stimulate their degradation mediated by RNA binding proteins and TOB1.

Recruitment of the Ccr4-Not to mRNA is not entirely dependent upon accessory factors linking the complex to RNA binding proteins. The Ccr4-Not complex is directly recruited to Nanos2, an RNA binding protein that is required for the development of germ cells and male fertility (Suzuki et al., 2010). The absence of Nanos2 leads to the accumulation of several Nano2 target mRNAs suggesting that its interaction with the Ccr4-Not complex causes mRNA degradation (Suzuki et al., 2010). The Ccr4-Not scaffold protein CNOT1 can interact with TTP protein, which directly binds to AREs present in mRNA sequences. This interaction mediates the rapid deadenylation and decay of the mRNA utilising the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylases (Sandler et al., 2011). Finally, cells lacking one copy of CNOT3 show evidence of defects in the mRNA decay pathway suggesting that CNOT3

may play a role in recruiting the Ccr4-Not complex to mRNA (Morita et al., 2011). Together this data indicates that Ccr4-Not subunits, other than the deadenylase enzymes, can function to recruit the complex to mRNA and stimulate its degradation.

1.4.3.3 Relationships with microRNA-mediated gene repression

The observation that miRNA binding can induce deadenylation which depends on the activity of the Ccr4-Not complex was originally made in cells from the fruit fly and is conserved in human cells (Chekulaeva et al., 2011). The *Drosophila* model indicated that GW182, a component of the RISC complex, recruits PABP, in turn recruiting the Ccr4-Not complex (Figure 1.9C) (Eulalio et al., 2008b; Huntzinger and Izaurralde, 2011; Zekri et al., 2009). The knockdown or overexpression of a dominant negative Caf1 deadenylase inhibits miRNA dependant mRNA degradation suggesting that the Ccr4-Not complex plays a pivotal role in driving miRNA gene silencing (Behm-Ansmant et al., 2006; Eulalio et al., 2008a).

Interestingly deadenylation induced by miRNAs involve both the Ccr4-Not as well as the Pan2-Pan3 complex. Recent publications show the recruitment of both complexes is mediated by the three GW182/TNRC6A-C paralogues, which interact with the Ago proteins through their N-terminal domain (Behm-Ansmant et al., 2006; Braun et al., 2011; Fabian et al., 2011; Fabian et al., 2009; Kuzuoglu-Ozturk et al., 2012; Zekri et al., 2009). The C-terminal domain of GW182/TNRC6s has the ability to induce translational repression and deadenylation when tethered to the 3' UTR of mRNA. Through a series of W-motifs (comprising the amino acids G/S/T W or W G/S/T) the C-terminus of GW182 interacts with CNOT1. Recruitment of both the Ccr4-Not deadenylases as well as the Pan2-Pan3 deadenylase is dependent on these W-motifs (Chekulaeva et al., 2011; Fabian et al., 2011; Kuzuoglu-Ozturk et al., 2012). Some debate still exists as to whether PABP is required for effective mRNA degradation. Conflicting evidence shows that PABP is not required or

is an essential co-activator of miRNA-mediated deadenylation. However in the latter case PABP must be displaced from the mRNA before deadenylation can occur (Fabian et al., 2011). To date, the link between miRNA-mediated degradation and deadenylation via the Ccr4-Not complex has been demonstrated, however the involvement of both the Caf1-type and Ccr4-type deadenylases has yet to be established.

1.4.4 Regulation of physiological processes and relevance for disease

The Ccr4-Not deadenylases are ubiquitously expressed however evidence suggests that some subunits are particularly important for specific physiological processes (Chen et al., 2011). Through phenotypic analysis of Caf1a (CNOT7) knockout mice, a link was shown between this enzyme and the correct differentiation of male germ cells and in bone formation in adult mice (Washio-Oikawa et al., 2007; Washio-Oikawa et al., 2006). It would be expected that Caf1a (CNOT7)^{-/-} mice appear normal due to the extensive redundancy it shares with Caf1b (CNOT8) (Aslam et al 2009). However Caf1a (CNOT7)^{-/-} mice display defects in the early stages of male germ cell differentiation and exhibit a higher bone mass and bone density as compared to wild type mice (Washio-Oikawa et al., 2007). This increased bone density phenotype is shared with TOB1 null mice suggesting that the role Caf1a (CNOT7) is playing when regulating bone formation is mediated by interactions with TOB1.

Further evidence suggests that Ccr4-Not components other than the deadenylases also possess specific physiological roles. CNOT3 knockout mice are not viable with loss of CNOT3 resulting in early embryonic death at the pre-implantation stage (Morita et al., 2011). Heterozygous CNOT3^{+/-} mice display defects in cardiac function and develop cardiomyopathy. CNOT3^{+/-} mice are protected against obesity and have a reduced fat content in adipocytes. Furthermore, it was identified that the deadenylase Ccr4b (CNOT6L) recruitment to specific genes was reduced in CNOT3^{+/-} mice. This

correlated with the observation that several metabolic mRNAs were up-regulated, poly(A) tails lengths increased and mRNA turnover reduced (Morita et al., 2011). Interestingly this demonstrates a possible link between CNOT3 and the recruitment of deadenylases to target mRNAs. Together these observations indicate that individual components of the Ccr4-Not complex have specific physiological roles in defined tissue or cell types and possess a significant impact on the progression of human disease.

1.4.5 The Role of the Ccr4-Not complex in transcription

The Ccr4-Not complex was first identified in yeast as a transcription factor through genetic selection experiments. There is no enzymatic connection between the Ccr4-Not complex and transcription but as demonstrated through protein interaction studies, the complex elicits an effect in the nucleus. The Ccr4-Not complex can bind to the general transcription machinery TFIID, TBP and TBP-association factors (Collart and Panasenko, 2012) and the distribution of TFIID on promoters is in part regulated by the Ccr4-Not complex (Deluen et al 2002). Evidence indicates that the complex has a role in regulating histone modifications. For example in yeast Not2 interacts with the histone acetyltransferase Gcn5 and the deubiquitination enzyme Ubp8 (Daniel and Grant, 2007). In humans CNOT2 can repress transcription by stimulating histone de-acetylation through recruiting HDAC3 to target genomic DNA (Jayne et al., 2006; Zwartjes et al., 2004). Furthermore CNOT1 can interact with nuclear receptors and is recruited to target promoters where it modulates transcription. Binding of CNOT1 to the ligand-binding domain of estrogen receptor alpha (ER α) is hormone-dependant and disruption of this interaction (i.e. through siRNA knockdown of CNOT1) leads to the deregulation of ER α target genes (Winkler et al., 2006). In agreement with a role in transcription the human Ccr4-Not complex shuttles between the nucleus and cytoplasm, however some of its impact on transcription may be indirect. The Ccr4-Not complex plays such a

wide role in the polyadenylation status of many mRNAs that it may fundamentally alter the protein levels of transcription regulators, chromatic factors, proteome proteins and the exosome all leading to indirect changes in transcription (Collart and Panasenko, 2012). The majority of the Ccr4-Not complex appears to be located in the cellular cytoplasm suggesting that the main role of the complex is related to its deadenylase activity of cytoplasmic mRNA.

1.5 The BTG/TOB protein family

1.5.1 Discovery of the BTG/TOB protein family

The human BTG/TOB proteins form a small family of six proteins which share a conserved N-terminal domain and anti-proliferative activity (Matsuda et al., 2001; Tirone, 2001; Winkler, 2010). BTG2 was discovered first by two laboratories: as the immediate/early response gene PC3 in rat PC12 cells upon stimulation with nerve growth factor, and as TIS21 in mouse 3T3 fibroblasts in response to treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Bradbury et al., 1991; Fletcher et al., 1991; Zhang et al., 2010). The identification of a new family of anti-proliferative genes was confirmed with the discovery of BTG1 (B-cell translocation gene 1), a gene involved in a chromosomal translocation associated with chronic lymphocytic leukemia (Bhattacharyya et al., 2006; Rouault et al., 1992). To add to this TOB1 was discovered as an interacting protein of the ErbB2 tyrosine-kinase receptor (HER2) that affects cell proliferation (Matsuda et al., 1996). The remaining three members BTG3 (ANA), BTG4 (PC3B) and TOB2 were identified based on sequence homology of the conserved N-terminal domain. The BTG/TOB family is unique to metazoans where most vertebrates contain all six BTG/TOB members, however only two isoforms are present in *D.melanogaster* and just one in *C.elegans* (Jia and Meng, 2007; Tirone, 2001).

1.5.2 Structure and defining characteristics

The conserved N-terminal BTG domain encompasses 104-106 amino acids and contains two regions of particularly high-sequence conservation known as Box A and Box B. This conserved region functions as a protein-protein interaction domain and is unique to the BTG/TOB family (Matsuda et al., 2001; Tirone, 2001). Through sequence alignments of the six members it is apparent that TOB1 and TOB2 are the most closely related followed by BTG1

and BTG2, with BTG3 and BTG4 more distantly related (Figure 1.10) (Winkler, 2010). Sequence alignments also demonstrate that the BTG/TOB C-terminal regions are far more diverse. TOB1 and TOB2 possess the largest C-terminal which contains a further protein interaction domain, the PAM motif, allowing it to bind with PABP (Ezzeddine et al., 2007; Funakoshi et al., 2007; Mauxion et al., 2008; Zhouravleva et al., 1995). BTG1 and BTG2 have the shortest C-terminal domain which contains a second conserved motif known as Box C, located adjacent to the BTG domain. This region is a protein-protein interaction domain and is required for BTG1 and BTG2 to interact with the protein methyltransferase PRMT1 (Lin et al., 1996).

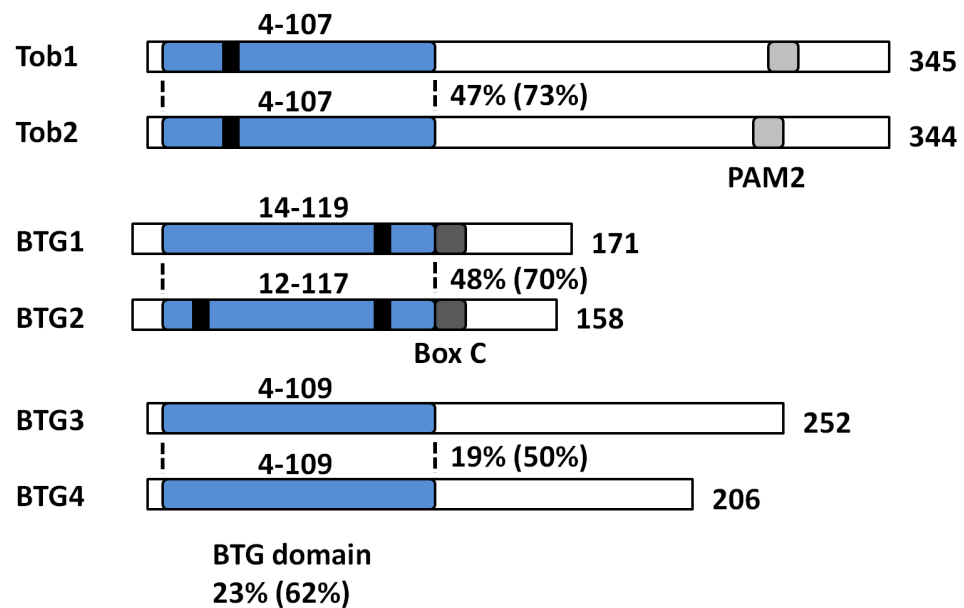


Figure 1.10 Schematic over view of the BTG/TOB family of proteins. Diagram base on amino acid sequences. BTG domain highlighted (blue), conserved PAM motif (light grey), Box C motif (dark grey) and LXXLL motif (black). Also shown is protein size (amino acids) and percentage of identical (similar) amino acids. Modified from Winkler, 2010.

The conserved N-terminal mediates the majority of the BTG/TOB protein interactions. In humans all members of the BTG/TOB family apart from BTG4 have been shown, in experiments, to interact with Caf1a (CNOT7) and Caf1b (CNOT8). However due to the similarity of the BTG4 N-terminal region compared to the other BTG/TOB members, an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) is expected (Ikematsu et al., 1999; Prevot et al., 2001; Rouault et al., 1998; Yoshida et al., 2001). The BTG domain is highly structured as demonstrated through the crystal structures of BTG2 and TOB1, whereas the C-terminal region is unstructured and does not crystallise (Horiuchi et al., 2009; Yang et al., 2008). For BTG2 the approximate dimensions of the protein are 49 x 40 x 29 Å and the structure consists of 5 α -helices and 4 β -strands forming 2 anti-parallel sheets (Figure 1.11) (Yang et al., 2008). Box A consists of a β -strand (β 1), a short α helix (α 3) and a section of the α 2 helix and the connecting loop between them. Box B is formed of two anti-parallel β -strands (β 2 and β 3) and the β 4 strand. The third domain, Box C, found in BTG1 and BTG2, is located just beyond Box B and is composed of an extending C-terminal loop and β -sheet (Horiuchi et al., 2009; Yang et al., 2008).

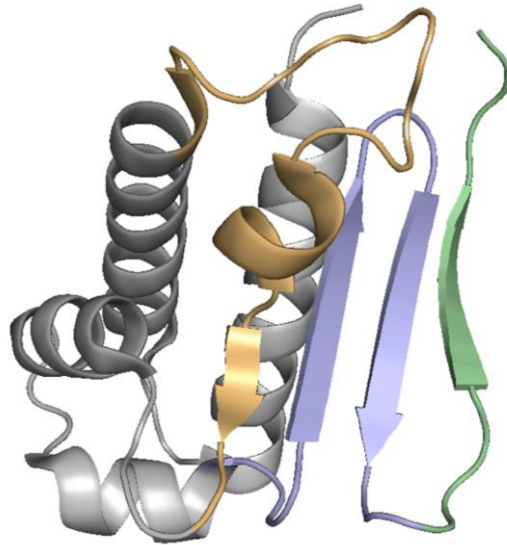


Figure 1.11 Structure of the BTG domain. Schematic representation of the crystal structure of BTG2 (PDB 3DJU). Indicated are the highly conserved Box regions: Box A (wheat), Box B (blue) and Box C (green).

1.5.3 BTG/TOB and mRNA turnover

The ability of BTG/TOB proteins to interact with Caf1a (CNOT7)/Caf1b (CNOT8) and (in the case of TOB1/TOB2) PABP suggests a role in mRNA deadenylation and turnover. These molecular interfaces are located on different faces of the protein indicating that TOB1/TOB2 may be capable of interacting with more than one target at once. Through analysis of the available crystal structure of the TOB1–Caf1a (CNOT7) complex and BTG2, it is apparent that both Box A and Box B domains come into close proximity to Caf1a (CNOT7) and may be required for the interaction. Furthermore amino acid substitutions within these domains are capable of disrupting this interaction (Horiuchi et al., 2009; Yang et al., 2008). This structural data allows a predicated model of BTG/TOB binding to Caf1a (CNOT7)/Caf1b (CNOT8). This interaction is away from the deadenylase catalytic site and leaves the C-terminal domain of the BTG/TOB proteins available to associate with other possible RNA-binding proteins (Winkler, 2010). The effect BTG/TOB proteins have on mRNA turnover is still under debate. The binding of BTG2 to Caf1a (CNOT7)/Caf1b (CNOT8) causes suppression of deadenylase activity *in vitro*. However suppression of deadenylase activity is not seen when the interaction is disrupted by amino acid substitutions, suggesting the interaction somehow modulates catalytic activity (Yang et al., 2008). TOB1 has also been shown to repress the deadenylase activity of the Ccr4-Not complex primarily through Ccr4a (CNOT6L), again *in vitro* (Miyasaka et al., 2008). In contrast a purified N-terminal fragment of TOB1 had no effect on the deadenylase activity of Caf1a (CNOT7) (Horiuchi et al., 2009).

In vivo transcriptional chase studies demonstrated TOB1 may act to increase mRNA deadenylation and co-localise to P-bodies, suggesting a role in mRNA turnover (Ezzeddine et al., 2007). Further *in vitro* evidence indicates that BTG2 may function as a general activator of mRNA decay in mammalian cells. Cloning the 3' UTR of a known BTG2 target gene into a reporter construct causes the mRNA half-life of that construct to significantly decrease,

indicating that BTG2 may affect mRNA stability. Interestingly in that study the half-life of the control reporter mRNA decreased by the same amount as the BTG target gene suggesting that BTG2 may function as a non-specific up-regulator of mRNA decay (Mauxion et al., 2008).

Evidence suggests that the anti-proliferative activity of the BTG/TOB proteins is mediated through their Caf1a (CNOT7)/Caf1b (CNOT8) interaction. Overexpression of TOB1 in COS-1 and HEK293 cells reduces cell proliferation. This inhibition of cell proliferation is relieved after the introduction of amino acid substitutions in Caf1a (CNOT7) that disrupt the TOB1-Caf1a (CNOT7) interaction (Horiuchi et al., 2009). Remarkably MCF-7 breast cancer cells not expressing Caf1a (CNOT7) also show reduced proliferation rates, but this is independent of an interaction with BTG/TOB proteins (Aslam et al., 2009).

1.5.4 BTG/TOB proteins as effectors in signalling pathways

The BTG/TOB proteins target and are utilised in a variety of different signalling pathways to alter a cell's proliferation rate. In addition to their interactions with mRNA processing factors, BTG/TOB proteins are also capable of interacting with a number of transcription factors to modulate their ability to bind their cognate DNA sequence elements. Both BTG1 and BTG2 can interact with Hoxb9, a homeobox DNA-binding transcription factor, through their extreme N-terminus (Prevot et al., 2000). This interaction enhances the ability of Hoxb9 to bind to its consensus DNA sequence. Thus this may increase the transcription rates of Hoxb9 target genes, which may contribute to the anti-proliferative function of BTG1 and BTG2 (Prevot et al., 2000; Tirone, 2001).

BTG1 and BTG2 are also able to interact with Protein Arginine Methyl-Transferase 1 (PRMT1), suggesting that BTG1/BTG2 could be involved in chromatin modifications (Lin et al., 1996). This interaction occurs within the

conserved Box C region, a β -sheet located at the protein's N-terminal. PRMT1 methylates the arginine three residue of histone H4, which facilitates the subsequent acetylation of histone H4 tails by p300. The p300 gene is a transcriptional co-activator responsible for chromatin remodelling and plays an important role in cell proliferation and differentiation. BTG2 favours differentiation of HL-60 cells through the gene-specific modification of arginine methylation and acetylation of histone H4. The current hypothesis suggests that BTG2 is modulating the recruitment of PRMT1 after arginine methylate and thus affect transcription (Passeri et al., 2006). BTG1 also contributes to muscle differentiation and represses myoblast proliferation through interactions with the TR1 α / β nuclear receptor and myogenic transcription factors (Busson et al., 2005; Rodier et al., 1999).

In MCF-7 cells (an estrogen receptor-expressing breast cancer cell line) BTG2 expression can be regulated both positively and negatively through signalling via members of the nuclear receptor family of transcription factors. Treatment of MCF-7 cells with retinoic acid activates BTG2 expression through direct binding of the retinoic acid receptor (RAR/RXR) heterodimers to three retinoic acid response elements (RARE) in the BTG2 promoter region (Donato et al., 2007). This leads to a reduction in cell proliferation rates. Conversely cell proliferation rates increase when MCF-7 cells are treated with estrogen. Activation of receptor ER α and its co-repressor REA leads to the repression of BTG2 expression and drives cell proliferation (Frasor et al., 2003). One method through which BTG1 and BTG2 have been shown to reduce cell proliferation rates is by mediating changes in Cyclin D1 expression. Upon retinoic acid treatment, the subsequent increase in expression of BTG2 leads to reduced Cyclin D1 transcription and decreased CDK4 activity (Donato et al., 2007; Guardavaccaro et al., 2000). Both Cyclin D1 and active CDK4 are required for cell cycle progression. BTG2 can also downregulate the expression of Cyclin D1, Cyclin E1 and reduce the phosphorylation of retinoblastoma (Rb) in response to oncogenic transformation. In embryonic mouse fibroblasts BTG2 plays a critical role in

suppressing transformation through oncogenic Ras by acting as a downstream effector of p53 (Boiko et al., 2006). BTG2 and BTG3 are direct transcriptional targets for p53, containing p53 binding motifs within their promoters and also play a role in the p53-mediated DNA damage response (Ou et al., 2007; Rouault et al., 1996).

The C-terminal region of TOB1 mediates interactions with a number of Smad transcription factors, altering their ability to bind to DNA (Tzachanis et al., 2001; Yoshida et al., 2000). TOB1 increases the binding affinity of Smad4 for its consensus DNA sequences and can enhance transcription of synthetic reporter constructs activated by Smad factors (Tzachanis et al., 2001). As a consequence TOB1 regulates the expression of Smad target genes, such as the cytokine IL-2 promoter in quiescent T-cells, which is negatively regulated by Smads. The inhibitory Smads like Smad6 and Smad7 demonstrate the strongest interaction with TOB1, however interactions with others Smads have been demonstrated (Yoshida et al., 2003a).

A further example of gene regulation through alterations in transcription factor binding is demonstrated by BTG3 and its interaction with E2F1, a transcription factor important for S-phase entry and cell cycle progression. BTG3 binds E2F1 through its N-terminal Box A region, which, in this case, inhibits DNA binding of the E2F1, thereby reducing the overall transcription rate of E2F1-responsive promoters (Ou et al., 2007). The E2F1 transcription factor is important for S-phase entry and inhibiting its action supports the general consensus that BTG/TOB factors inhibit S-phase progression.

Post-translation modifications such as phosphorylation and ubiquitination can alter the activity of BTG/TOB proteins. Several serine and threonine residues in the C-terminal domain of TOB1 are subject to phosphorylation by the Erk1 and Erk2 kinases (Maekawa et al., 2002; Matsuda et al., 1996; Suzuki et al., 2002; Suzuki et al., 2001). These kinases are part of the MAP signalling pathway which is activated in NIH3T3 cells upon growth factor stimulation. Phosphorylation of TOB1 causes its deactivation and a

subsequent increase in Cyclin D1 and CDK4 activity relieving TOB1's repression on cell proliferation (Suzuki et al., 2002). The phosphorylation of BTG2 at Ser-147 and Ser149 also occurs via Erk1 and Erk2 and induces binding of BTG2 to PIN-1, a peptidyl-prolyl cis/trans isomerases (Hong et al., 2005). This interaction leads to cell death with increased mitochondrial depolarisation. The dysregulation of PIN-1 has been connected to many disease states, in particular its up-regulation with tumorigenesis and cancer, however the physiological implications of the BTG2-PIN-1 interaction remain unclear (Brenkman et al., 2008; Ryo et al., 2002; Winkler, 2010)

1.5.5 The role of BTG/TOB protein in cancer and tumourigenesis

The BTG/TOB proteins have been identified as important factors in the prevention of cancer and tumourigenesis (Table 1.3). BTG2 is a direct transcriptional target for the tumour suppressor p53 and Ras-mediated transformation is impaired by TOB1 activity (Boiko et al., 2006; Suzuki et al., 2002). Indeed upon stimulation by growth factors TOB1 is targeted for phosphorylation by the Ras signalling components Erk1 and Erk2, causing TOB1 to lose its anti-proliferative activity. TOB1 knockout mice develop spontaneous carcinogenesis at a higher frequency than wild-type mice (Yoshida et al., 2003a). This increase in tumour formation is accelerated with the combined removal of p53 suggesting that TOB1 works synergistically with p53 to prevent tumour formation (Yoshida et al., 2003a). Mice lacking BTG3 display a similar phenotype with an increased incidence of lung tumours (Yoneda et al., 2009). Conversely expression of BTG/TOB proteins can protect against tumour formation. When BTG2 is over expressed in a medulloblastoma transgenic mouse model, the appearance of this form of cancer is reduced (Farioli-Vecchioli et al., 2007). Furthermore induced expression of TOB1 inhibits tumour growth of intraperitoneal injected pancreatic cancer cells (Yanagie et al., 2009).

Direct involvement of BTG/TOB proteins in cancers has also been identified in human clinical cancer samples. BTG1 and BTG2 are frequently found to be mutated in non-Hodgkin lymphomas. Such mutations are seemingly present in a mutually exclusive manner compared to p53 mutations, suggesting a causative role as a component of the p53 pathway in this type of cancer (Morin RD, 2011; Waanders et al., 2012). Expression of BTG1 can be used as a biomarker to monitor remission of patients suffering from acute myeloid leukaemia because BTG1 expression is only detected in normal cells or cells from patients in complete remission (Cho et al., 2004; Rouault et al., 1992). It is frequently observed in lung and thyroid cancer samples that TOB1 is inactivated by phosphorylation or its expression is reduced (Ito et al., 2005; Yoshida et al., 2003a). In particular an increase in inactive phosphorylated TOB1 correlated to tumour grade in a panel of lung cancer samples (Iwanaga et al., 2003). Down-regulation and impaired expression of BTG2 is often seen in a variety of cancers including prostate cancer, breast cancer and clear renal cell carcinomas (Ficazzola et al., 2001; Kawakubo et al., 2006; Kawakubo et al., 2004; Struckmann et al., 2004). Interestingly a shift in subcellular localisation of BTG2 correlates with tumour grade where BTG2 expression shifts from a nuclear localisation pattern to cytoplasmic (Kawakubo et al., 2006). This pattern of impaired expression in carcinomas is also seen for BTG3, the expression of which is reduced by promoter inactivation via DNA methylation (Majid et al., 2009). Together the evidence indicates that the inactivation or impaired expression of the BTG/TOB proteins contribute to carcinogenesis and tumour progression.

Gene	Cancer		Reference
TOB1	Lung	Spontaneous tumor formation [§]	Yoshida <i>et al.</i> , 2003a.
	Lung	Decreased expression/increased phosphorylation [†]	Iwanaga <i>et al.</i> , 2003
	Lymph node	Spontaneous tumor formation [§]	Yoshida <i>et al.</i> , 2003a
	Liver	Spontaneous tumor formation [§]	Yoshida <i>et al.</i> , 2003a
	Breast	Reduced expression in human breast cancer cell lines	Helms <i>et al.</i> , 2009
	Thyroid	Decreased mRNA expression [†]	Ito <i>et al.</i> , 2005
	Pancreatic	Induced expression inhibits tumorigenesis in nude mice	Yanagie <i>et al.</i> , 2009
BTG1	Leukemia	Reduced/undetectable expression [†]	Cho <i>et al.</i> , 2004
	Lymphoma	Somatic mutations [†]	Morin <i>et al.</i> , 2011
BTG2	Breast	Reduced expression and relocalization (nuclear to cytoplasm) [†]	Kawakubo <i>et al.</i> , 2006
	Renal	Reduced mRNA levels [†]	Stuckman <i>et al.</i> , 2004
	Prostate	Low/undetectable mRNA levels [†]	Ficazzola <i>et al.</i> , 2001
	Brain	Induced expression inhibits medulloblastomas (transgenic mice)	Farioli-Vecchiolo <i>et al.</i> , 2007
	Lymphoma	Somatic mutations [†]	Morin <i>et al.</i> , 2011
BTG3	Lung	Increased lung tumor formation [§]	Yoneda <i>et al.</i> , 2009
	Lung	Reduced expression in adenocarcinoma samples [†]	Yoneda <i>et al.</i> , 2009
	Renal	Reduced mRNA expression [†]	Majid <i>et al.</i> , 2009
BTG4	Colon	Reduced mRNA expression [†]	Toyota <i>et al.</i> , 2008

Table 1.3 Relationship of BTG/TOB expression and cancer. § Observations made using mouse knock-out models. † Observation made using human clinical cancer samples and biopsies. To date, no changes in TOB2 expression are reported in human cancer samples.

1.5.6 Role of BTG/TOB protein in bone morphology

TOB1 and BTG2 are implicated in the signalling of TGF-family members through Smad transcription factors. Bone morphogenic protein (BMP), a member of the TGF-family, activates Smad transcription factors. This signalling is repressed in the presence of TOB1. In osteoblast cells TOB1 co-localises with Smad 1, 5 and 8 transcription factors in nuclear bodies upon BMP2 stimulation causing a repression of the Smad target genes negatively regulating osteoblast proliferation and differentiation (Yoshida et al., 2000). Interestingly BTG2 appears to enhance Smad-regulated transcription in response to BMP signalling, which regulates vertebral patterning (Park et al., 2004).

The generation of mice containing null alleles of TOB1, TOB2, BTG2 and BTG3 uncovered a role for these proteins in bone formation and resorption. Mice lacking TOB1 are apparently normal but display increased bone volume and bone density due to enhanced bone formation (Yoshida et al., 2003b). As described previously Caf1a (CNOT7) null mice also display a similar increase in bone density (Washio-Oikawa et al., 2007). Osteoclast parameters remain unchanged for both TOB1^{-/-} and Caf1a (CNOT7)^{-/-} mice suggesting that TOB1 may act through its interaction with Caf1a (CNOT7) when regulating bone formation. Surprisingly TOB2 null mice display the opposite phenotype with decreased bone mass due to an increased number of differentiated osteoclast cells (Ajima et al., 2008). Currently there is no evidence that TOB2 regulates Smad transcription factors like other family members TOB1 and BTG2. TOB2 affects bone formation through an interaction with the vitamin D receptor to reduce the expression of RANKL, a vitamin D induced gene, which increases the number of differentiated osteoclast cells.

Together this data demonstrates that the BTG/TOB proteins can regulate bone formation and are therefore required for more processes than simply the prevention of cancer through their anti-proliferative properties.

1.6 Study Aims

The BTG/TOB proteins are known anti-proliferative proteins that have been shown to interact with a wide variety of different proteins. This study will aim to further investigate the interactions between BTG/TOB proteins and the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylase subunits of the Ccr4-Not complex. In particular the work will focus on the interactions between the BTG/TOB proteins BTG2 and TOB1 and the Caf1a (CNOT7)/Caf1b (CNOT8) enzymes and the importance of these interactions for the anti-proliferative activities of the BTG/TOB proteins.

To address these questions, essential amino acids located in BTG2 or TOB1 required for the interaction with Caf1a (CNOT7) and Caf1b (CNOT8) will be identified, guided by multiple sequence alignments and structural information. These amino acids will be substituted using site-directed mutagenesis and screened to identify those that disrupt the BTG2/TOB1 - Caf1a (CNOT7)/Caf1b (CNOT8) interaction. Successful amino acid substitutions that disrupt the interactions will be used to investigate the role of the BTG/TOB – Caf1a (CNOT7)/Caf1b (CNOT8) interactions on cell proliferation of MCF-7 cells. Finally an RNA tethering approach will be used to correlate the anti-proliferative activities of BTG/TOB proteins to their roles in regulating deadenylation and mRNA abundance of a reporter mRNA.

1.7 Experimental approaches

1.7.1 Yeast two-hybrid

The budding yeast *Saccharomyces cerevisiae* is a powerful model used to study eukaryotic protein interactions. Due to the ease of transformation and quick doubling time the yeast two-hybrid assay can be used to quickly screen multiple protein interactions. Furthermore the transcriptional control and post-translation protein modifications of *S.cerevisiae* are well conserved in mammals. Together this makes *S.cerevisiae* a powerful and widely used tool for initial protein-protein interaction screening.

This method takes advantage of the ability to separate the DNA binding domain (DBD) and transcriptional activation domain (AD) of transcription factors. The DBD can bind to DNA without activating transcription; the AD domain cannot bind to DNA and recruit RNA Pol II alone. Both the DBD and AD domains must be brought together onto the promoter to initiate transcription, normally of a reporter gene like β -Galactosidase. These two fragments of the transcription factor are fused to two target proteins. If the target proteins interact they bring together the DBD and AD domain onto the β -Galactosidase promoter and initiate transcription (Figure 1.12A). If the two target proteins do not interact or if that interaction has been disrupted by amino acid substitutions then transcription will be prevented because the AD domain will not be brought to the promoter (Figure 1.12A). The primary drawback to the yeast two-hybrid system is the high incidence of intrinsic transcriptional activation caused by the protein being tested. To that end all proteins must be tested individually to ensure they are not capable of stimulating β -Galactosidase transcription alone. In this project the yeast two-hybrid system will be used to screen amino acid substitutions that aim to disrupt the interaction between BTG2 and TOB1 with the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8). Co-immuno precipitation assays performed in HEK293T cells were used to confirm that any interaction disruption was conserved in human cells.

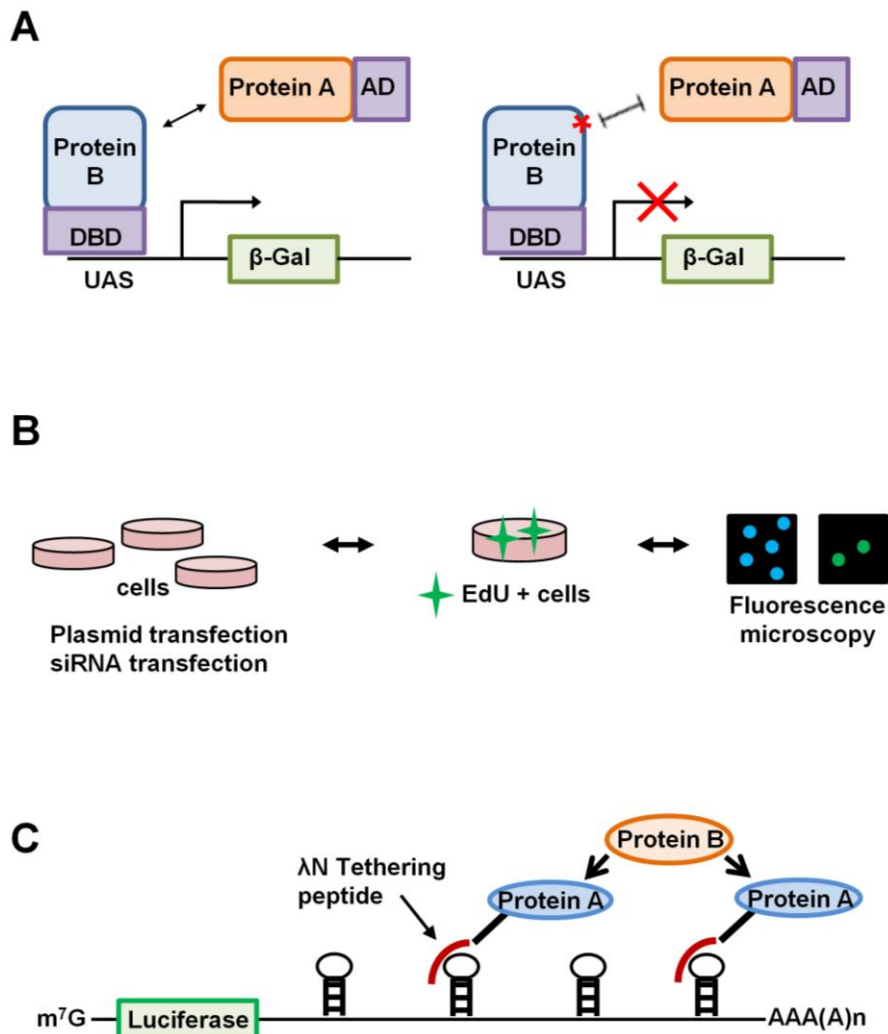


Figure 1.12 Schematic representation of the experimental techniques used in this thesis. (A) Yeast two-hybrid. AD, Gal4 activation domain; BD, Gal4 DNA binding domain. When an interaction occurs between protein A and protein B, the Gal4-BD and Gal4-AD are brought in close proximity on the β -Galactosidase promoter thereby inducing transcription (left panel). In the absence of an interaction, β -Galactosidase transcription is not initiated (right panel). **(B) EdU labelling of cells in S-phase.** Mammalian cells are seeded onto glass cover slips and transfected with plasmid DNA or siRNA. After 48 h the thymidine analogue EdU is added to the cell media and is incorporated into newly synthesised DNA. The cells are fixed onto the cover slips and the incorporated EdU detected via coupling of Alexa Fluor 488 azide using 'click' chemistry. The number of cells in S-phase are counted using a Zeiss LSM 510 Meta microscope and compared to total cells determine via a Hoechst staining. **(C) RNA tethering assay.** Upon expression of protein A fused to the λ N peptide it will bind to the 5 BoxB motifs located in the renilla luciferase mRNA 3' UTR. Protein A can then recruit other factors to the mRNA 3' UTR that may affect mRNA translation or stability.

1.7.2 S-phase labelling assay to monitor cell proliferation

In this study the effect of BTG/TOB expression on cell proliferation was assessed. In particular experiments were conducted to determine if the interaction between BTG/TOB proteins and Caf1a (CNOT7)/Caf1b (CNOT8) was required for their anti-proliferative effect. The BTG/TOB proteins are known anti-proliferative proteins and have been shown to target the G1 to S-phase cell cycle check point (Donato et al., 2007; Guardavaccaro et al., 2000; Hata et al., 2007). To observe changes in mammalian cell proliferation rates the S-phase labelling assay was utilised, which monitors the number of cells in S phase. The assay labels newly synthesised DNA making it an accurate method of monitoring cell proliferation. Here a thymidine analogue EdU (5-ethynyl-2'-deoxyuridine) is added to the cell media for a pre-determined time. Any cells entering S-phase during this period will incorporate EdU into their newly synthesised DNA (Figure 1.12B). The EdU detection occurs through a click reaction between an azide and alkyne, catalysed by copper. The EdU contains the alkyne which is detected upon addition of an Alexa Fluor dye containing azide. This Alexa Fluor dye causes all cells in S-phase to fluoresce green under fluorescent microscopy. The assay is based on the standard BrdU (bromo-deoxyuridine) thymidine analogue but holds some advantages. BrdU requires the DNA to be denatured before antibody detection, the azide used to detect EdU is small and can access the DNA without the need for it to be denatured. This means milder conditions can be used for EdU detection and the process is quicker and more accurate.

To determine the numbers of cells in S-phase, cells are fixed onto glass cover slips, EdU is detected and cells are counter stained with Hoechst that binds indiscriminately to DNA. Using fluorescent microscopy the number of cells in S phase are counted, followed by the total number of cells visualised by the Hoechst stain (Figure 1.12B). This allows for the percentage of cells in S-phase to be counted for any particular sample.

1.7.3 RNA tethering reporter assay

TOB1 and TOB2 contain PAM2 motifs that allow them to bind to PABP. This suggests that TOB1/TOB2 may be present in the complex of proteins that bind to an mRNA poly(A) tail (Ezzeddine et al., 2007). This poses the question: can the BTG/TOB proteins recruit the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8) to an mRNA and stimulate poly(A) tail removal and mRNA decay? To study this a modified luciferase reporter experiment was conducted using plasmids donated by Prof N Gehring (Gehring et al., 2008). The assay utilises the RNA binding properties of the bacteriophage lambda-antiterminator protein N (lambda N-peptide). This 22 amino acid peptide is fused to a target protein of interest (here the BTG/TOB proteins) allowing it to bind to specific RNA motifs. A renilla luciferase plasmid was modified to include the corresponding RNA motifs in its 3' UTR. These Box B sequences form hairpin loop structures that bind to the lambda N-peptide and therefore cause the target protein to be tethered to the luciferase mRNA 3' UTR (Figure 1.12C). This reporter system can be used to monitor changes in translation and mRNA stability in response to the presence of BTG/TOB proteins bound to mRNA.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Bacterial growth and transformation

2.1.1 Reagents, stock solutions and buffers for use in bacterial methods:

Luria-Bertoni (LB) media and agar: 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, pH 7.2 (NaOH), for LB agar include 15g/L bacteriological agar. The medium was sterilised by heat using a Prestige medical 2100 classic bench-top autoclave and stored at room temperature.

Ampicillin 1000x stock solution: 100mg/ml in 50% ethanol/water. Sterilised by filtration (0.22 µm pore size). Stored at -20°C

Chloramphenicol 1000x stock solution: 34mg/ml in 100% ethanol. Sterilised by filtration (0.22 µm pore size). Stored at -20°C

Kanamycin 1000x stock solution: 50mg/ml in H₂O. Sterilised by filtration (0.22 µm pore size). Stored at -20°C

TBF1 buffer: 100mM CaCl₂, 100mM RbCl, 50mM MgCl₂, 30mM KCl, 15% glycerol, pH 5.8 (0.2M glacial acetic acid). Sterilised by heat using a Prestige medical 2100 classic bench-top autoclave and stored at 4°C

TBF2 solution: 75mM CaCl₂, 10mM RbCl, 10mM MOPS, 15% glycerol, pH 6.5 (1M KOH). Sterilised by heat using a Prestige medical 2100 classic bench-top autoclave and stored at 4°C

1M IPTG: Isopropyl β-2-thiogalactopyranoside, in H₂O. Sterilised by filtration (0.22 µm pore size). Stored at -20°C

X-Gal solution: a solution of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (50mg/ml in dimethylformimide) was purchased from Promega. Stored at -20°C

2.1.2 Culture of *E. coli* DH5 α

The bacterial strain *E. coli* DH5 α was used for all DNA manipulations. To obtain single colonies the bacteria were streaked onto agar plates (containing the appropriate antibiotics) and grown at 37°C. Once colonies had formed the plates were stored at 4°C for up to several weeks. Liquid cultures were inoculated with single colonies and grown in into universal screw-cap tubes containing LB medium (with appropriate antibiotics) and incubated at 37°C, 200rpm for 12-24 h. For larger cultures glass conical flasks were used.

2.1.3 Preparation of *E. coli* competent cells

E. coli DH5 α were made competent for transformation using rubidium chloride/calcium chloride. A single *E. coli* colony from an LB plate was used to inoculate 5ml of LB medium and grown overnight at 37°C, 200rpm. The following morning the culture was diluted 1:100 in LB medium and grown at 37°C to an optical density (OD₆₀₀) of 0.5. The culture was incubated on ice for 10 min in pre-chilled 50ml falcon tubes, cells were then harvested by centrifugation at 1100g, 4°C for 10 min. Cell pellets were re-suspended gently with 20ml of ice cold TBF1 and centrifuged again at 1100g, 4°C for 10 min. Each cell pellet was then gently re-suspended in 2ml of ice cold TBF2, the cell suspensions were then combined and distributed into 200 μ l aliquots in microfuge tubes before being snap frozen in liquid nitrogen. Competent cells were stored at -80°C.

2.1.4 Transformation of competent cells

Between 50 and 100 μ l of competent cells were used for each transformation. When transforming plasmids, <500ng of DNA was used to give a good transformation yield, when transforming ligations or mutagenesis reactions, 10 μ l of ligation reaction mix was used. Competent *E. coli* cells and DNA were mixed in pre-chilled round bottomed universal tubes and incubated on ice for

5-20 min. The transformation mix was heat shocked by incubation at 42°C for 90 sec followed by incubation on ice for 2 min. One millilitre of LB medium (pre-warmed at 37°C) was added to the cells and they were incubated at 37°C, 200rpm for 1 h. For plasmid transformation 100µl of the cell suspension was spread onto LB agar plates (containing the appropriate antibiotic). For ligations or mutagenesis transformations the cells suspension was centrifuged at 3000g for 2 min, 900µl of supernatant removed and the cells re-suspending in the remaining 100µl of LB. All the re-suspended cells were then spread onto LB agar plates (containing the appropriate antibiotic). For blue/white screening (for *E. coli* transformed with derivatives of the pBluescript II KS(+) cloning vector), the cells were plated along with 17µl of X-Gal (50mg/ml, Promega) and 4µl of 1M IPTG.

2.2 Molecular biology

2.2.1 Reagents, stock solutions and buffers for use in molecular biology methods:

5x TBE: 40mM Tris base, 40mM boric acid, 1mM EDTA, pH 8.0, stored at room temperature

6x Ficoll loading dye: 16% Ficoll, 0.2% orange G in H₂O, stored at room temperature.

TE Buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA

Oligonucleotides: Lyophilised primers were dissolved in TE to give a 100mM solution and stored at -20°C.

2.2.2 Small scale plasmid DNA preparation

Typically, Macherey-Nagel plasmid mini prep kits (Cat# 740588.250) were used for the small scale preparation of plasmid DNA. A single bacterial colony was picked and used to inoculate either 2ml (for small scale) or 5ml (for use in mammalian cell transient transfections and yeast transformations) of LB containing the appropriate antibiotic and grown overnight in an orbital shaker at 37°C, 200rpm. All of the overnight culture was pelleted by centrifugation in a microfuge tube at 4000g for 2 min. The plasmid DNA was then extracted according to the manufacturer's instructions, details of which can be found in the Macherey-Nagel plasmid mini prep handbook. Small scale plasmid extractions were eluted in 50µl of elution buffer pre-warmed to 70°C, or in 100µl of pre-warmed buffer when DNA was isolated from 5ml. All plasmid DNA was assessed using a Nanodrop ND-1000 spectrophotometer to check the concentration and purity before being stored at -20°C.

2.2.3 Large scale plasmid DNA preparation

For the production of large quantities of high quality plasmid DNA for transient mammalian transfections, the Sigma GenElute HP maxi prep kit was used. A single *E. coli* colony containing the desired plasmid was picked and used to inoculate 5ml of LB (containing the appropriate antibiotic), this culture was grown for 4-6h in an orbital shaker at 37°C, 200rpm. All 5ml of this culture was used to inoculate 200ml of LB containing the appropriate antibiotic and the culture grown overnight in an orbital shaker at 37°C, 200rpm. The overnight culture was pelleted by centrifugation at 4000g for 15min. The cell pellet was re-suspended and subjected to manufacturer's standard protocol. Typically, the plasmid DNA was eluted in 500µl of elution buffer and stored at -20°C.

2.2.4 Determination of DNA/RNA concentration

All DNA was quantified by spectrophotometry using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). The A_{260}/A_{280} ratio values were calculated to estimate sample purity. A ratio between 1.8 and 2.0 was deemed acceptable for DNA samples, while a ratio between 2.0 and 2.2 was expected for RNA.

2.2.5 Agarose gel electrophoresis

DNA was fractionated by size using 0.8%-2% agarose gels (w/v) as appropriate to the size of the DNA fragment being resolved. To the gels, 0.5µg/ml of ethidium bromide was added. Agarose gels were made and run with 0.5x TBE buffer. DNA samples were loaded in a 1x Ficoll sample buffer. DNA gels were run at 80V for between 45 min and 2 h depending upon the sample being run. Following electrophoresis gels were visualised by transillumination with ultraviolet light and documented using either a Gel-Doc 2000 (Bio-Rad) and BioRad Quantity One computer software or using a Fujifilm LAS-4000 imager reader and the accompanying computer software (Fujifilm, Japan).

2.2.6 DNA extraction and purification from agarose gel

To purify DNA fragments from gels, DNA bands were excised from the agarose gel using a surgical scalpel. Purification was conducted according to the manufacturer's protocol provided in the Macherey-Nagel Gel/PCR purification kit (Cat# 740609.50). Briefly, the gel slice was dissolved at 65°C in binding buffer (100µl of binding buffer per 100µg of gel). Samples were applied to the spin columns, centrifuged briefly in a bench top microfuge and the flow-through discarded. Columns were washed twice with Wash Solution I and subsequently, the DNA was eluted in 50µl of the pre-heated 65°C elution buffer provided. For small DNA fragments (< 500bp) used in cloning

the DNA was eluted in 30µl of pre-heated 65°C elution buffer to increase the DNA concentration.

2.2.7 Annealing of DNA oligos

To anneal complementary DNA oligos 1µl of each oligo (100µM stock in TE buffer) was incubated with 3µl of 10x T4 DNA ligase buffer and 25µl of H₂O. The sample was placed into the peqlab Primus 96 advanced thermal cycler at 85°C and allowed to cool slowly at 1°C per minute until the temperature reached 20°C. The oligos were used in restriction digests or blunt end ligation reactions.

2.2.8 Restriction enzyme digestion of DNA

Plasmid DNA was digested with restriction endonucleases in order to generate compatible ends for cloning and to verify newly created plasmids. All restriction endonucleases and corresponding buffers were supplied by NEB and used according to the manufacturer's instructions.

To verify plasmids after ligation reactions, 100ng of plasmid DNA was digested in 10µl total reaction volume; to digest large quantities of DNA for cloning and ligations 5µg of plasmid DNA was digested in 50µl total reaction volume. Digests lasted 1-5h depending on the amount of DNA and were conducted at the optimal temperature for the enzyme used.

2.2.9 Removal of 5' phosphate group from linearised plasmid DNA

The removal of 5' phosphate groups from restriction enzyme digested plasmid was conducted to prevent DNA from self ligating in ligation reactions. The 5' phosphate group was removed using NEB Antarctic Phosphatase, 1µl of enzyme was added to the digested plasmid DNA (1-5µg of plasmid DNA) and incubated at 37°C for 30 min. A further 1µl of enzyme was

added and the sample and incubated for a further 30 min at 37°C. To deactivate the enzyme, the mixture was incubated at 65°C for 5 min.

2.2.10 Ligation of DNA fragments

Recombinant plasmids were created by ligating DNA fragments using T4 DNA ligase (NEB). Ligation reactions were set up by mixing approximately 100ng of digested vector with a 3-fold molar excess of insert along with 0.5µl of T4 DNA ligase in 1x T4 DNA ligase buffer and a final reaction volume of 10µl. Reactions were left overnight at room temperature.

2.2.11 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed using peqlab Primus 96 advanced thermal cycler. PCR was used for the generation of DNA fragments for cloning and the screening of recombinant plasmids.

For the screening of newly generated plasmids (colony PCR), single clones from a ligation reaction were used to inoculate 500µl of LB with the appropriate antibiotic and grown for 4-6h in an orbital shaker at 37°C, 200rpm. A standard Taq Polymerase PCR reaction was set up as shown in Table 2.1. The primers used are shown in Tables 2.3 and 2.4. Samples were initially denatured at 95°C for 3 min followed by 25 cycles each consisting of a denaturing step of 95°C for 20 sec, annealing at 55°C for 30 sec, then elongation at 72°C for 1 min/kb. A final elongation step for 5min at 72°C was included at the end. To check the insert was the correct size, 10µl of the reaction was loaded onto a 1% agarose gel.

For the synthesis of blunt end DNA fragments for cloning, Phusion DNA polymerase (Finnzymes) with 5' - 3' proofreading ability was used. A standard reaction was set up as shown in Table 2.2. The primers used are shown in Tables 2.3 and 2.4. Samples were initially denatured at 95°C for 3

min followed by 30 cycles consisting of a denaturing step of 95°C for 10 sec, annealing at 50°C for 30 sec, then elongation at 68°C for 1 min/0.5 kb. A final elongation step for 10 min at 68°C was included at the end. To check newly generated PCR fragments were the correct size, 10µl of the reaction was run on a 1% agarose gel.

Volume	Component/Reagent
2µl	Bacterial culture
1.0µl	10mM forward primer (Invitrogen), stock at 100mM in TE
1.0µl	10mM reverse primer (Invitrogen), stock at 100mM in TE
2.5µl	10x Taq Pol buffer (NEB)
1.0µl	10mM dNTP mix (NEB)
0.25µl	Taq DNA Polymerase (NEB)
17.25µl	nuclease-free H ₂ O

Table 2.1 Standard Taq DNA polymerase PCR reaction

Volume	Component/Reagent
x µl	100ng DNA template
1.0µl	10mM forward primer (Invitrogen), stock at 100mM in TE
1.0µl	10mM reverse primer (Invitrogen), stock at 100mM in TE
10.0µl	5x Phusion HF buffer (Finnzyme)
1.0µl	10mM dNTP mix (NEB)
0.5µl	Phusion High Fidelity DNA Polymerase (Finnzyme)
up to	nuclease-free H ₂ O

Table 2.2 Standard Phusion DNA polymerase reaction

2.2.12 Site-directed mutagenesis

For site-directed mutagenesis, overlapping primers with the desired mutations were designed (Table 2.5) using the PrimerX programme (<http://www.bioinformatics.org/primerx>) using the primer design protocol optimised for the Stratagene Quikchange protocol. Reactions were set up as shown in Table 2.2. Reactions were initially incubated at 95°C for 3 min followed by 30 cycles consisting of a denaturing step of 95°C for 10 sec, annealing for 30 sec, then elongation at 68°C for 1 min/0.5 kb. A final elongation step for 10 min at 68°C was included at the end. The annealing temperature could be varied between 40 and 60°C to optimise the reaction efficiency.

To remove template DNA from site directed mutagenesis reactions, the enzyme DpnI was used. One microlitre of DpnI was added to each 50µl mutagenesis reaction and incubated at 37°C for 2 h. Ten microlitres of the reaction was then loaded and run on a 1% agarose gel. The remaining sample was then used to transform *E. coli* DH5α before DNA isolation and sequencing to confirm the presence of the desired mutations.

2.2.13 List of Primers

Name	Sequence
pBD-Gal4 Seq primer 5'	GTGCGACATCATCATCGGAAG
pAD-Gal4 Seq primer 5'	AGGGATGTTTAATACCACTAC
M13 rev	CACACAGGAAACAGCTATGACCAT
T3	ATTAACCCTCACTAAAG
T7 Fw	TAATACGACTCACTATAGGG
T7 Rv	GCTAGTTATTGCTCAGCGG

Table 2.3 Primers used for DNA sequencing

Name	Sequence
CNOT7-XbaI Rv	CGACTCTAGACTATGACTGCTTGTT
CNOT8-XbaI Rv	CGACTCTAGACTACTGCTGCATGTT
CNOT8-MluI Rv	ACGCGTCTACTGCTGCATGTT
BTG2 XhoI Fw	CCTCGAGATGAGCCACGGGAAGGGAACCG
BTG2 BamHI Fw	GGATCCATGAGCCACGGGAAGGGAACCG
BTG2 EcoRI Fw	AAAAAGAATTCATGAGCCACGGGAAGGGAACCG
HA-BTG2 FW	GGATCCCGCTTACCCATACGATGTTCCAGATTACGCTATGA GCCACGGGAAGGGAACCG
HA-BTG2 [λ N] fw	CAGCACCTCGAGGCTTACCCATACGATGTTCCAGATTACG CTATGAGCCACGGGAAGGGAAC
TOB1-XbaI Rv	CGACTCTAGACTAGTTAGCCATAAC
TOB1 EcoRI Fw	AAAAAGAATTCATGCAGCTTGAAATCCAAGTAGC
HA-TOB1 [λ N] fw	CAGCACCTCGAGGCTTACCCATACGATGTTCCAGATTACG CTATGCAGCTTGAAATCC
HA BamHI Bottom	GATCAGCGTAATCTGGAACATCGTATGGGTAAGCTT
HA BamHI Top	GATCAAGCTTACCCATACGATGTTCCAGATTACGCT
PRMT1 fw	GGATCCATGGCGGCAGCCGAGGCCGCGA
PRMT1 rv	GTCGACACCGACTACCGGATGCGCTGA
PABPC1 fw	GGATCCATGAACCCAGTGCCCCCAGCT
PABPC1 rv	GTCGACTTAAACAGTTGGAACACCGGT
Gal4-HA FW	AATTGTACCCATACGATGTTCCAGATTACGCTGGATCCGCC CGGGAATTCCCGTCGG
Gal4-HA RV	TCGACCGACGGGAATTCCCGGGCGGATCCAGCGTAATCTGG AACATCGTATGGGTAC

Table 2.4 Primers used for DNA cloning

Name	Sequence
BTG2_H53A FW	CAGAGCACTACAAACACGCCTGGTTTCCCGAAAAGC
BTG2_H53A RV	GCTTTTCGGGAAACCAGGCGTGTTTGTAGTGCTCTG
BTG2_H53A_long FW	CAGAGCACTACAAACACGCCTGGTTTCCCGAAAAGCCG
BTG2_H53A_long RV	CGGCTTTTCGGGAAACCAGGCGTGTTTGTAGTGCTCTG
BTG2_Y65A FW	GTCCAAGGGCTCCGGCGCCCGCTGCATTTCGCATC
BTG2_Y65A RV	GATGCGAATGCAGCGGGCGCCGGAGCCCTTGGAC
BTG2_D75A FW	CAACCACAAGATGGCCCCCATCATCAGCAG
BTG2_D75A RV	CTGCTGATGATGGGGGCCATCTTGTGGTTG
BTG2_W103A FW	CAGCGAGCTGACCCTGGCGGTGGACCCCTATGAG
BTG2_W103A RV	CTCATAGGGGTCCACCGCCAGGGTCAGCTCGCTG
BTG2_D105A FW	GACCCTGTGGGTGGCCCCCTATGAGGTGTC
BTG2_D105A RV	GACACCTCATAGGGGGCCACCCACAGGGTC
BTG2_E108A FW	GTGGGTGGACCCCTATGCAGTGTCTACCGCATTG
BTG2_E108A RV	CAATGCGGTAGGACACTGCATAGGGGTCCACCCAC
BTG2_E115A FW	CTACCGCATTGGGGCGGACGGCTCCATCTG
BTG2_E115A RV	CAGATGGAGCCGTCCGCCCCAATGCGGTAG
BTG2_E115Along FW	CCTACCGCATTGGGGCAGACGGCTCCATCTGCGTCTTG
BTG2_E115Along RV	CAAGACGCAGATGGAGCCGTCTGCCCCAATGCGGTAGG
BTG2 S147 149A FW	CTGCTGGGCCGGAGCGCCCCCGCCAAGAACTACGTG
BTG2 S147 149A RV	CACGTAGTTCTTGGCGGGGGCGCTCCGGCCCAGCAG
Tob1 H43A FW	GAAGAAATATGAAGGGGCCTGGTATCCTGAAAAGC
Tob1 H43A RV	GCTTTTCAGGATAACCAGGCCCCTTCATATTTCTTC
Tob1 H45A FW	GAAATATGAAGGGCACTGGGCACCTGAAAAGCCATACAAAG
Tob1 H45A RV	CTTTGTATGGCTTTTCAGGTGCCCAGTGCCCTTCATATTTTC

Tob1 F55A FW	CATACAAAGGATCGGGGGCTAGATGTATACACATAGG
Tob1 F55A RV	CCTATGTGTATACATCTAGCCCCGATCCTTTGTATG
Tob1 D65A FW	CATAGGGGAGAAAGTGGCCCCAGTGATTGAACAAG
Tob1 D65A RV	CTTGTTCAATCACTGGGGCCACTTTCTCCCCTATG
Tob1_W93A FW	CACAGGATCTTAGTGTTGCGATCGACCCATTTGAG
Tob1_W93A RV	CTCAAATGGGTGCGATCGCAACACTAAGATCCTGTG
Tob1 D95A FW	CTTAGTGTTTGGATCGCCCCATTTGAGGTTTC
Tob1 D95A RV	GAAACCTCAAATGGGGCGATCCAAACACTAAG
Tob1 E98A FW	GTTTGGATCGACCCATTTGCGGTTTCTTACCAAATTGG
Tob1 E98A RV	CCAATTTGGTAAGAAACCGCAAATGGGTGCGATCCAAAC
Tob1 E105A FW	CTTACCAAATTGGTGCAAAGGGACCAGTGAAG
Tob1 E105A RV	CTTCACTGGTCCCTTTGCACCAATTTGGTAAG
Tob1 S125 154 164A FW	CTCATCAGTGTCCAGCGCTCCAGCGCCTCCTTTTGGTCAC TCTGCTGCTGTAGCCCCTACCTTCATGC
Tob1 S125 154 164A RV	GCATGAAGGTAGGGGCTACAGCAGCAGAGTGACCAAAGGA GGCGCTGGAGCGCTGGACACTGATGAG
7E247FW	GAGAGAAATGTTCTTTGCCGATCATATTGATGATGCC
7E247REV	GGCATCATCAATATGATCGGCAAAGAACATTTCTCTC
7Y260FW	CAAATATTGTGGTCATTTGGCTGGCCTTGGTTCTGGTTC
7Y260REV	GAACCAGAACCAAGGCCAGCCAAATGACCACAATATTTG
8E247FW	GAAAGAGTTGTTTTTTGCGGACAGCATTGATGATG
8E247REV	CATCATCAATGCTGTCCGCAAAAAACAACCTCTTTC
8Y260FW	CTGTGGGCGGCTCGCTGGCTTAGGCACAG
8Y260REV	CTGTGCCTAAGCCAGCGAGCCGCCACAG
CNOT7 D40A FW	GAAAATATAATTACGTTGCTATGGCCACCGAGTTTCCAGGT GTGGTTGC
CNOT7 D40A RV	GCAACCACACCTGGAAACTCGGTGGCCATAGCAACGTAATT ATATTTTC
CNOT8 D40A	GTTATATTGCCATGGCCACAGAATTTCCAGG
CNOT8 D40A RV	CCTGGAAATTCTGTGGCCATGGCAATATAAC

Table 2.5 Primers used for site-directed mutagenesis.

2.3 Yeast two-hybrid analysis

2.3.1 Reagents, stock solutions and buffers for use in yeast culture

YPD medium: 10g/L yeast extract, 20g/L glucose, 20g/L bacteriological peptone. For YPD agar, 20g/L bacteriological agar was added. The medium was sterilised by heat using a Prestige medical 2100 classic bench-top autoclave and stored at 4°C.

Yeast selective complete (SC) medium: 6.7g/L yeast nutrient broth, 20g/L glucose, 1.4g/L amino acid drop out mix (minus histidine, uracil, tryptophan and leucine). For selective agar plates, 20g/L bacteriological agar was added. The medium was sterilised by heat using a Prestige medical 2100 classic bench-top autoclave and stored at 4°C.

Histidine solution: stock 20mg/ml in H₂O (add 4.3ml per 1L of medium). After sterilisation by filtration (0.22 µm pore size), the solution was stored at room temperature.

Uracil: stock 5mg/ml in H₂O (add 17.2ml per 1l of medium). After sterilisation by filtration (0.22 µm pore size), the solution was stored at room temperature.

Single-stranded DNA: Herring Sperm single-stranded DNA (2 mg/ml, Abcam, ab46666) was heated to 98°C for 5min and placed on ice before use. Stored at -20°C.

Lithium acetate solutions: LiAc solutions (1M and 0.1M; Sigma) were prepared and sterilised by filtration (0.22 µm pore size). Stored at room temperature.

PEG-3350 solution: a solution containing 50% (w/v) polyethylene glycol (PEG-3350; Sigma) was sterilisation by filtration (0.22 µm pore size). The solution was stored at room temperature for up to several months.

SDS loading buffer (4x): 50% upper buffer, 40% glycerol, 1% β -mercaptoethanol, 1.25% Bromophenolblue (0.1%), 0.25% H₂O, stored at -20°C. Small aliquots were stored short-term at 4°C.

2.3.2 Culture of *Saccharomyces cerevisiae* strain YRG2

The yeast strain *S. cerevisiae* YRG2 (Stratagene; stored at -80°C in YPD containing 50% glycerol) was used for all yeast two-hybrid analyses. To obtain single colonies the yeast was streaked onto YPD agar plates and grown at 30°C. Once colonies had formed (2-4 days) the plates were stored at 4°C for several weeks. Liquid cultures were inoculated with large single colonies and grown in universal screw-cap tubes containing YPD or SC medium and incubated at 30°C, 200rpm for 12-24 h. For larger cultures glass conical flasks were used.

2.3.3 Transformation of YRG2 cells

Derivatives of plasmids pBD-GAL4 Cam and pAD-GAL4-2.1 (Stratagene) were used for all yeast two-hybrid assays. Yeast YRG2 cells were first made competent through a heat shock treatment. Competent cells were always used on the day they were made. One large single yeast colony was used to inoculate 10ml of YPD medium and grown overnight at 30°C and 200rpm, this culture was then used to inoculate 50ml of YPD medium to give an OD₆₀₀ of 0.2. This culture was incubated at 30°C, 200rpm until the OD₆₀₀ had increased to 0.8 (3-5 h) at which point the culture was centrifuged to pellet the yeast at 3000g for 5 min. The supernatant was removed and the cells carefully re-suspended in 25ml of sterile water followed by another centrifuge step of 3000g for 5 min. The water was removed and the yeast re-suspended in 1ml of 0.1M lithium acetate and transferred to a 1.5ml microfuge tube before being centrifuged at 4000g for 15 sec. The supernatant was removed and the cells re-suspended to a final volume of 500 μ l using

0.1M lithium acetate (requiring the addition of approximately 350 μ l 0.1M lithium acetate). One 50ml culture provided sufficient cells for ten transformations. The cell suspension was equally divided (\sim 50 μ l) into ten 1.5ml microfuge tubes, the cells pelleted as described above and the supernatant removed.

Volume	Component/Reagent
50 μ l	500ng (combined) plasmid DNA diluted in H ₂ O
240 μ l	50% polyethylene glycol (PEG-3350)
36 μ l	1M lithium acetate
25 μ l	Single-stranded DNA (2 mg/ml)

Table 2.6 Mixture for yeast transformation

The 50% PEG solution was first pipetted onto the yeast cell pellet followed by a mix of the other transformation components. Herring sperm ssDNA, (Abcam, ab46666) was boiled for 5 min and quickly chilled on ice before use (Table 2.6). After the addition of the all components, the transformation mix was vortexed until the pellet was completely resuspended. The yeast transformation mix was then incubated for 30 min at 30°C, 200rpm followed by 20 min incubation at 42°C in a water bath. The samples were then centrifuged at 4000g for 15 sec and the yeast pellet re-suspended in 500 μ l of sterile H₂O. From this cell suspension 100 μ l was placed on SC selective plates (without tryptophan and leucine) and incubated at 30°C for 3-5 days until colonies formed.

2.3.4 Yeast two-hybrid β -galactosidase assay

For each transformation, three independent yeast clones were tested. Yeast colonies were streaked into small rectangles (approximately 0.5 x 1.0cm) on SC selective medium agar plates. After 48-72 h the resulting rectangle of yeast was used to inoculate 2ml of YPD medium. After incubation at 30°C, 200rpm for 24 h, one millilitre of the yeast culture was used to obtain the OD₆₀₀ value as a measure of the culture density. For dense cultures with an OD₆₀₀ >1, the sample was diluted 10-fold with SC medium. From the remaining culture, 25µl was added to 25µl of β -Glo reagent (Promega) in a white 96 well flat bottom plate and incubated in the dark for 30min at room temperature. β -Galactosidase luminescence was measured for 1 sec using a Berthold Orion microplate luminometer and Simplicity 4.02 software. For each yeast culture, the β -galactosidase activity was tested in duplicate. The β -galactosidase activities were normalised using the OD₆₀₀ values.

2.3.5 Western blot analysis of yeast cell lysates

To confirm expression of AD-Caf1a (CNOT7), AD-Caf1b (CNOT8), BD-HA-BTG2 and BD-HA-TOB1, protein lysates were produced and subjected to western blot analysis. Individual yeast colonies were streaked into small rectangles and cultured in 2ml to SC medium. One millilitre of yeast culture was transferred to a 1.5ml microfuge tube and centrifuged at 3000g for 2 min. The resulting cell pellet was re-suspended in 100µl of H₂O. After the addition of 100µl of 0.2M NaOH, the cells were vortexed and incubated for 5 min at room temperature. The cells were centrifuged again at 3000g for 2 min, the supernatant was removed and the pellet re-suspended in 50µl of 1x SDS loading buffer. The samples were boiled at 98°C for 5 min before being analysed by SDS-PAGE or stored at -80°C until required.

2.4 Cell culture

2.4.1 Reagents, stock solutions and buffers for use in tissue culture

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

HEPES-buffered saline: 50mM HEPES-KOH, 280mM NaCl, 1mM Na₂HPO₄, pH 7.1

DMEM complete: DMEM (Sigma, D6546), 10% fetal calf serum (FCS; Biosera S1900-500, lot-S0611951900), 100units/ml penicillin and 100units/ml streptomycin (PAA, P11-010), glutamine (200mM; Lonza, BE17-60SE)

Doxycycline (1000x): stock 1.0mg/ml in H₂O, working concentration 1.0µg/ml, stored at -80°C.

Blasticidin solution: stock 100mg/ml in H₂O (Invivo Gen), stored at -20°C.

Zeocin solution: stock 100mg/ml in H₂O (Invivo Gen), stored at -20°C.

Trypsin/EDTA solutions: 10x concentrated solutions of phosphate-buffered saline containing 0.5% trypsin and 0.2% EDTA were purchased from (PAA, L11-003) stored at -20°C. 1x solutions obtained by the addition of PBS were stored at 4°C

2.4.2 Maintenance of cell lines

Cell culture was performed in a Class II biological safety cabinet under aseptic conditions.

Cell Line	Origin
HEK293T	Human embryonic kidney
U2OS	Human osteosarcoma
MCF-7	Human mammary epithelium

Table 2.7 Mammalian cell lines used

2.4.3 Routine maintenance of cell lines

Adherent cells (Table 2.7) were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100units/ml penicillin and 100units/ml streptomycin (complete DMEM), in a humidified incubator at 37°C and 5% CO₂. When cells were 70-80% confluent, they were washed with phosphate buffered saline (PBS; pre-warmed at 37°C) and incubated with 1-2ml of trypsin/EDTA for 1-5 min (dependent upon cell type) at 37°C. Trypsin was deactivated by the addition of 10ml complete DMEM and the cells were resuspended into a single cell suspension. The cell suspension was centrifuged at 250g for 5 min to pellet the cells, the supernatant removed and then the pellet re-suspended in complete DMEM for dilution and plating.

2.4.4 Freezing cells for long term storage

To place cells into liquid nitrogen storage, cells were detached from a confluent 75cm² flask using trypsin transferred to a 15ml Falcon tube and centrifuged at 250g for 5 min. After centrifugation, the cell pellet was re-suspended in 2ml of complete DMEM containing 10% dimethylsulfoxide

(DMSO) and 1ml aliquots of the cell suspension were transferred into cryovials. The cryovials were then placed at -80°C in a Mr. Frosty cryocontainer (NALGENE Labware), which allows slow freezing of -1°C per minute. After one to several days, cryovials were placed in liquid nitrogen for long-term storage.

2.4.5 Retrieving cells from liquid nitrogen storage

To culture cells from liquid nitrogen storage they were first thawed quickly by incubation in a 37°C water bath, and then added to 9ml of complete DMEM medium (pre-warmed at 37°C). To allow for the removal of the DMSO, cells were pelleted by centrifugation at 250g for 5 min. The supernatant was discarded and the cell pellet re-suspended in 10ml of complete DMEM medium (pre-warmed at 37°C). The cell suspension was transferred to a 75 cm² flask and placed in the humidified incubator (5% CO₂ and 37°C). After 24 h, the medium was changed, and the cells were passaged between 48 and 96 h after retrieval from liquid nitrogen.

2.4.6 Generation of MCF-7 TR stable cells lines

To generate cell lines that can be induced to express (mutant) BTG2, we used MCF-7 TR cells, which express the Tet repressor protein following stable integration of plasmid pcDNA6/TR (Invitrogen).

MCF-7 TR cells were transfected with the appropriate vector (Table 2.8) using the calcium phosphate precipitation method. Cells were seeded at 60% confluency into 6 cm (diameter) culture dishes 24 h prior to transfection. Cells were washed with PBS before the addition of fresh DMEM medium. After the preparation of a 0.25M CaCl₂ (pH 7.7) solution (100µl) containing six micrograms of plasmid DNA, 100µl of HEPES-Buffered Saline was added with gentle agitation. The mixture was left at room temperature to precipitate for 20 min, before being added to the cells. After transfection

(24 h), cells were washed with PBS and fresh DMEM medium added. Forty eight hours after transfection the plates were split into five 10cm (diameter) plates in medium containing zeocin (600µg/ml) to select for transformants. Cells were first washed with PBS, then 1ml of trypsin was added and the plate and incubated for 5 min at 37°C. Once cells had detached from the plate, the cell suspension was aspirated several times to generate a single cell suspension, followed by centrifugation at 250g for 5 min. The supernatant was discarded and the pellet re-suspended in 10ml of DMEM containing zeocin (600µg/ml). To each culture plate (10cm diameter) containing 8ml of complete medium containing zeocin (600µg/ml), 2ml of cell suspension was added. Plates were kept in a humidified incubator (37°C, 5% CO₂) for approximately three weeks until the antibiotic selection was complete and single colonies had formed. During this time the cells were washed with PBS and the medium replaced every 3-4 days. All cell lines were derived from MCF-7 TR cells containing stably integrated plasmid pcDNA6/TR. All cells were routinely maintained in complete DMEM medium containing 10% tetracycline-free FCS, 5µg/ml blasticidin, and 300µg/ml zeocin.

Cell Line	Vector transfected
MCR7 TR pcDNA4-TO	pcDNA4-TO
MCF-7 TR BTG2	pcDNA4-TO-HA-Flag-BTG2
MCF-7 TR BTG2 53A	pcDNA4-TO-HA-Flag-BTG2 53A
MCF-7 TR BTG2 103A	pcDNA4-TO-HA-Flag-BTG2 103A
MCF-7 TR BTG2 105A	pcDNA4-TO-HA-Flag-BTG2 105A

Table 2.8 Stable cells lines containing a doxycycline-inducible expression cassette.

2.5 Immunoprecipitation analysis

2.5.1 Reagents, stock solutions and buffers for use in co-immunoprecipitation

Cell lysis buffer containing 0.2% NP-40: 50mM 1M Tris-HCl pH8.0, 150mM NaCl, 5mM MgCl₂, 0.5mM EDTA, 5% glycerol, 0.2% NP-40, 1mM DTT. After addition of one Roche complete protease inhibitor cocktail tablet to 50ml, the lysis buffer was stored at -20°C.

1M Dithiothreitol (DTT): dissolved in H₂O, stored at -20°C.

SDS loading buffer (4x): 50% upper buffer, 40% glycerol, 1% β-mercaptoethanol, 1.25% Bromophenolblue (0.1%), 0.25% H₂O, stored at -20°C. Small aliquots were stored short-term at 4°C.

Agarose beads: protein A-agarose beads and protein G-agarose beads were purchased from Roche and stored at 4°C.

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

2.5.2 Preparation of protein lysates for immunoprecipitation

HEK293T cells were seeded at 80% confluency in culture dishes (6cm diameter) and after 24 hours, transfected with the two appropriate expression vectors using the JetPEI transfection reagent. For each vector, 2.5μg plasmid (5μg total plasmid) was added to 10μl of JetPEI following instructions in the JetPEI handbook. The DNA/JetPEI mixture was incubated for 30 min before being added drop wise to the cells. Forty eight hours after transfection, cells were harvested by repeated pipetting of the medium directly onto the cells. The cell suspension was centrifuged at 4000g, 4°C for 5min and the pellet re-suspended in 1ml of cold PBS (4°C) to wash the cells. The centrifugation step was repeated and the cell pellet re-suspended in 500μl of cold cell lysis buffer containing 0.2% NP-40. The lysates were then

transferred to microfuge tubes and freeze/ thawed twice (-80°C), followed by centrifugation at 16,000g for 5 min at 4°C. Supernatants were stored at -80°C until required.

2.5.3 Preparation of antibody-agarose beads and immunoprecipitation

For each immunoprecipitation 10µl of protein A and 10µl of protein G agarose beads were required. Bead volumes were scaled up depending upon the number of samples being tested. To prepare agarose beads, 10µl of protein A and 10µl protein G agarose beads were mixed together in a 1.5ml microfuge tube and centrifuged at 3000g for 30 sec. The supernatant was discarded and the pellet resuspended in 500µl PBS. This wash step was repeated twice before the appropriate antibody was added to the beads (Table 2.9). One microgram of antibody was added per 10µl of beads (2µg per immunoprecipitate) and the volume brought up to 500µl with PBS. The 1.5ml microfuge tube containing the agarose bead/antibody mixture was packed into a 50ml falcon tube and left on a rotating shaker for 1 h at room temperature. Following antibody incubation the agarose beads/antibody mixture was centrifuged at 3000g for 30 sec and the supernatant removed carefully with a gel loading tip. The agarose beads were washed with 500µl of PBS and this step repeated three times followed by a final wash in cell lysis buffer containing 0.2% NP40. From this point on all the samples were kept on ice. To the antibody coated agarose beads in a 1.5ml microfuge tube, 450µl of protein lysate was added the microfuge tube packed into a 50ml falcon tube and the mixture incubated overnight at 4°C on a rotating shaker. The lysate/bead mixtures were centrifuged at 3000g for 30 sec and the supernatant carefully discarded using a gel loading tip. Beads were then washed three times with 500µl of lysis buffer containing 0.2% NP40. After the final wash, 10µl PBS and 10µl 4x SDS loading buffer was added to the bead and the sample was boiled for 5 min to denature the protein-antibody

complexes. The samples were then subjected to western blot analysis or stored at -80°C until needed.

2.5.4 List of antibodies used for co-immunoprecipitation

Primary Antibody	Dilution	Supplier
Anti-Flag M2	1µg per 10µl of agarose beads	Sigma (F1805)
Anti-HA	1µg per 10µl of agarose beads	Roche (1 867 423)

Table 2.9 List antibodies used for co-immunoprecipitation

2.6 EdU labelling of S-phase cells

To look at the effect of BTG/TOB proteins on cell proliferation, the Click-iT EdU Imaging kit (Invitrogen) was used to label cells in S-phase. All conditions were tested in biological triplicate.

2.6.1 Reagents, stock solutions and buffers for use in EdU labelling

Lysis buffer containing 1% NP40: 50mM 1M Tris-HCl pH8.0, 150mM NaCl, 5mM MgCl₂, 0.5mM EDTA, 5% glycerol, 1% NP-40, 1mM DTT. The lysis buffer was stored at -20°C after the addition of one Roche complete protease inhibitor cocktail tablet to 50ml buffer.

4% paraformaldehyde (PFA): 4% PFS in H₂O w/v, stored at -20°C.

0.5% Triton X-100: 0.5% Triton X-100 in PBS, stored at 4°C

3% Bovine serum albumin (BSA): 3% BSA in PBS, stored at 4°C

Mounting medium: 90% glycerol, 10% PBS

MG132 (1000x): 1mg/ml powder was dissolved in DMSO and stored at -80°C.

siRNA: siRNA's purchased from Dharmacon, stock at 20nmol in 1x siRNA buffer (Dharmacon, B-002000-UB-015), stored at -20°C.

DMEM complete: DMEM (Sigma, D6546), 10% fetal calf serum (FCS; Biosera S1900-500, lot-S0611951900), 100units/ml penicillin and 100units/ml streptomycin (PAA, P11-010), glutamine (200mM; Lonza, BE17-60SE)

Serum Free Media: DMEM, (Sigma, D6546)

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

2.6.2 Cell seeding and transient transfection for EdU labelling

Working in a class II biological safety cabinet, square glass cover slips (22mm x 22mm, 0.13-0.17mm thick) were sterilised by immersion in 100% ethanol before being passed through a flame. The cover slips were placed into 6-well culture plate. Cells were seeded (n=180,000 per well) in 2ml DMEM onto the coverslips. After 24 h, cells were at 70% confluency and either transfected using GeneJuice transfection reagent or BTG2 expression was induced by the addition of doxycycline (1µg/ml) to the culture medium. For transfected cells 1µg of plasmid DNA was diluted in 100µl of serum free media and mixed with 3µl of GeneJuice reagent as stated in the GeneJuice handbook. Genejuice/DNA mixture was incubated for 15 min before being added drop wise to the cells. One sample was transfected with a GFP containing plasmid to monitor transfection efficiency. Four hours after transfection, cell medium was removed and replaced with fresh medium to reduce cell toxicity. Forty eight hours after transfection cells were subjected to Click-iT EdU labelling. Each transfection was carried out in triplicate.

2.6.3 Cell seeding and siRNA transfection for EdU labelling

Working in a class II biological safety cabinet, square glass cover slips (22mm x 22mm, 0.13-0.17mm thick) were sterilised by immersion in 100% ethanol before being passed through a flame. The cover slips were placed into 6-well culture plate. Cells were seeded (n=100,000 per well) in 2ml DMEM onto the coverslips. After 24 h, cells were at 40% confluency and transfected using Interferin following the produce protocol. Briefly, 5nM of siRNA (Table 2.10) was added to 200µl of serum free media followed by 8µl of Interferin, the siRNA/Interferin mixture was vortexed and incubated for 10 min before being added drop wise to the cells. After 24 h the cell medium was removed and replaced with fresh 37°C complete DMEM, 72 h after siRNA knockdown Click-iT EdU labelling preformed. Each transfection was carried out in triplicate.

siRNA	Product code
siControl	On-TARGET Plus Control non-targeting pool siRNA D-001810-10-05
siBTG1	On-TARGET Plus Human BTG1 L-011598-00
siBTG2	On-TARGET Plus Human BTG2 L-012308-00
siTOB1	On-TARGET Plus Human TOB1 L-010560-00
siTOB2	On-TARGET Plus Human TOB2 L-019434-00

Table 2.10 siRNAs used for S phase analysis. All siRNA's purchased from Dharmacon and stored 20nmol

2.6.4 Click-iT EdU labelling

Forty eight hours after transfection or induction of BTG2 expression, 1ml of medium per well was removed and replaced with 1ml of fresh medium containing 20 μ M EdU to give a final working concentration of 10 μ M EdU per well. The GFP plasmid transfected cells were not incubated with EdU and stored at 4°C until the cell fixing step. MCF-7 cells were incubated with EdU for 2h, U2OS cells for 30 min in a humidified incubator at 37°C and 5% CO₂. After labelling, medium containing EdU was removed and the cover slips were washed twice with 1ml of PBS. Cells were fixed though a 15 min incubation with 1ml of 4% PFA and washed twice with 3% BSA in PBS. Cover slips were then incubated with 1ml of ice cold 0.5% Triton X100 (diluted in PBS) for 20 min to permeabilise the cells before being washed twice with 1ml of 3% BSA in PBS. The Click-iT reaction cocktail was prepared as stated in the Click-iT handbook but all component volumes were reduced by a factor of ten to allow 50 μ l of cocktail per coverslip. When making the Click-iT reaction cocktail master mix, 50 μ l extra mix was made to allow for pipetting error. Fifty microlitre of Click-iT reaction cocktail was spotted onto parafilm in a humidification chamber and the coverslips placed face down onto the solution. This was incubated for 30 min at room temperature in the dark. Coverslips were then returned to a 6-well culture plate and washed twice with 1ml of 3% BSA in PBS followed by a wash with 1ml of PBS. To stain (nuclear) DNA, the PBS was removed and 1ml of Hoechst stain (5 μ g/ml) was added to each coverslip and incubated in the dark at room temperature for 10 min. From this point onwards the coverslips were shielded from bright light as much as possible. After removal of the Hoechst stain, the coverslips were washed twice with 1ml of PBS. To mount the coverslips onto standard glass microscope slides, 50 μ l of mounting medium was spotted onto the slide before the coverslip was lowered onto it face down. The coverslips were sealed with clear nail varnish and slides were kept in the dark until imaging.

2.6.5 Calculating the number of cells in S-phase

All samples were tested in biological triplicate and experiments repeated at least twice. For each coverslip, the numbers of cells in S-phase were counted at three randomly selected areas on the coverslip using a Zeiss LSM 510 Meta microscope and argon laser for excitation at 488 nm. Following the S-phase nuclei count, the total numbers of nuclei were counted for each area using the mercury bulb detecting Hoechst stained nuclei. On average 300 nuclei were counted for each coverslip. The number of nuclei in S-phase and the total number of nuclei were calculated for each coverslip. For each sample, the average number of cells in S-phase and the standard error of the mean were calculated from the biological triplicates. The GFP plasmid transfect samples were counted via the same method to monitor transfection efficiency, which typically varied between 50-70%.

2.6.6 Western blot analysis for Click-iT EdU labelling

To confirm expression of BTG2 and TOB1, cells were seeded as stated previously for the Click-iT EdU protocol. For the BTG2 stable cell lines, 48 h after doxycycline treatment, the cell medium was removed and cells washed with 1ml PBS. Cells were then scraped using a 1ml tip, collected in 500µl cold (4°C) PBS and transferred to a pre-chilled 1.5ml microfuge tube. Cell suspensions were centrifuged at 16,000g for 3 min at 4°C. Cell pellets were re-suspended in 100µl of 1% NP-40 lysis buffer. Cell lysates were freeze/thawed (-80°C) before a second centrifugation at 16,000g for 5 min at 4°C. Cell lysates were then subjected to western blot analysis.

For transiently transfected cells 48 h after transfection 1ml of cell media was removed and replaced with DMEM containing the protease inhibitor MG132. Four hours after addition of MG132 protein lysates were produced as described for BTG2 stable cell lines. Cell pellets were re-suspended in 50µl of 1% NP-40 lysis buffer.

2.7 Immunofluorescence microscopy

2.7.1 Reagents, stock solutions and buffers for use in immunofluorescence

4% paraformaldehyde (PFA): 4% PFS in H₂O w/v, stored at -20°C.

0.5% Triton X-100: 0.5% Triton X-100 in PBS, stored at 4°C

3% Bovine serum albumin (BSA): 3% BSA in PBS, stored at 4°C

10% Bovine serum albumin (BSA): 10% BSA in PBS, stored at 4°C

Mounting medium: 90% glycerol, 10% PBS

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

2.7.1 Preparation of mammalian cells for immunofluorescence

Working in a class II biological safety cabinet, square glass cover slips (22mm x 22mm, 0.13-0.17mm thick) were sterilised by immersing in 100% ethanol before being passed through a flame. The cover slips were placed into 6-well culture plates. MCF-7 TR BTG2 cells were seeded (n=180,000 cells per well) in 2ml of DMEM onto the coverslips. After 24 h cells, were at 70% confluency and BTG2 expression was induced by the addition of doxycycline (1µg/ml) to the medium. Forty eight hours after the addition of doxycycline, medium was removed and the cover slips washed twice with 1ml PBS. To fix the cells 1ml 4% paraformaldehyde (4% PFA) was added to the coverslips and incubated at room temperature for 15 min followed by two washes with 1ml of PBS. After fixing, coverslips were stored at 4°C for 1-4 days or used immediately for permeabilisation and antibody incubation.

2.7.2 Immunofluorescence microscopy

Cover slips were incubated with ice cold 0.5% Triton X-100 for 20 min to permeabilise the cells and washed twice with 1ml of PBS. Cells were then treated by the addition of 1ml of 10% bovine serum albumin (BSA) in PBS and incubated for 1 h at 37°C. Cover slips were then incubated with primary antibody at the appropriate dilution (Table 2.11). To this end, 50µl of antibody solution was spotted onto parafilm in a humidified chamber and the coverslips placed face down onto the solution. Primary antibodies were diluted in 3% BSA in PBS and incubated for 1 h at 37°C. After primary antibody incubation coverslips were transferred back to the 6-well culture plate and washed three times 5 min with 1ml PBS on an orbital shaker at 60rpm. Coverslips were then incubated with 50µl of secondary antibody as described previously in the humidified chamber. Secondary antibodies were diluted in 3% BSA in PBS and incubated for 1 h at 37°C before coverslips were transferred back to the 6-well culture plate and washed three times for 5 min with 1ml PBS on an orbital shaker at 60rpm. To stain DNA, 1ml of Hoechst stain at 5µg/ml was added to each coverslip and incubated in the dark at room temperature for 10 min. From this point on coverslips were shielded from bright light as much as possible. After removal of the Hoechst stain, the coverslips were washed twice with 1ml PBS. Cover slips were then mounted onto standard glass microscope slides: 50µl of mounting medium was spotted onto the slide before the coverslip was lowered onto it face down. The coverslips were sealed with clear nail varnish and slides were kept in the dark until imaging. Slides were imaged using a Zeiss LSM 510 Meta microscope and HeNe laser for excitation at 594 nm. For each slide, two representative Z-stack images were taken.

2.7.3 List of antibodies used for immunofluorescence

Primary Antibodies	Dilution ^a	Supplier
Anti-BTG2	1/500	Sigma (AV33561)
Anti-Flag	1/500	Sigma (F1805)
Anti-HA	1/500	Roche (1 867 423)
Secondary Antibodies	Dilution ^a	Supplier
Alexa Fluor 488		
Anti-Mouse	1/1000	Invitrogen (A414666)
Anti-Rat	1/1000	Invitrogen (A21212)
Alexa Fluor 594		
Anti-Mouse	1/1000	Invitrogen (A21203)
Anti-Rabbit	1/1000	Invitrogen (A408192)

Table 2.11 Antibodies used for immunofluorescence. ^aAntibodies were diluted in PBS containing 3% BSA.

2.8 Analysis of cell cycle regulators

To determine if the expression of cell cycle regulatory proteins was changed due to BTG2 expression, relative protein and mRNA levels were assessed using western blot and qPCR analysis.

2.8.1 Reagents, stock solutions and buffers used in analysis of cell cycle regulators

Cell lysis buffer containing 1% NP-40: 50mM 1M Tris-HCl pH8.0, 150mM NaCl, 5mM MgCl₂, 0.5mM EDTA, 5% glycerol, 1% NP-40, 1mM DTT. After addition of one Roche complete protease inhibitor cocktail tablet to 50ml, the lysis buffer was stored at -20°C.

1M Dithiothreitol (DTT): dissolved in H₂O, stored at -20°C.

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

TE Buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA

2.8.2 Cell seeding, transfection and protein extraction for the analysis of cell cycle regulatory protein expression.

To assess change in protein levels of cell cycle regulatory proteins, MCF-7 TR BTG2 stable cell lines were used. Working in a class II biological safety cabinet, MCF-7 TR BTG2 stable cell lines were seeded into 6-well culture plates at 200,000 cells per well. Cell medium was replaced three days after seeding with fresh medium containing doxycycline (1.0µg/ml). Seven days after seeding, cells were washed with 1ml PBS and harvested by scraping the cells in 500µl of PBS. After transfer microfuge tubes, the cell suspension was centrifuged at 16,000g, 4°C for 5 min and the pellet re-suspended in 100µl of cell lysis buffer containing 1% NP-40. The lysate was freeze/thawed once

(-80°C), followed by centrifugation at 16,000g for 5 min at 4°C. Samples were subjected to western blot analysis or stored at -80°C until required.

2.8.3 Cell seeding, transfection and RNA extraction for the analysis of cell cycle regulatory protein expression.

For assessing changes in mRNA levels, MCF-7 cells or MCF-7 TR BTG2 stable cell lines were seeded into 6-well culture plates at 180,000 cells per well. After 24 h, cells were at 70% confluency and either transfected using GeneJuice (following GeneJuice manual) or BTG2 expression induced via the addition of doxycycline (10µg/ml) to the cell medium. For transient transfection the cell medium was preplaced with fresh medium four hours after transfection to reduced cell toxicity.

Forty eight hours after transfection total RNA was harvested using the E.N.Z.A Total RNA extraction kit (OMEGA Bio Tek, VWR). Cells were lysed with 350µl of TRK lysis buffer directly in the 6-well culture plate. Gentle pipetting of the cell lysate helped to disrupt all the cells and this was then transferred to a 1.5ml microfuge tube. The RNA extraction was then conducted as stated in the kit protocol, RNA is purified on a column and eluted in 50µl of DECP-treated water. RNA samples were kept on ice at all times and stored at -80.

2.8.4 Production of cDNA for qPCR analysis to determine the levels of mRNA for cell cycle regulatory proteins

Quantitative PCR (qPCR) was conducted to investigate the changes in mRNA levels of target genes. SuperScript III (Invitrogen) was used for cDNA synthesis as detailed in Table 2.12. Reactions were incubated in a peqlab Primus 96 advanced thermal cycler at 70°C for 5 min before being transferred onto ice for a further 1 min.

To this sample (reaction mix 1), a second master mix was added containing the reverse transcriptase and its corresponding buffer as shown in Table 2.13. This was again placed in a peqlab Primus 96 advanced thermal cycler and incubated at 55°C for 60 min, followed by incubation at 70°C for 15 min to deactivate the enzyme. Resulting cDNA samples were diluted 1:5 with TE prior to use, and stored at -20°C until required. The qPCR analysis was conducted as described in section 2.10.2

Volume	Component/Reagent
x µl	150-300ng RNA template
0.4 µl	primer VN(dT) ₂₀
0.5µl	10mM dNTPs
up to 7.0µl	RNase-free H ₂ O

Table 2.12 Superscript cDNA synthesis reaction mix 1

Volume	Component/Reagent
7.0µl	Reaction mix 1
2.0 µl	First Strand buffer
0.5µl	SuperScript III
0.5µl	0.1M DTT

Table 2.13 Superscript cDNA synthesis reaction mix 2

2.9 RNA tethering assay

2.9.1 Reagents, stock solutions and buffers for use in RNA tethering assays

TE Buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA

DMEM complete: DMEM (Sigma, D6546), 10% fetal calf serum (FCS; Biosera S1900-500, lot-S0611951900), 100units/ml penicillin and 100units/ml streptomycin (PAA, P11-010), glutamine (200mM; Lonza, BE17-60SE)

Serum free media: DMEM (Sigma, D6546)

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (1x PBS without Ca²⁺, Mg²⁺; PAA, H15-002)

siRNA: siRNA's purchased from Dharmacon, stock at 20nmol in 1x siRNA buffer (Dharmacon, B-002000-UB-015), stored at -20°C.

2.9.2 Cell seeding and transfection for RNA tethering assay

Working in a class II biological safety cabinet, MCF-7 cells were seeded into 12-well culture plates at 80,000 cells per well. After 24 hours, cells were at 70% confluency and co-transfected using JetPEI transfection reagent. For each transfection 1µg of pRL-5boxB, 1µg of pCIλN plasmid and 4µl of JetPEI reagent was used (following instructions in the JetPEI manual). The DNA/JetPEI mixture was incubated for 30 min before being added drop wise to the cells. Four hours after transfection, cell medium was removed and replaced with fresh medium to reduce cell toxicity caused by JetPEI. Twenty four hours after transfection, cells were harvested for the luciferase assay.

2.9.3 Cell seeding and transfection for combined siRNA knockdown/RNA tethering assay

Working in a class II biological safety cabinet, MCF-7 cells were seeded into 12-well culture plates at 40,000 cells per well. After 24 hours, cells were at 40% confluency and subjected to siRNA transfection using Interferin to knockdown desired genes. A total of 10nM siRNA (Table 2.14) was added to each well (5nM of siRNA1 and 5nM of siRNA2) in a total volume of 1ml DMEM. Twenty four hours after siRNA transfection, medium was removed and replaced with fresh medium. Forty eight hours after siRNA transfection, cells were transfected using GeneJuice transfection reagent. For each transfection 0.25µg of pRL-5boxB, 0.5µg of pCIλN plasmid and 3µl of Genejuice reagent was used (following instructions in the GeneJuice manual). The DNA/Genejuice mixture was incubated for 15 min before being added drop wise to the cells. Four hours after transfection, medium was replaced with fresh medium to reduce cell toxicity caused by the transfection reagent. Twenty four hours after transfection cells were harvested for the luciferase assay. Each transfection was carried out in triplicate.

siRNA	Product code
siControl	siGENOME Control siRNA, none targeting siRNA #1 D-001210-01-20
siCNOT1 D1	siGENOME duplex KIAA1007 D-015369-01
siCNOT1 D2	siGENOME duplex KIAA1007 D-015369-02
siCNOT3 D1	siGENOME duplex CNOT3 D-020319-01
siCNOT3 D2	siGENOME duplex CNOT3 D-020319-02
siCNOT7 D1	siGENOME duplex CNOT7 D-012897-01
siCNOT8 D2	siGENOME duplex CNOT8 D-018791-02
siCNOT6 (new)	siGENOME siRNA Human KIAA1194 D-019101-01
siCNOT6L (new)	siGENOME siRNA Human CNOT6L D-016411-02

Table 2.14 siRNAs used in RNA tethering assay. All siRNA's purchased from Dharmacon and stored 20nmol

2.9.4 RNA tethering reporter assay

Twenty four hours after transfection, cells were harvested for the luciferase assay. First, cells were washed with 1ml PBS and then 100µl of 1x cell lysis buffer (Luciferase cell lysis buffer, NEB B3321S) was added directly to the cells in the 12-well culture plate. The plates were then placed at -80°C for 15 min until completely frozen. Samples were then thawed and mixed by pipetting to aid the lysis process before transfer into 1.5ml microfuge tubes. Samples were then centrifuged at 16,000g for 5 min at 4°C and stored at -80°C or used immediately.

The Gluc assay solution (BioLux Gaussia Luciferase Assay Kit, NEB E3300S/L) was prepared according to the manufacture's instructions. The BioLux GLuc substrate was diluted (1:100) with BioLux GLuc assay buffer and mixed by gentle inverting. In a white opaque flat bottom 96 well plate, 10µl of cell lysate was added and the plate was placed into a Berthold Orion microplate luminometer. After injection of 50µl of GLuc assay solution, chemiluminescence was measured for 2 sec after a 5 sec delay. Each sample was tested in duplicate

A Bradford assay was conducted for all samples (section 2.11.2) to determine total protein concentrations, which was used to normalise the luciferase activity.

2.9.5 Cell seeding and transfection for mRNA analysis

Working in a class II biological safety cabinet, MCF-7 cells were seeded into 6-well culture plates at 180,000 cells per well. After 24 hours, cells were at 70% confluency and subjected to DNA transfection using JetPEI transfection reagent. For each transfection 1.5µg of pRL-5boxB, 1.5µg of pCIλN plasmid and 4µl of JetPEI reagent was used (following instructions in the JetPEI manual). The DNA/JetPEI mixture was incubated for 30 min before being added drop wise to the cells. Four hours after transfection, medium was

replaced to reduce cell toxicity. Forty eight hours after transfection total RNA was extracted and used for cDNA production and qPCR analysis.

2.9.6 Preparation of RNA

Total RNA was extracted using the RNeasy Plus mini kit (Qiagen, cat no. 74134) including treatment with RNase-free DNase (Qiagen, cat no. 79254). Forty eight hours after transfection; cells were lysed by addition of 350µl RLT lysis buffer directly in the well. Gentle mixing of the cell lysate by pipetting helped to disrupt the cells. After transfer of the lysate to a 1.5ml microfuge tube, RNA extraction was conducted as stated in the manufacturer's protocol. The RNase-free DNase was added directly to the column and incubated at room temperature for 15 min before RNA elution using 50µl of DECP-treated water. RNA samples were stored at -80.

2.9.7 Production of cDNA

For the production of cDNA free from plasmid DNA contamination the QuantiTect Reverse Transcriptase was used (Qiagen, cat no. 205310). A reaction mix was set up as shown in Table 2.15 and incubated in a peqlab Primus 96 advanced thermal cycler at 42°C for 2 min before being transferred onto ice for a further 1 min.

To this sample (reaction mix 1) a second master mix was added containing the reverse transcriptase and its corresponding buffer as shown in Table 2.16. This mixture was placed in a peqlab Primus 96 advanced thermal cycler and incubated at 42°C for 15 min, followed by incubation at 95°C for 3 min to deactivate the enzyme. Resulting cDNA samples were diluted 1:5 with TE prior to use, and stored at -20°C until required.

Volume	Component/Reagent
x µl	300ng RNA template
1.0µl	gDNA wipeout buffer x7
up to 7.0µl	RNase-free H ₂ O

Table 2.15 RNA tethering assay: cDNA synthesis reaction mix 1

Volume	Component/Reagent
7µl	Reaction mix 1
2.0µl	Reverse transcriptase buffer x5
0.5µl	Reverse transcriptase
0.5µl	RT primer mix

Table 2.16 RNA tethering assay: cDNA synthesis reaction mix 2

2.10 RNA analysis

2.10.1 Reagents, buffers and stock solutions for use in RNA analysis

TE: *TE Buffer*: 10mM Tris-HCl pH 8.0, 1mM EDTA

Oligonucleotides: Lyophilised primers were dissolved in TE to give a 100mM solution and stored at -20°C.

2.10.2 Quantitative PCR analysis

All qPCR reactions were performed using Brilliant II SYBR Green QPCR master mix (Stratagene), or Sensi mix SYBR LoX-ROX (Bioline) all samples were tested in technical triplicate using primers specific for the gene being tested and separately with primers for a housekeeping gene (either ACTB, GAPDH or 36B4), which were used for normalisation. A master mix was produced containing the appropriate primers (Table 2.18) and the Brilliant II SYBR Green master mix (Table 2.17).

Volume	Component/Reagent
1.0µl	cDNA template
5.0µl	Brilliant II SYBR Green QPCR or Sensi mix SYBR LoX-ROX master
0.05µl	Oligo 1 (10mM)
0.05µl	Oligo 2 (10mM)
3.9µl	RNase free H ₂ O

Table 2.17 qPCR reaction set-up

The qPCR master mix was pipetted into the appropriate wells of a 96-well plate before the cDNA was added. The 96-well plate was placed into the Stratagene Mx3005P qPCR machine and subjected to the following program, 10 min 95°C, 40 cycles at 15 sec 95°C, 60 sec 60°C, followed by melt curve analysis of one cycle of 1 min 95°C, 30 sec 55°C, 30 sec 95°C.

In order to calculate the initial template quantity of the mRNA being tested the samples were compared against reference cDNA produced from MCF-7 cells. Reference cDNA was produced using the standard E.N.Z.A RNA extraction and cDNA production methods (section 2.8.2, 2.8.3) from an untreated population of cells. To make reference cDNA the standard cDNA production protocol was followed but 1µg of RNA was used. The reference cDNA was serially diluted 1:10 to produce a standard curve with 5 points from 10^0 to 10^{-4} . These samples were run on the qPCR in duplicate along with an RNase free H₂O control sample. The H₂O control sample was used to confirm that there is no RNA, DNA or cDNA contamination in the reaction mix or primers. The duplicate standard samples were averaged before plotting as a standard curve using the MxPro RT-PCR software. This standard curve was then used to identify initial cDNA copy numbers in the samples being tested.

2.10.3 List of primers used for qPCR

Name	Sequence
RT BTG1 [1] Fw	TGCAGACCTTCAGCCAGAG
RT BTG1 [1] Rv	ATCCCTTGCATGGCTTTTC
RT BTG2 [1] Fw	GCGAGCAGAGGCTTAAGGT
RT BTG2 [1] Rv	GGGAAACCAGTGGTGTTTGTA
RT BTG3 Fw	GAAACCCAGTTCGGTGACTG
RT BTG3 Rv	GCCATTCCCTCGATACATTC
RT BTG4 Fw	GCCTCATCAGACGTTTCCTC
RT BTG4 Rv	AAGGGCTGTTTCAAGTTTTCAA
CCND1 Fw	TGTCCTACTACCGCCTCACA
CCND1 Rv	AGGGCTTCGATCTGCTC
p21 Fw	TCACTGTCTTGTACCCTTGTG
p21 Rv	GGCGTTTGGAGTGGTAGAAA
p27 Fw	CTGCAACCGACCATTCTTCTACT
p27 Rv	CTTCTTGGGCGTCTGCTC
RT TOB1 [1] Fw	TCTGTATGGGCTTGGCTTG
RT TOB1 [1] Rv	TGTTGCTGCTGTGGTGGT
RT Tob2 Fw	AGTCCCCACTCCCTTTCAG
RT Tob2 Rv	GACGAAGTGCTAATCTCCCTGTA

RT hRenLucif [1] Fw	ATGGGTAAGTCCGGCAAGA
RT hRenLucif [1] Rv	CCAAGCGGTGAGGTACTTGT
RT GAPDH Fw	TCTGGTAAAGTGGATATTCTTG
RT GAPDH Rv	CATGGTGATGGGATTTCC
RT Actin Fw	AGAAAATCTGGCACCACACC
RT Actin Rv	AGAGGCGTACAGGGATAGCA
RT 36B4 Fw	GTGTTGACAATGGCAGCAT
RT 35B4 Rv	GACACCCTCCAGGAAGCGA

Table 2.18 Primer used for quantitative PCR. Primers were designed spanning exon-exon junctions (where possible) using the Roche Universal ProbeLibrary Assay Design Center.

2.11 Protein analysis

2.11.1 Reagents, stock solutions and buffers for use in protein analysis

Upper buffer (4x): 0.5M Tris base, 0.4% SDS, pH6.8; stored at room temperature

Lower Buffer (4x): 1.5M Tris base, 0.4% SDS, pH8.8; stored at room temperature

Running buffer (10x): 0.25M Tris Base, 1.0% SDS, 1.92M glycine; stored at room temperature

Transfer buffer (10x): 0.25M Tris Base, 1.92M glycine; stored at room temperature

SDS loading buffer (4x): = 50% upper buffer, 40% glycerol, 1% β -mercaptoethanol, 1.25% Bromophenolblue (0.1%), 0.25% H₂O; stored stock at -20°C. Small aliquots were stored short-term at 4°C

Tris buffer saline supplemented with 0.05% Tween-20 (TBST): = 50mM Tris-HCl pH 7.8, 150mM NaCl, 0.1% Tween-20. Stored at room temperature.

10% APS: 10% ammonium persulphate (APS) in H₂O

2.11.2 Bradford assay to determine protein concentration

A Bradford assay was used to determine protein concentration of cell lysates. The Bio-Rad protein reagent was allowed to warm up to room temperature and diluted 1:5 with H₂O before use. A protein standard curve was produced using BSA dissolved in water. Standards at 0.1mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml and 1.0mg/ml were stored at -20°C. Ten microlitres of protein sample was added to 990 μ l of 1x Bradford reagent, vortexed and incubated for 1 min at room temperature. Samples were transferred to 1ml cuvettes and the absorbance read at OD₆₀₀. The standard curve was obtained

by linear regression analysis using Microsoft Excel and used to calculate the protein concentration of the samples.

2.11.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The Invitrogen X-Cell SureLock Mini Cell system was used for SDS-PAGE analysis. Gels were prepared as shown in Table 2.19 based on the molecular mass of the protein of interest. Separating gels were cast in gel cassettes (Invitrogen) leaving ~1.5cm of room at the top for the stacking gel to be cast. The separating gel was cast first, 500µl of isopropanol carefully added on top and allowed to set for 30 min. The isopropanol was removed by inverting the gel and draining onto tissue paper before the stacking gel was applied and the appropriate comb inserted. The gels left to set for a further 30 min before use or stored for several days at 4°C. Before use the comb was removed and the wells washed with 1x running buffer. Protein extracts were denatured by boiling for 5 min in 1x SDS sample buffer immediately prior to loading. Gels were run in 1x running buffer at 180V for approximately 1-1.5 h.

2.11.4 Western blotting and immunodetection

Proteins separated on SDS-PAGE gels for western blotting were transferred to nitrocellulose (Whatman Protran 0.45µm) membranes using the Invitrogen X-Cell SureLock Mini Cell system for 1 h at 25V in transfer buffer. The membrane was then blocked in TBST containing 5% dried milk for 1 h at room temperature while mixing on a platform shaker. Subsequently, the membrane was transferred to a 50ml Falcon tube containing 5ml of the appropriate primary antibody solution (for working concentrations and diluents see Table 2.20) and incubated overnight at 4°C on a rotating shaker. Membranes were then washed three times with TBST for 5 min and then incubated with the corresponding secondary antibody (Table 2.21) for 1 h at

room temperature on a platform shaker at 60rpm. Another 3 x 5 min TBST wash cycle was applied before immunodetection. This involved incubation of the membrane with 500 μ l of ECL western blotting substrate (Pierce) for 2 min prior to imaging using a Fujifilm LAS-4000 system and image reader computer software (Fujifilm, Japan).

	Resolving Gel			Stacking Gel
	8%	10%	12%	4%
Molecular mass resolved	>80kDa	80-30kDa	<30kDa	
40% acrylamide: bisacrylamide (29:1)	1.6ml	2.0ml	2.4ml	300 μ l
4x Lower buffer	2.0ml	2.0ml	2.0ml	-
4x Upper Buffer	-	-	-	750 μ l
H ₂ O	4.4ml	4.0ml	3.6ml	1950 μ l
10% APS	80 μ l	80 μ l	80 μ l	60 μ l
TEMED	8 μ l	8 μ l	8 μ l	6 μ l

Table 2.19 Preparation of SDS PAGE gels

2.11.5 List of western blot antibodies

Primary Antibodies	Dilution	Supplier
Anti-Actin	1/1000 ^a	Sigma (A5441)
Anti-BTG2	1/1000 ^a	GeneTex (GTX1082795)
Anti-BTG2	1/1000 ^a	Sigma (AV33561)
Anti-BTG2	1/1000 ^a	Santa Cruz (sc-33775)
Anti-CDK4	1/1000 ^a	Cell Signalling (#2906)
Anti-CDK6	1/1000 ^a	Cell Signalling (#3136)
Anti-CNOT1	1/1000 ^a	Proteintech (14276-1AP)
Anti-CNOT3	1/1000 ^a	Abnova (4B8)
Anti-CNOT7	1/500 ^a	Eurogentec ^d
Anti-CNOT7 + CNOT8	1/500 ^a	Eurogentec ^d
Anti-CNOT8	1/500 ^a	Eurogentec ^d
Anti-CNOT6	1/500 ^a	Eurogentec ^e
Anti-CNOT6L	1/500 ^a	Eurogentec ^e
Anti-CNOT6 + CNOT6L	1/500 ^a	Eurogentec ^e
Anti-CyclinD1 (DCS6)	1/1000 ^a	Cell Signalling (#2926)
Anti-CyclinD3	1/1000 ^a	Cell Signalling (#2936)
Anti-DDK	1/1000 ^a	OriGene (TA100011)
Anti-Flag M2	1/1000 ^a	Sigma (F1805)
Anti-Gal4 DBD	1/1000 ^a	Santa Cruz (sc-510)
Anti-Gal4 TA	1/1000 ^a	Santa Cruz (sc-1663)
Anti-HA	1/1000 ^c	Roche (1 867 423)
Anti K-Alpha tubulin	1/5000 ^c	Sigma (WH0010376M1)
Anti-p15 INK4B	1/1000 ^b	Cell Signalling (#4822)
Anti-p16 INK4A	1/1000 ^b	Cell Signalling (#4824)
Anti-p21 Waf1	1/1000 ^a	Cell Signalling (#2946)
Anti-p27 Kip1	1/1000 ^a	Cell Signalling (#2552)
Anti-Tob1	1/1000 ^a	Santa Cruz (sc-33192)
Anti-V5 probe	1/1000 ^a	Santa Cruz (sc-81594)
Anti-γtubulin	1/1000 ^a	Santa Cruz (sc-7396)

Table 2.20 Primary antibodies used for western blotting. ^aAntibodies were diluted in TBST containing 5% dried milk powder. ^bAntibodies were diluted in TBST containing 5% BSA. ^cAntibodies were diluted in TBST. ^dAntibodies were generated through a custom order (Eurogentec project SY4043). ^eAntibodies were generated through a custom order (Eurogentec project SY4049).

Secondary Antibodies	Dilution	Supplier
Anti-Mouse HRP	1/1000	Santa Cruz (sc-2314)
Anti-Rat HRP	1/1000	Santa Cruz (sc-2006)
Anti-Rabbit HRP	1/1000	Santa Cruz (sc-2004)
Anti-Goat HRP	1/1000	Santa Cruz (sc-2020)

Table 2.21 Secondary antibodies used for western blotting. Antibodies were diluted in TBST containing 5% dried milk powder.

Chapter 3

Identification of amino acids mediating the interaction between BTG/TOB proteins and the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8)

Chapter 3: Identification of amino acids mediating the interaction between BTG/TOB proteins and the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8)

3.1 Introduction

The BTG/TOB family of proteins contains six members and all (apart from BTG4) have been shown to interact with Caf1a (CNOT7) and Caf1b (CNOT8), two deadenylase enzymes that form part of the Ccr4-Not complex. The interaction has been mapped to the structurally conserved BTG domain as illustrated by the crystal structure of TOB1 in complex with Caf1a (CNOT7) (Horiuchi et al., 2009). The crystal structure of BTG2 demonstrated the conserved structure of the BTG domain between BTG2 and TOB1 (Yang et al., 2008). During the course of this work a number of amino acid residues in BTG2 have been recognised as essential for the BTG2-Caf1a (CNOT7) interaction. These amino acids, Gly-64, Tyr-65, Trp-103 and Asp-105 are situated within the conserved Box A and Box B regions of the BTG domain (Yang et al., 2008). However, no specific amino acid residues in TOB1 have been identified as required for its interaction with Caf1a (CNOT7). Due to the level of sequence conservation between BTG2 and TOB1 it can be hypothesised that the equivalent residues in TOB1 may play a similar role in conferring the Caf1a (CNOT7) interaction. Conversely, amino acids located in Caf1a (CNOT7) have been identified that are required for its interaction with BTG2 and TOB1. Replacement of Lys-203 for alanine in Caf1a (CNOT7) disrupts the Caf1a (CNOT7)-TOB1 interaction (Horiuchi et al., 2009). This substitution possibly prevents an interaction between two amino acids in close proximity, Lys-203 of Caf1a (CNOT7) and Trp-93 of TOB1. Furthermore, two additional amino acids in Caf1a (CNOT7) have been identified as required for its interaction with BTG2, namely Glu-247 and Tyr-260 (Aslam et al., 2009). All of which can be used as tools to discover specific amino acid in BTG2 and TOB1 that are required for the Caf1a (CNOT7) interaction.

3.2 Identification of candidate residues of BTG2 and TOB1 required for the interaction with Caf1a (CNOT7) and Caf1b (CNOT8)

To identify potential amino acids required for the interaction between BTG2 and Caf1a (CNOT7), the protein sequences of the six BTG/TOB members were aligned using Clustalw2 (Figure 3.1). The alignment was used to identify conserved residues across the protein family likely to be required for the interaction with Caf1a (CNOT7) and Caf1b (CNOT8). The BTG domain contains two highly conserved regions known as Box A consisting of a β -strand (β 1), a short α helix (α 3) and a section of the α 2 helix and Box B which is formed from two anti-parallel β -strands (β 2 and β 3).

To further analyse the potential role of the Box A and Box B amino acids in mediating the Caf1a (CNOT7)/Caf1b (CNOT8) interaction, the orientation of several selected amino acids of BTG2 and TOB1 was analysed using the published crystal structure of the BTG domain of TOB1 in complex with Caf1a (CNOT7) (PDB accession number 2D5R). Amino acid side chains that protrude towards Caf1a (CNOT7) were inspected for interactions with the deadenylase and evaluated as potential candidates for experimental validation (Figure 3.2A). The structure of TOB1 N138 (the N-terminal 138 residues of TOB1 comprising the BTG domain) interacting with Caf1a (CNOT7) confirms that TOB1 interacts via its BTG domain utilising its Box A and Box B domains. In addition, by aligning the crystal structure of BTG2 (PDB accession number 3DJU) with that of TOB1 N138-Caf1a (CNOT7), a model was produced of the BTG2-Caf1a (CNOT7) interaction. The structural alignments and analysis was performed using PyMOL molecular modelling software. Initially, charged amino acids with complex side chains, which may be involved in ionic or hydrogen bonding, were focused upon. In addition to this PDBePISA (Protein Interface Surface Assemblies) analysis was conducted on the TOB1-Caf1a (CNOT7) crystal structure (PDB accession number 2D5R) to characterise the interactions between amino acids in TOB1 and Caf1a (CNOT7). The analysis

highlighted accessible and interfacing amino acids and residues likely to cause hydrogen bonding or disulphide bridges. During the course of this work additional amino acids were identified as required for the interaction between BTG2 and Caf1a (CNOT7), namely, Tyr-65, Trp-103, Asp-105 and Gly-64 (Yang et al., 2008). From the combined analysis several amino acids were identified for experimental validation (Table 3.1).

Amino acid in BTG2	Conserved position in TOB1	Location in BTG domain
His-53	His-43	Box A
Tyr-65	Phe-55	Box A
Asp-75	Asp-65	Between Box A and Box B
Tyr-103	Tyr-93	Box B
Asp-105	Asp-95	Box B
Glu-108	Glu-98	Box B
Glu-115	Glu-105	Box B

Table 3.1 List of selected amino acids with a potential role in mediating the interaction with Caf1a (CNOT7)/Caf1b (CNOT8)

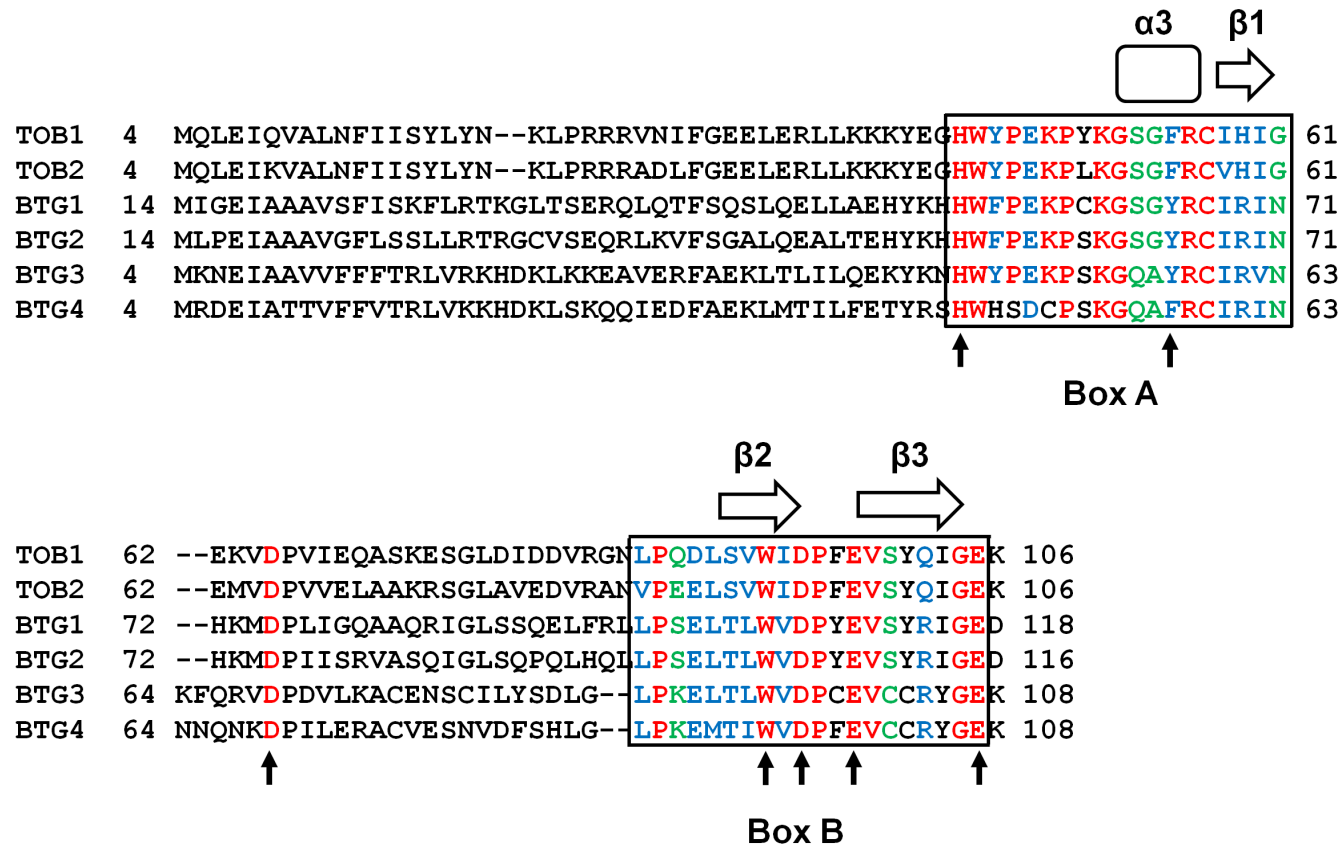


Figure 3.1 Sequence alignment of the human BTG/TOB proteins. The alignment of the conserved BTG domain was made using Clustalw2. Highlighted are the highly conserved regions Box A and Box B and their secondary structure (Yang et al 2008). In red are conserved amino acid residues, blue are highly similar and green are moderately similar residues. Conserved amino acids outside the Box A and Box B regions are not highlighted. The arrows indicate the amino acids substituted for alanine to create interaction mutants.

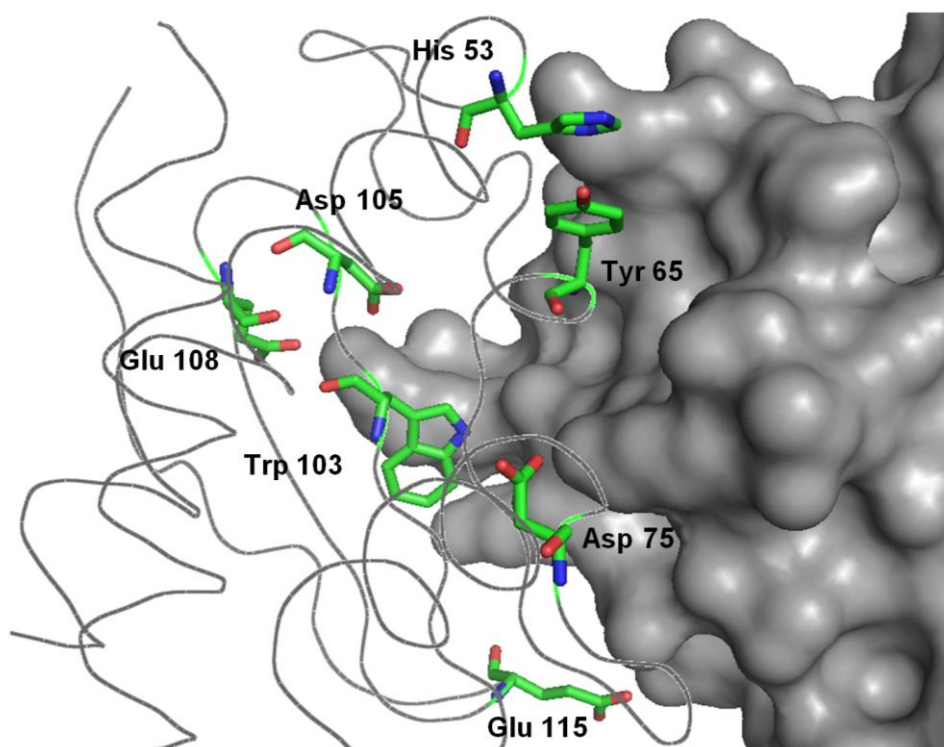


Figure 3.2 Structural analysis of the BTG2-Caf1a (CNOT7) interaction surface.

Amino acids predicted to be required for the BTG2-Caf1a (CNOT7) interaction and subject to experimental validation are shown in green. BTG2 is represented as a light grey ribbon, Caf1a (CNOT7) as a dark grey surface. This model was based on a Caf1a (CNOT7)-Tob1 structure (PDB 2D5R) in which BTG2 (PDB 3DJU) was aligned with the TOB1 N138 structure. All representations were generated using PyMOL.

3.3 Generation of vectors for yeast two-hybrid interaction analysis

To verify the role of the identified residues in the interaction between BTG2, TOB1 and Caf1a (CNOT7)/Caf1b (CNOT8) yeast two-hybrid analysis was performed. The amino acids being investigated were substituted for alanine to evaluate their importance for the interaction with Caf1a (CNOT7) and Caf1b (CNOT8). Alanine was used because it has a small, uncharged side chain and is relatively chemically inert compared to the amino acids suspected to play a role in the interaction.

The BTG2 cDNA was PCR amplified to include an EcoRI restriction site and the HA epitope tag at the 5' end and a XhoI restriction site at the 3' end. The restriction digested BTG2 cDNA was cloned into the yeast expression vector pBD-Gal4 Cam via the EcoRI and SalI restriction sites. This plasmid produces the Gal4 DNA binding domain fused to the N-terminus of BTG2 (Figure 3.3A). The HA epitope tag was introduced to facilitate western blot detection. The TOB1 cDNA was cloned via the same method into plasmid pBD-Gal4 Cam. The CNOT7 cDNA was PCR amplified to include XhoI restriction sites at the both its 5' and 3' ends. The restriction digested CNOT7 cDNA was cloned into the yeast expression vector pAD-Gal4-2.1, which fuses the Gal4 transcriptional activation domain to the N-terminus of CNOT7 (Figure 3.3B). The Gal4 AD-CNOT7 fusion protein was detected using antibodies against the Gal4 AD domain. The CNOT8 cDNA was cloned via the same method in to pAD-Gal4-2.1. All cDNA sequences were confirmed by DNA sequencing.

To introduce the desired point mutations in BTG2 and TOB1, DNA oligos were designed containing the relevant modified codons and used for site directed mutagenesis. Plasmids containing the desired mutations were identified through DNA sequencing.

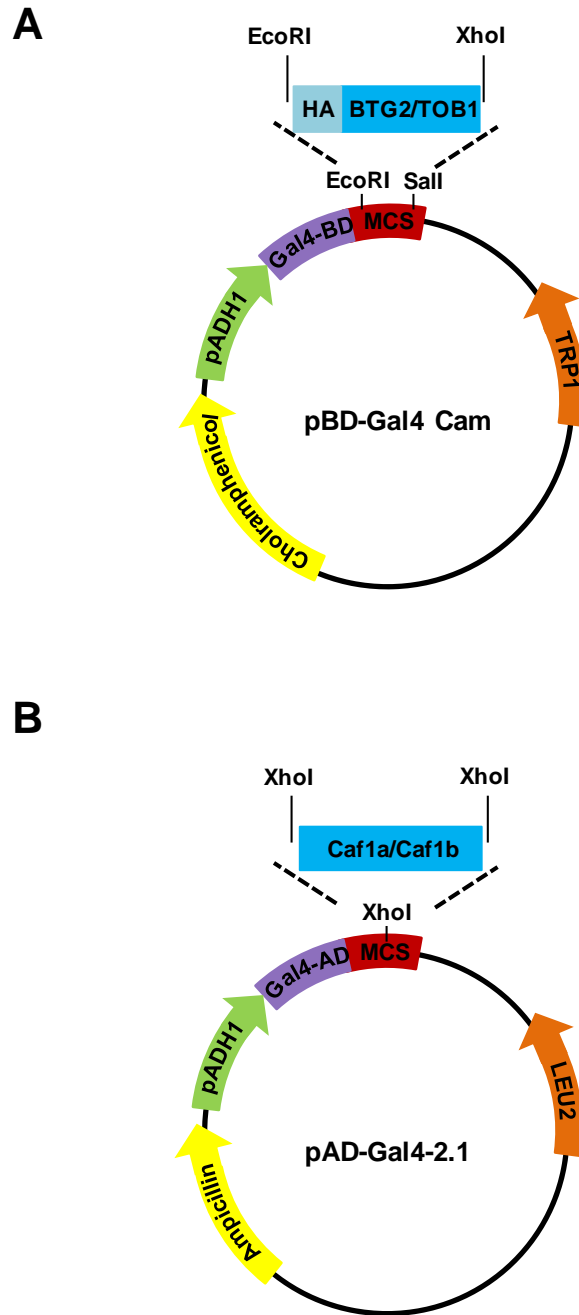


Figure 3.3 Generation of plasmids for yeast-two-hybrid interaction analysis.

(A) Gal4 DNA binding domain hybrids. The BTG2 and TOB1 cDNAs were amplified by PCR. The 5' primer included an EcoRI restriction site followed by a sequence encoding the HA epitope. The 3' primer contained a stop codon followed by an XhoI site. The cDNA fragments were ligated into yeast expression vector pBD-Gal4 Cam via the EcoRI and SalI restriction sites. **(B) Gal4 activation domain hybrids.** The CNOT7 and CNOT8 cDNAs were amplified by PCR using XhoI sites included in both the 5' and 3' primers. The cDNA fragments were ligated into the yeast expression vector pAD-Gal4-2.1.

3.4 Identification of residues required for the BTG2 and TOB1 interaction with Caf1a (CNOT7)/Caf1b (CNOT8) through yeast two-hybrid analysis

To ascertain if any of the amino acids identified are required for the interaction between BTG2 and TOB1 with Caf1a (CNOT7)/Caf1b (CNOT8) yeast two-hybrid analysis was preformed. The appropriate yeast two-hybrid vectors were transformed into yeast strain YRG2 and grown on media without leucine or tryptophan to select transformants. Three colonies were subjected to the β -galactosidase assay for each BTG2/TOB1 amino acid substitution.

Transformation of the yeast with pAD-Gal4 and pBD-Gal4-HA confirmed the absence of background β -galactosidase activity (Figure 3.4B). Transformation of pAD-Gal4 and pBD-Gal4-HA-BTG confirmed that BTG2 fused to the DNA binding domain was alone not capable of inducing β -galactosidase expression (Figure 3.4B). Upon an interaction between BTG2 fused to the Gal4 DNA binding domain and Caf1a (CNOT7) fused to the Gal4 transcriptional activation domain β -galactosidase expression was induced (Figure 3.4A and 3.4B). Of the seven BTG2 constructs containing amino acid substitutions three, H53A, E108A and E115A, retained the ability to interact with Caf1a (CNOT7) and stimulate β -galactosidase expression. The remaining four amino acid substitutions Y65A, D75A, W103A and D105A did not induce β -galactosidase expression and therefore were incapable of interacting with Caf1a (CNOT7) (Figure 3.4A and 3.4B). From the yeast-cultures used in the β -Galactosidase assay, protein was extracted for western blot analysis to confirm expression of Caf1a (CNOT7) and the various BTG2 mutants (Figure 3.4C).

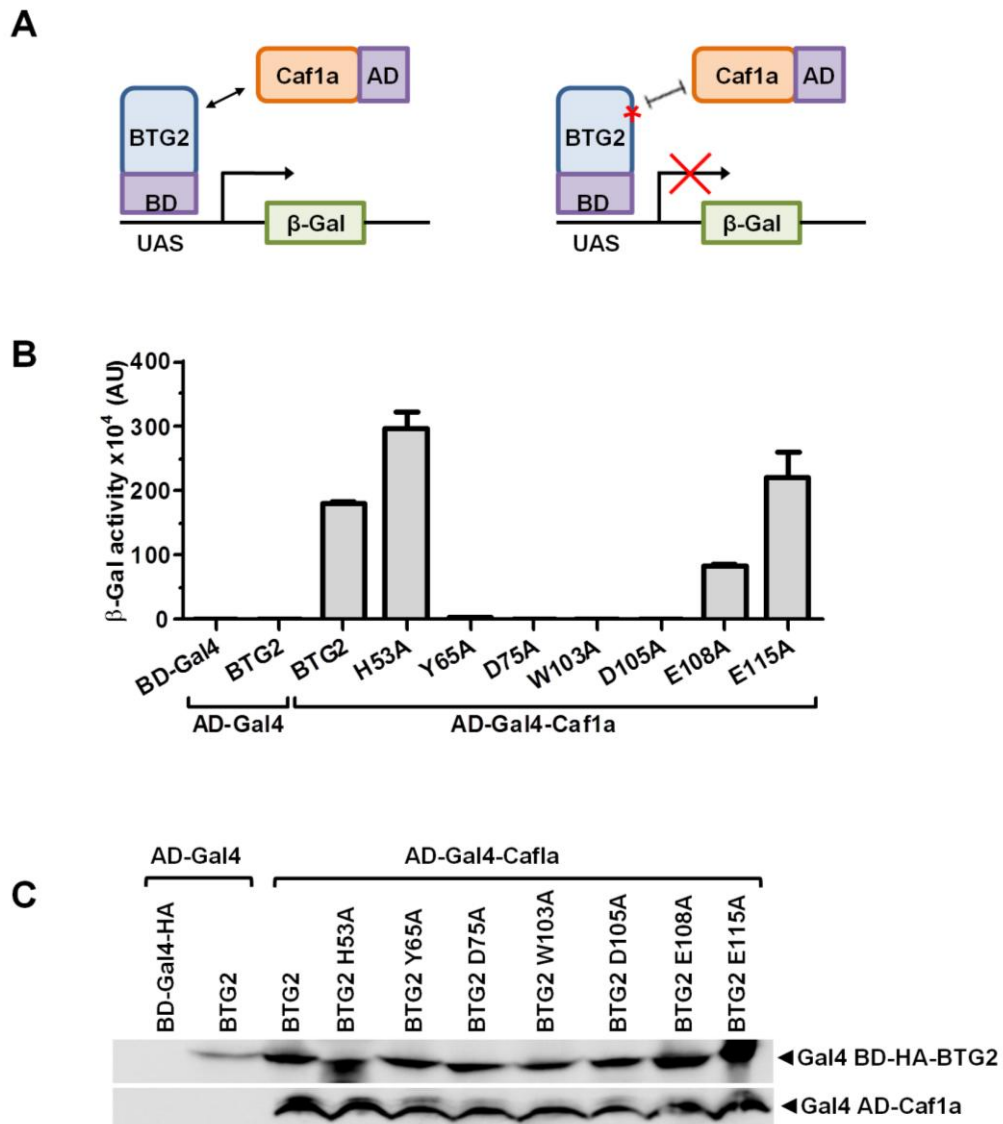


Figure 3.4 Yeast two-hybrid analysis of the BTG2-Caf1a (CNOT7) interaction.

(A) Schematic representing the yeast two-hybrid experiment. AD, Gal4 activation domain; BD, Gal4 DNA binding domain. When an interaction occurs between BTG2 and Caf1a (CNOT7), the Gal4-BD and Gal4-AD are brought in close proximity on the β -Galactosidase promoter thereby inducing transcription (left panel). In the absence of an interaction, β -Galactosidase transcription is not initiated (right panel). **(B)** YRG2 yeast cells were transformed with the indicated vectors; pAD-Gal4 or pAD-Gal4-CNOT7 and pBD-Gal4-HA or pBD-Gal4-HA-BTG2. β -Galactosidase activity was normalised against total protein. Experiments were carried out in triplicate. Error bars represent the standard error of the mean. **(C)** Western blots analysis showing expression of Gal4-BD-HA-BTG2 and Gal4-AD-Caf1a (CNOT7). Anti-HA antibodies were used to detect Gal4-BD-HA-BTG2, anti-Gal4-AD antibodies were used to detect Gal4-AD-Caf1a (CNOT7).

Yeast two-hybrid analysis was also performed to investigate the interaction between Caf1b (CNOT8) and the BTG2 mutants. As expected the interaction between BTG2 fused to the Gal4 DNA binding domain and Caf1b (CNOT8) fused to the Gal4 transcriptional activation domain induced β -galactosidase expression (Figure 3.5A and 3.5B). Two of the seven BTG2 mutants, E108A and E115A retained the ability to interact with Caf1b (CNOT8) and stimulate β -galactosidase expression. The remaining five amino acid substitutions, H53A, Y65A, D75A, W103A and D105A, all failed to induce β -galactosidase expression and therefore were incapable of interacting with Caf1b (CNOT8) (Figure 3.5A and 3.5B). This is in contrast to the BTG2-Caf1a (CNOT7) interaction where BTG2 H53A retained the ability to interact with Caf1a (CNOT7). From the yeast cultures used in the β -galactosidase assay I also extracted protein for western blot analysis to confirm expression of Caf1b (CNOT8) and the various BTG2 mutants (Figure 3.5C).

The yeast two-hybrid analysis identified four BTG2 mutants, Y65A, D75A, W103A and D105A, which were unable to interact with Caf1a (CNOT7) and Caf1b (CNOT8). The H53A amino acid substitution of BTG2 was only capable of disrupting the interaction with Caf1b (CNOT8). Both amino acid changes E108A and E115A did not disrupt the interaction between BTG2 and Caf1a (CNOT7) or Caf1b (CNOT8).

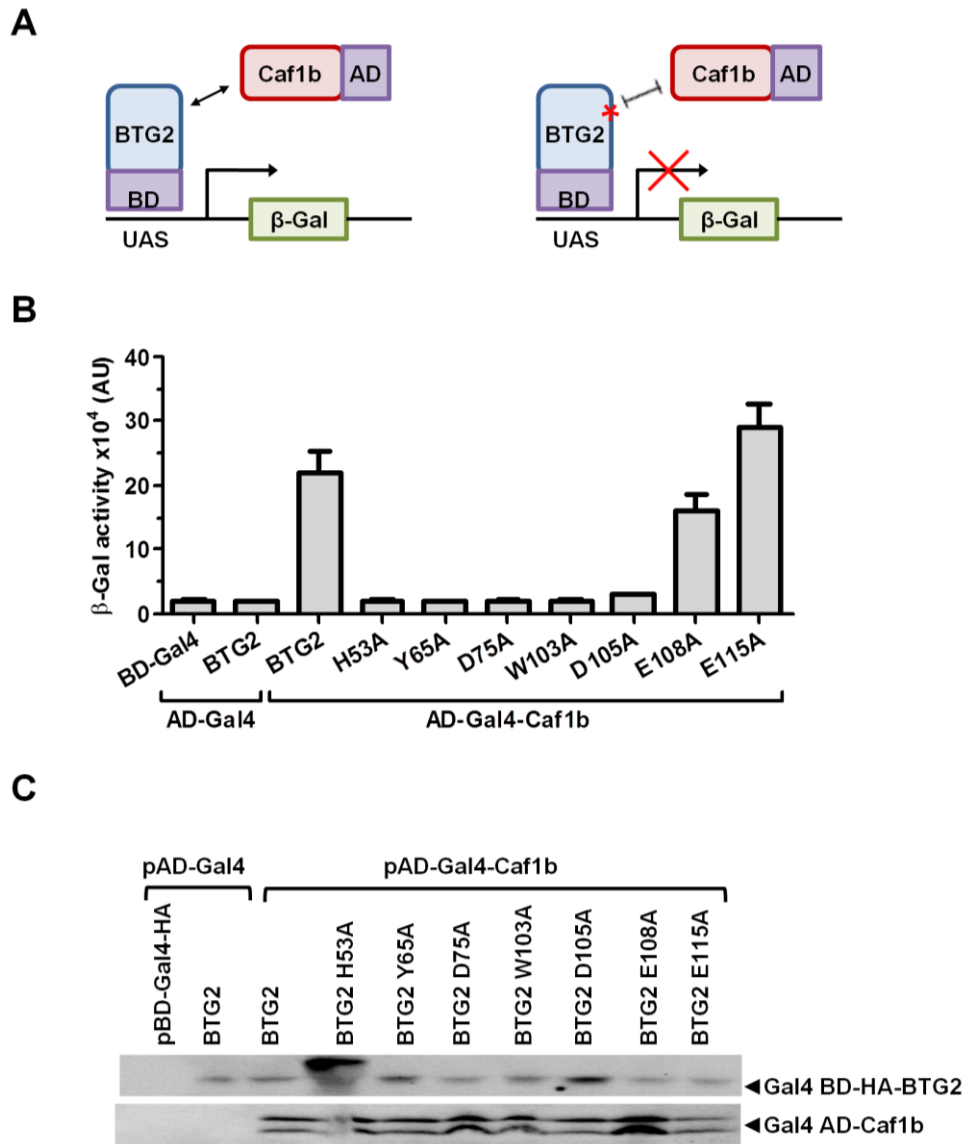


Figure 3.5 Yeast two-hybrid analysis of the BTG2-Caf1b (CNOT8) interaction.

(A) Schematic representing the yeast two-hybrid experiment. AD, Gal4 activation domain; BD, Gal4 DNA binding domain. When an interaction occurs between BTG2 and Caf1b (CNOT8), the Gal4-BD and Gal4-AD are brought in close proximity on the β -Galactosidase promoter thereby inducing transcription (left panel). In the absence of an interaction, β -Galactosidase transcription is not initiated (right panel). **(B)** YRG2 yeast cells were transformed with the indicated vectors; pAD-Gal4 or pAD-Gal4-CNOT8 and pBD-Gal4-HA or pBD-Gal4-HA-BTG2. β -Galactosidase activity was normalised against total protein. Experiments were carried out in triplicate. Error bars represent the standard error of the mean. **(C)** Western blots analysis showing expression of Gal4-BD-HA-BTG2 and Gal4-AD-Caf1b (CNOT8). Anti-HA antibodies were used to detect Gal4-BD-HA-BTG2, anti-Gal4-AD antibodies were used to detect Gal4-AD-Caf1b (CNOT8).

To ascertain if the equivalent amino acid substitutions in TOB1 are also capable of disrupting the interaction with Caf1a (CNOT7) and Caf1b (CNOT8), further yeast two-hybrid analysis was conducted (Figure 3.6A). All of the amino acids substituted in BTG2 are conserved in TOB1, apart from Tyr-65 which in TOB1 is Phe-55, a structurally similar amino acid. The TOB1 yeast two-hybrid vectors were co-transfected into yeast strain YRG2 along with either pAD-CNOT7 or pAD-CNOT8. Transformation of the YRG2 yeast with pAD-Gal4 and pBD-Gal4-HA confirmed the absence of background β -galactosidase activity while transformation of pAD-Gal4 with pBD-Gal4-HA-TOB1 confirmed that TOB1 fused to the DNA binding domain was alone not capable of inducing β -galactosidase expression (Figure 3.6B and 3.6C). As expected transformation of yeast strain YRG2 with TOB1 fused to the Gal4 DNA binding domain and Caf1a (CNOT7) or Caf1b (CNOT8) fused to the Gal4 transcriptional activation domain induced β -Galactosidase expression. This confirms that TOB1 is capable of interacting with Caf1a (CNOT7) and Caf1b (CNOT8). Two of the seven TOB1 mutants E98A and E105A retained the ability to interact with Caf1a (CNOT7) and Caf1b (CNOT8) and stimulated β -galactosidase expression. The remaining five mutants H43A, F55A, D65A, W93A and D95A did not induce β -galactosidase expression and therefore were incapable of interacting with Caf1a (CNOT7) or Caf1b (CNOT8) (Figure 3.6B and 3.6C). This was in contrast to the BTG2-Caf1a (CNOT7) interaction analysis where amino acid substitution H53A did not disrupt the interaction between BTG2 and Caf1a (CNOT7). However, the equivalent substitution in TOB1 (H43A) did disrupt the interaction with Caf1a (CNOT7).

In summary, the yeast two-hybrid analysis identified four amino acid substitutions that disrupt the interaction between BTG2/TOB1 and Caf1a (CNOT7) and Caf1b (CNOT8) (Table 3.2).

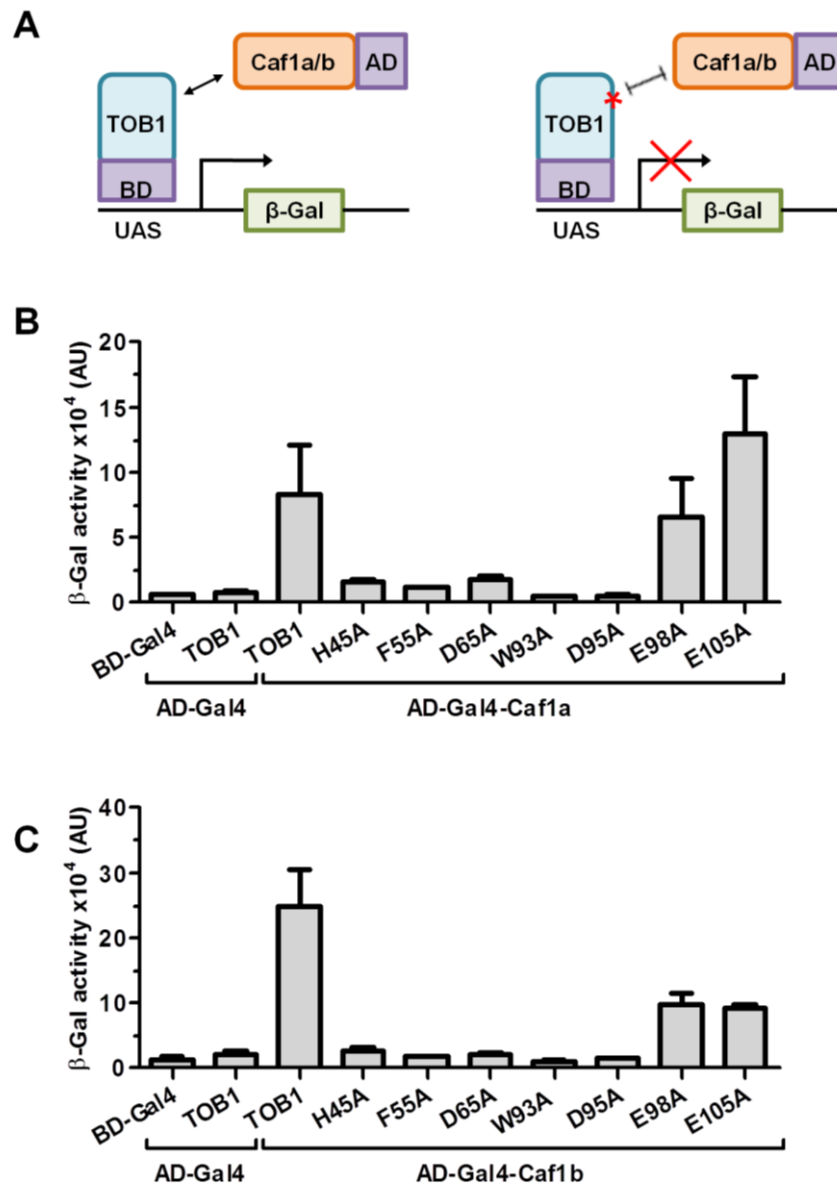


Figure 3.6 Yeast two-hybrid analysis of the TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction. **(A)** Schematic representing the yeast two-hybrid experiment. AD, Gal4 activation domain; BD, Gal4 DNA binding domain. When an interaction occurs between TOB1 and Caf1a (CNOT7) or Caf1b (CNOT8), the Gal4-BD and Gal4-AD are brought in close proximity on the β -Galactosidase promoter thereby inducing transcription (left panel). In the absence of an interaction, β -Galactosidase transcription is not initiated (right panel). **(B)** YRG2 yeast cells were transformed with the indicated vectors; pAD-Gal4 or pAD-Gal4-CNOT7 and pBD-Gal4-HA or pBD-Gal4-HA-TOB1. β -Galactosidase activity was normalised against total protein. **(C)** YRG2 yeast cells were transformed with the indicated vectors; pAD-Gal4 or pAD-Gal4-CNOT8 and pBD-Gal4-HA or pBD-Gal4-HA-TOB1. β -Galactosidase activity was normalised against total protein. All experiments were carried out in triplicate. Error bars represent the standard error of the mean.

Amino acid in BTG2	Conserved position in TOB1	Location in BTG domain
Tyr-65	Phe-55	Box A
Asp-75	Asp-65	Between Box A and Box B
Tyr-103	Tyr-93	Box B
Asp-105	Asp-95	Box B

Table 3.2 List of identified amino acids required in mediating the interaction with Caf1a (CNOT7) and Caf1b (CNOT8)

3.5 Generation of mammalian BTG2 and TOB1 expression vectors

To verify that the amino acid substitutions that disrupt the BTG2-Caf1a (CNOT7) interaction in the yeast two-hybrid analysis are also capable of disrupting the interaction in mammalian cells, co-immunoprecipitation analysis was performed. To this end, expression vectors containing Flag and HA-tagged versions of the BTG2 cDNA were generated. To introduce the BTG2 cDNA into the pcDNA3-Flag plasmid, BTG2 was amplified by PCR to include XhoI restriction sites at the 5' and 3' ends. This PCR fragment was ligated into the SmaI restriction site of the pBluescript II KS(+) cloning vector. The pBluescript-BTG2 plasmid was digested with BamHI and EcoRV to release the BTG2 cDNA, which was ligated into pcDNA3-Flag (digested with BamHI-EcoRV). To introduce the Flag-BTG2 cDNA into plasmid pcDNA4-TO, the pcDNA3-Flag-BTG2 plasmid was digested with HindIII and EcoRV to release the Flag-BTG2 cDNA. This fragment was ligated into pcDNA4-TO plasmid through the HindIII and EcoRV restriction sites (Figure 3.7A).

To introduce the BTG2 cDNA into the plasmid pCMV5-HA, plasmid pBluescript-BTG2 was digested with XhoI to release the BTG2 cDNA. This cDNA fragment was ligated into the SalI restriction site of pCMV5-HA (Figure 3.7B). To introduce the desired point mutations into BTG2, DNA oligos were designed containing the relevant modified codons and used for site directed mutagenesis. Plasmids containing the desired mutations were identified through DNA sequencing. Expression plasmids containing epitope-tagged CNOT7 are described in Aslam et al (2009).

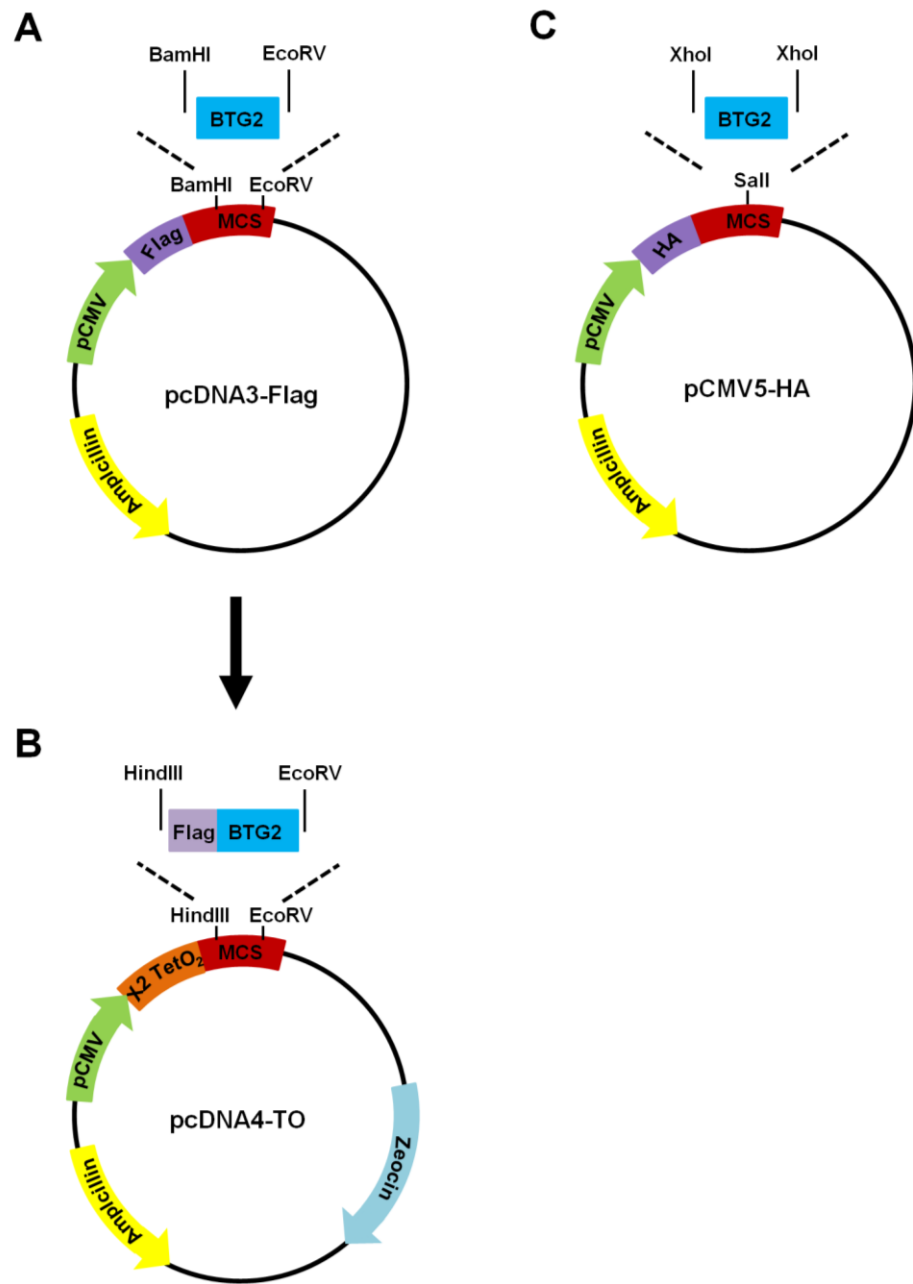


Figure 3.7 Generation of Flag-BTG2 and HA-BTG2 expression vectors. The human BTG2 cDNA was amplified by reverse transcriptase PCR using total RNA isolated from MCF-7 cells as a template. Primers included XhoI restriction sites 5' of the ATG start codon and after the 3' stop codon. This PCR fragment was ligated into the cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-BTG2. **(A)** The BTG2 cDNA was excised with BamHI and EcoRV and subcloned into plasmid pcDNA3-Flag. **(B)** Subsequently, the Flag-BTG2 fragment was subcloned from pcDNA3-Flag-BTG2 into pcDNA4-TO using the HindIII and EcoRV restriction sites. **(C)** Or the BTG2 cDNA was excised from pBluescript-BTG2 with XhoI and subcloned into plasmid pCMV5-HA via the SalI restriction site.

3.6 Confirmation of the amino acids required for the BTG2-Caf1a (CNOT7) interaction through co-immunoprecipitation analysis

To confirm the role of the selected amino acids in mediating BTG2-Caf1a (CNOT7) interactions in the context of mammalian cells, immunoprecipitation analysis was conducted. To distinguish BTG2 containing amino acid substitutions from endogenous BTG2, epitope tagged variants of BTG2 and Caf1a (CNOT7) were used. The appropriate mammalian expression plasmids were co-transfected into HEK293T cells before total cell lysates were produced. Western blot analysis of the total cell lysates was performed before the immuno-precipitation step to confirm equal expression of HA-CNOT7. Wild-type Flag-BTG2 and Flag-BTG2 containing amino acid substitutions were precipitated using the anti-Flag coated beads. As expected, no precipitation of HA-Caf1a (CNOT7) was demonstrated in the absence of Flag-BTG2 (Figure 3.8). By contrast, Flag-BTG2 was able to immuno-precipitate HA-Caf1a (CNOT7). Of the seven BTG2 proteins containing amino acid substitutions, E108A and W115A maintained the BTG2-Caf1a (CNOT7) interaction. A weak signal of HA-Caf1a (CNOT7) was seen upon immunoprecipitation of BTG2 H53A. The remaining four amino acid substitutions, Y65A, D75A, W103A and D105A, did not co-precipitate HA-Caf1a (CNOT7) indicating that these amino acids are important for the BTG2-Caf1a (CNOT7) interaction (Figure 3.8).

To confirm the co-immunoprecipitation result, a reciprocal co-immunoprecipitation assay was performed using Flag-Caf1a (CNOT7) and HA-BTG2. HEK293T cells were co-transfected with pcDNA3-Flag-CNOT7 and appropriate pCMV5-HA-BTG2 plasmids. Western blot analysis of the total cell lysates was performed before the immuno-precipitation step to confirm equal expression of Flag-Caf1a (CNOT7) and HA-BTG2. As expected, no immunoprecipitation of HA-BTG2 was seen in the absence of Flag-Caf1a (CNOT7) (Figure 3.9). By contrast, Flag-Caf1a (CNOT7) was able to immunoprecipitate wild type HA-BTG2.

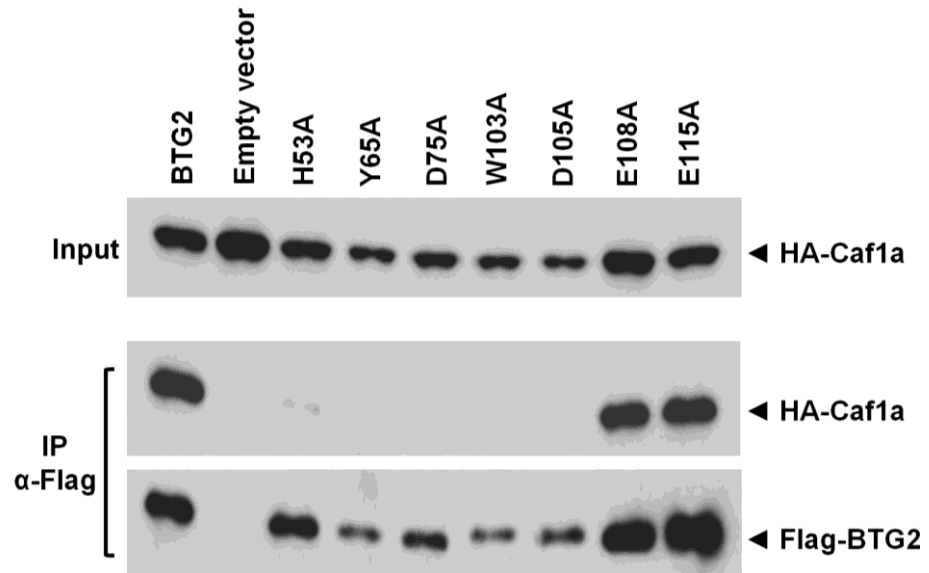


Figure 3.8 Analysis of the Flag-BTG2-HA-Caf1a (CNOT7) interaction by co-immunoprecipitation. HEK293T cells were transfected with pCMV5-HA-CNOT7 and pcDNA4-TO-Flag-BTG2, or empty vector. Anti-Flag coated agarose beads were used to pull down Flag-BTG2. The eluted protein samples were loaded on to SDS PAGE gels and western blot analysis was conducted using anti-Flag and anti-HA antibodies. The input sample contained 2.5% of total protein as compared to the amount used for the immuno-precipitation.

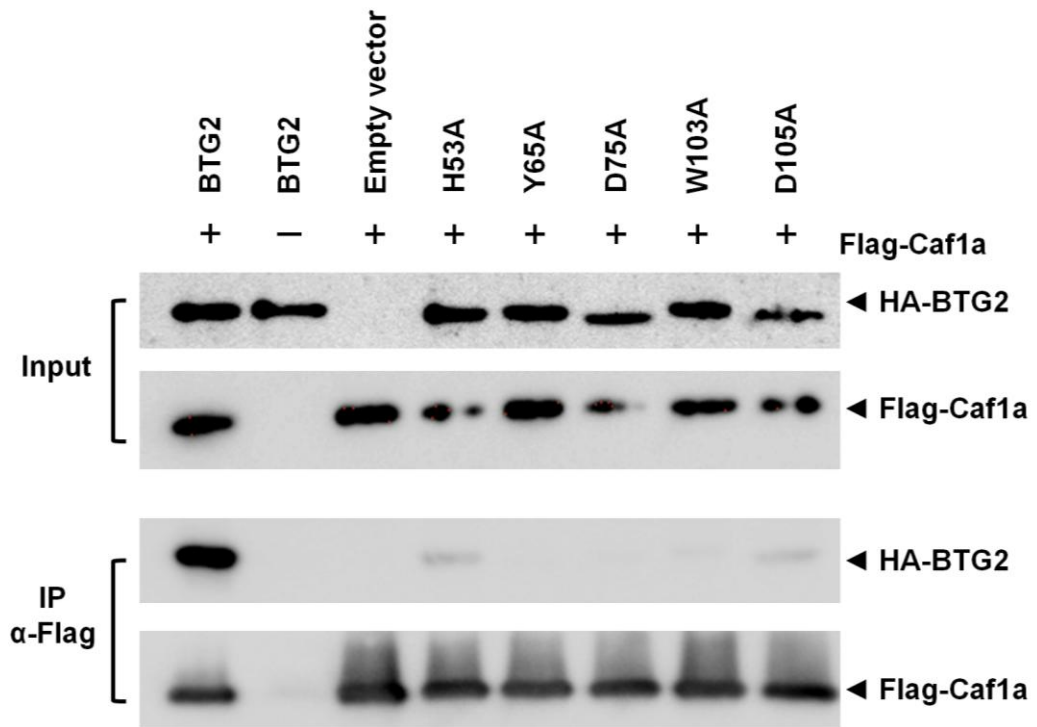


Figure 3.9 Co-immunoprecipitation assay using HA-BTG2 and Flag-Caf1a (CNOT7). HEK293T cells were transfected with pcDNA3-Flag-CNOT7 and pCMV5-HA-BTG2, or empty vector. Anti-Flag coated agarose beads were used to pull down Flag-Caf1a (CNOT7). The eluted protein samples were loaded on to SDS PAGE gels and western blot analysis was conducted using anti-HA and anti-Flag antibodies. The input sample contained 2.5% of total protein as compared to the amount used for the immuno-precipitation.

A weak signal was seen when BTG2 H53A or BTG2 D105A was expressed. The remaining three amino acid substitutions Y65A, D75A and W103A, did not co-immunoprecipitate with Flag-Caf1a (CNOT7) indicating the importance of these amino acids (Figure 3.9).

Together, the results presented in this chapter demonstrated that the amino acid substitutions Y65A, D75A and W103A can prevent the interaction between BTG2 and Caf1a (CNOT7)/Caf1b (CNOT8). The equivalent amino acid substitutions in Tob1, F55A, D65A and W93A also disrupt the TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interactions.

3.7 Discussion

The experiments described in this chapter identify three amino acids in BTG2 and TOB1 that are required for the interactions with Caf1a (CNOT7) and Caf1b (CNOT8). These amino acids are Tyr-65, Asp-75 and Trp-103 of BTG2 and Phe-55, Asp-65 and Trp-93 of TOB1. These findings are corroborated by data published during this study by (Yang et al., 2008), who also demonstrated that amino acid substitutions Y65A, W103A, D105A, and the combined amino acid change G64A/W103A disrupted the BTG2-Caf1a (CNOT7) interaction.

Residues Tyr-65 of BTG2 and Phe-55 of TOB1 are positioned in the conserved Box A region and come into close proximity with Caf1a (CNOT7). However, after further analysis of the crystal structure of the TOB1-Caf1a (CNOT7) complex, these amino acids do not appear to form any specific interaction with amino acid side chains of Caf1a (CNOT7). I predict that Y65A/F55A may be required for maintaining the helical secondary structure of the Box A region through stacking interactions with the aromatic side chain of His-53 of BTG2 or His-43 of TOB1, respectively (Figure 3.10A). Therefore, the amino acid substitutions Y65A of BTG2 and F55A of TOB1 may disrupt the secondary structure of Box A thus preventing BTG2 and TOB1 to form interactions with Caf1a (CNOT7) and Caf1b (CNOT8).

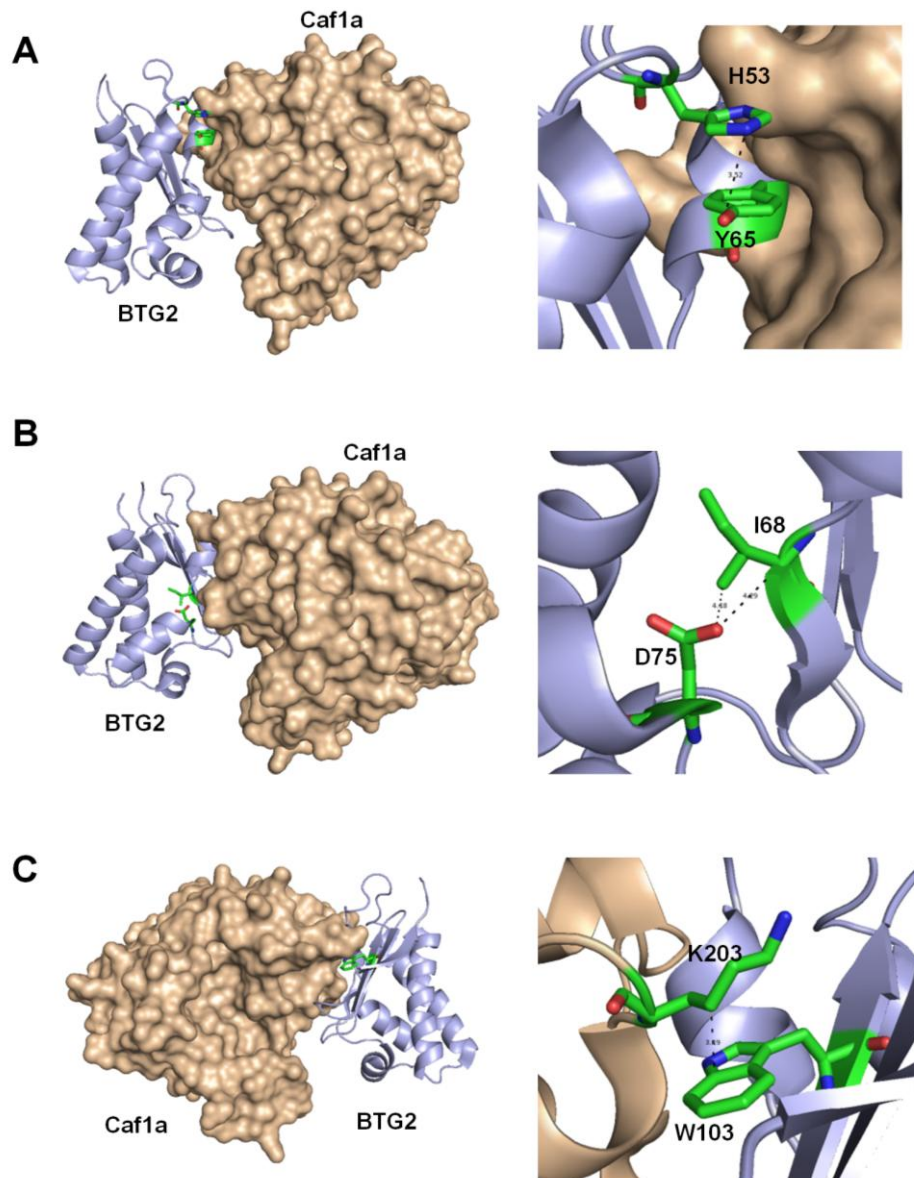


Figure 3.10 Structural analysis of the amino acids required for the interaction between BTG2 and Caf1a (CNOT7). (A) Tyr-65 and His-53 of BTG2 are highlighted. The aromatic ring of Tyr-65 may interact through stacking interactions with the aromatic ring of His-53 to maintain the helical structure of Box A. (B) Asp-75 and Ile-68 of BTG2 are highlighted. Asp-75 is not located in either Box A or Box B but may interact with Ile-68 or the protein backbone to maintain the helical structure of Box A. (C) Trp-103 of BTG2 and Lys-203 of Caf1a (CNOT7) are highlighted. Trp-103 of BTG2 is predicted to interact with the aliphatic part of Lys-203 of Caf1a (CNOT7). Light blue, BTG2 (PDB accession number 3DJU); Wheat, Caf1a (CNOT7) (PDB accession number 2D5R). The BTG2 backbone was aligned with TOB1. All representations were generated using PyMOL.

Asp-75 in BTG2 and Asp-65 in TOB1 are conserved across all members of the BTG/TOB family. However, these residues are not located within one of the highly conserved regions of the BTG domain but in the linker region between Box A and Box B. It is located adjacent to a proline residue indicating that it may be required to form a loop or turn in the protein structure. It may also form hydrogen bonds with the α -helix backbone to maintain the secondary structure of the BTG domain (Figure 3.10B). I predict that the amino acid changes D75A of BTG2 and D65A of TOB1 cause misfolding of the BTG domain thus preventing the interaction with Caf1a (CNOT7).

Trp-103 in BTG2 and Trp-93 in TOB1 are located in the middle of the Box B region, in the centre of a β -sheet with its aromatic side chain protruding towards Caf1a (CNOT7). This side chain may form direct interactions with the aliphatic portion of the Lys-203 side chain of Caf1a (CNOT7) (Figure 3.10C). Furthermore, PDBePISA analysis indicated that that TOB1 Trp-93 is fully buried in Caf1a (CNOT7) where it is capable of hydrogen bonding. The position of the side chain suggests that it is required for a direct interaction with Caf1a (CNOT7). It is likely that substituting aromatic amino acid like tryptophan for alanine, would disrupt any interaction between the residues Trp-103 of BTG2/Trp-93 of TOB1 and Lys-203 of Caf1a (CNOT7)/Caf1b (CNOT8).

Co-immunoprecipitation was not used to confirm the disruption of the TOB1-Caf1a (CNOT7) interaction as a result of the amino acid substitutions due to difficulties in detecting TOB1 via western blot. However, due to the high level of sequence and structural conservation between N-terminal domains of BTG2 and TOB1 (Figure 3.11A and 3.11B) and the reproducibility of the yeast two-hybrid results, we assume that the TOB1 W93A amino acid substitution will disrupt the TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interactions in mammalian cells.

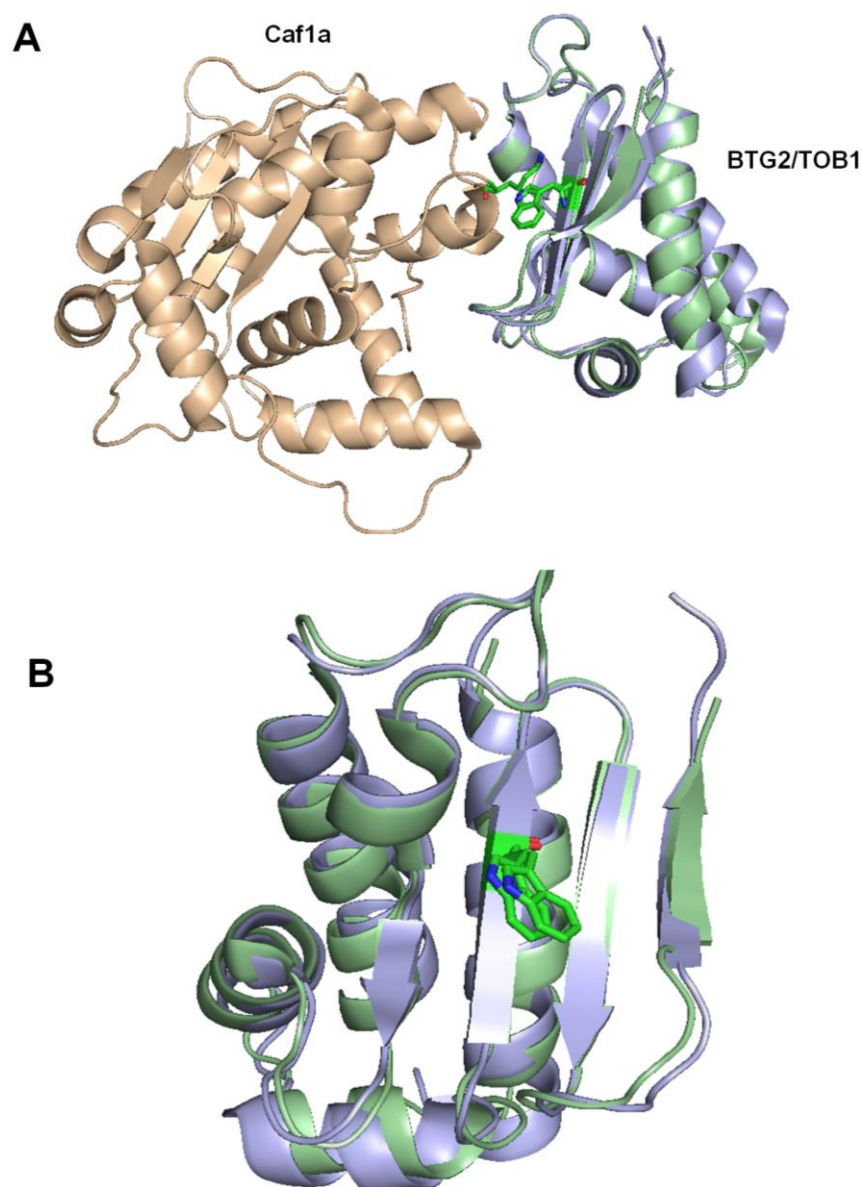


Figure 3.11 Structural alignments of the BTG domains of BTG2 and TOB1.

(A) The structure of the BTG domain of TOB1-Caf1a (CNOT7) (PDB accession number 2D5R). The predicted BTG2-Caf1a (CNOT7) interaction was produced by aligning BTG2 (PDB accession number 3DJU) with the TOB1 N138 backbone. **(B)** Schematic representation of the structures of the BTG domains of BTG2 and TOB1. BTG2 and TOB1 were aligned to confirm the identical positioning of Trp-103 of BTG2 and Trp-93 of TOB1. Wheat, Caf1a (CNOT7); light blue, BTG2; light green, TOB1; Lys-203 of Caf1a(CNOT7), Trp-103 of BTG2 and Trp-93 of TOB1 are highlighted in green. All representations were generated using PyMOL.

During the course of this work several further amino acid residues were identified as required for the TOB1/TOB2-Caf1a (CNOT7) interaction. Combined substitution of three leucine residues (Leu-32, Leu-35 and Leu-36) located close to the amino terminus of TOB1/TOB2 for glycine disrupts the TOB1/TOB2-Caf1a (CNOT7) interaction (Ezzeddine et al., 2012). Interestingly, PDBePISA analysis suggests that Leu-32 is an inaccessible residue and that Leu-35 and Leu-36 are not favourable candidates to form protein-protein interactions. Further data by Ezzeddine et al (2012), identified amino acids Glu-62, Met-63 and Asp-65 as required for the TOB1/TOB2-Caf1a (CNOT7) interaction. Interestingly, yeast two-hybrid analysis conducted in this thesis also identified TOB1 Asp-65 as required for the TOB1-Caf1a (CNOT7) interaction. I preferred the use of the single amino acid substitutions W103A of BTG2 and W93A of TOB1 over the triple amino acid substitutions used by Ezzeddine et al (2012), because the latter may have a more dramatic effect on protein structure.

On the basis of this interaction analysis, amino acid substitutions W103A of BTG2 and W93A of TOB1 were selected to evaluate the functional importance of the interactions between BTG2/TOB1 and Caf1a (CNOT7)/Caf1b (CNOT8). These interaction mutants can be used to investigate the importance of the interaction for the anti-proliferative activity of BTG2 and TOB1.

Chapter 4

**The activity of BTG2/TOB1 is mediated
through interactions with Caf1a (CNOT7)
and Caf1b (CNOT8)**

Chapter 4: The activity of BTG2/TOB1 is mediated through interactions with Caf1a (CNOT7) and Caf1b (CNOT8)

4.1 Introduction

The anti-proliferative activity of the BTG/TOB proteins has been demonstrated in a number of different cell lines. Over-expression of BTG1 and BTG2 in WEHI-231 cells causes a significant decrease in the rate of cell proliferation (Hata et al., 2007). Interestingly, over-expression of TOB2 and BTG3 in the same cell line only has a marginal effect on cell proliferation indicating that the BTG/TOB proteins may function cell specifically (Hata et al., 2007). The expression of BTG/TOB proteins causes an increase in the number of cells in G1 suggesting they act upon the G1 to S-phase cell cycle transition (Ikematsu et al., 1999; Rouault et al., 1992). After expression of BTG1 and BTG2 in WEHI-231 the number of cells in G1 significantly increases, which is paralleled by a drop in the number of cells in S-phase (Hata et al., 2007). Progression into S-phase is seen as the “point of no return” beyond which the cell is committed to DNA replication and division. Cell cycle progression is regulated by the activity of cyclin proteins and their corresponding cyclin-dependant kinases (CDK). For progression into S-phase the expression of S-phase specific genes such as Cyclin E1 and c-Myc must be induced. During G1, transcription of S-phase promoters is repressed by a transcription factor complex containing hypo-phosphorylated retinoblastoma protein (Rb), HDAC and E2F. To enter S-phase two cell cycle kinase-complexes, CDK4/6-Cyclin D and CDK2-Cyclin E work together to phosphorylate Rb, which displaces it from the transcription factor complex and relieves transcription repression (Figure 4.1). Hence, the levels and activity of these two cell cycle kinase-complexes directly lead to the transcriptional activation of S-phase promoting genes (Malumbres and Barbacid, 2009; van den Heuvel and Dyson, 2008).

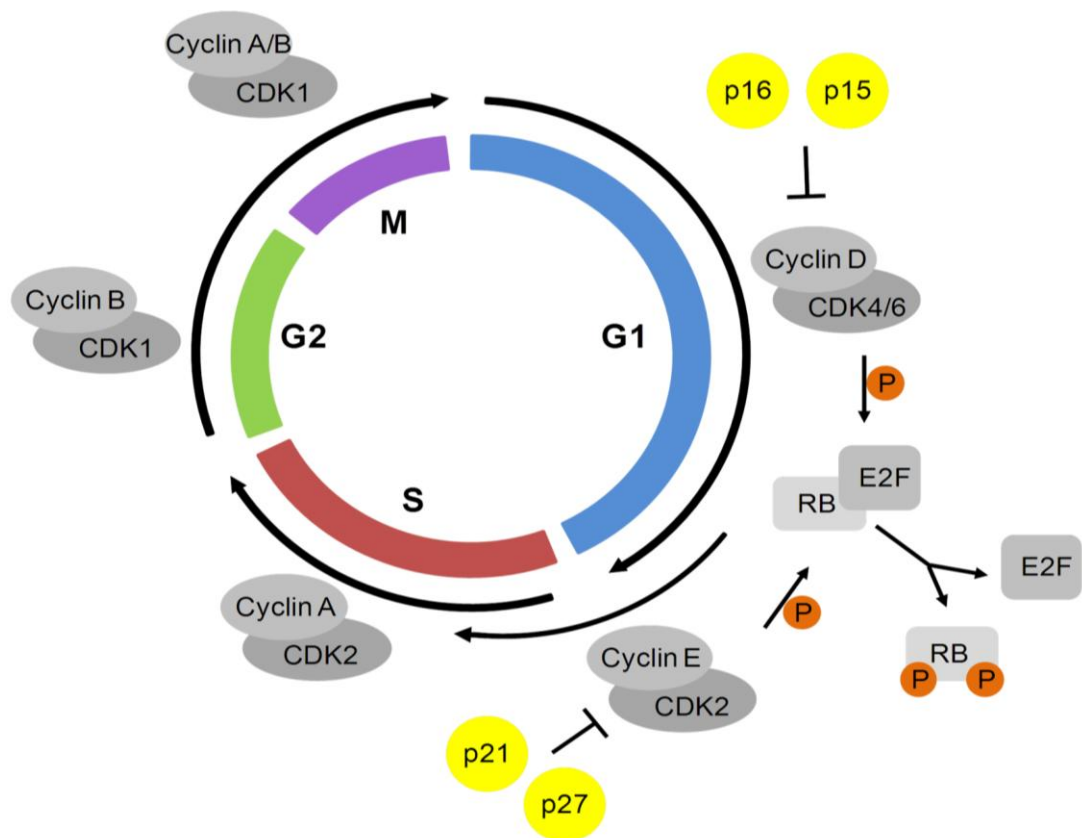


Figure 4.1 Model of the mammalian cell cycle. Cell cycle progression is driven by different cyclin dependant dependent kinase (CDK)/Cyclin complexes. Core components involved in the regulation of the G1 to S-phase transition are indicated. The CDK4/6-CyclinD1 and CDK2-CyclinE complexes act to phosphorylate the Retinoblastoma (Rb) protein causing it to dissociate from its transcription factor complex E2F and relieve repression of S-phase promoting genes. Both CDK4/6-CyclinD1 and CDK2-CyclinE complexes are inhibited by p15 (INK4A), p16 (INK4B) and p21 (Cip1/Waf1), p27 (Kip1) respectively.

Regulation of the G1 check point responds to signalling molecules such as growth factors and cellular stress such as DNA damage, both known inducers of BTG/TOB expression. The induction of BTG2 through p53 signalling causes an arrest in cell cycle progression, specifically at the G1-S check point through reduced Cyclin D1 transcription and CDK4 activation (Donato et al., 2007; Guardavaccaro et al., 2000). Similarly, inhibition of TOB1 activity through growth factor stimulation leads to an increase in Cyclin D1 transcription driving cell proliferation (Suzuki et al., 2002).

One unresolved question in the field is the link between the BTG/TOB anti-proliferative activity and their interactions with the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylase subunits of the Ccr4-Not complex. To date, different conclusions have been drawn based on mutagenesis studies to disrupt the BTG/TOB-Caf1a (CNOT7)/Caf1b (CNOT8) interaction. Expression of Caf1a (CNOT7)/Caf1b (CNOT8) is required for efficient transition from G1 to S phase in MCF-7 cells (Aslam et al., 2009). Interestingly, introduction of amino acid substitutions in Caf1a (CNOT7)/Caf1b (CNOT8) that prevent binding to BTG2 suggest that Caf1a (CNOT7)/Caf1b (CNOT8) does not required an interaction with BTG2 to facilitate cell division. Conversely, it was suggested that TOB1 requires an interaction with Caf1a (CNOT7) to be anti-proliferative in HEK293 and COS-1 cells (Horiuchi et al., 2009). However, again, in the study the BTG/TOB-Caf1a (CNOT7)/Caf1b (CNOT8) interaction was disrupted by substituting amino acid in Caf1a (CNOT7). Based on these reports, it is difficult to determine whether the loss of BTG2/TOB1 activity shown by Aslam et al (2009) and Horiuchi et al (2009) is due to prevention of binding with Caf1a (CNOT7) or simply due to a loss of proper Caf1a (CNOT7) function.

A second outstanding question is the role phosphorylation plays in regulating the BTG2 and TOB1 anti-proliferative activity (Hong et al., 2005; Suzuki et al., 2002; Suzuki et al., 2001). Phosphorylation of TOB1 contributes to the

progression of thyroid cancer and is often present in early lung carcinomas where it heightens cancer progression (Ito et al., 2005; Iwanaga et al., 2003; Yoshida et al., 2003a). This evidence suggests that phosphorylation of TOB1 leads to reduced anti-proliferative activity.

Together, the current published data gives contradictory conclusions as to the requirement of the BTG/TOB- Caf1a (CNOT7)/Caf1b (CNOT8) interaction for BTG/TOB anti-proliferative activity and whether phosphorylation is capable of regulating that activity. To address these questions BTG2 and TOB1 containing amino acid substitutions that prevent the Caf1a (CNOT7)/Caf1b (CNOT8) interaction or phosphorylation were over expressed in MCF-7 cells. From this their effect on cell proliferation was investigated further. Initially the activity of the six BTG/TOB proteins in MCF-7 cells was determined to identify the most relevant members to investigate further. Utilising the BTG2/TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction mutants identified in Chapter 3 the importance of this interaction for BTG/TOB anti-proliferative activity was assessed. Furthermore the importance of phosphorylation as a mechanism for regulating BTG/TOB activity was investigated through amino acid substitutions designed to prevent phosphorylation.

During the course of this work Ezziddine et al (2012) disrupted the TOB1/TOB2 interaction with Caf1a (CNOT7)/Caf1b (CNOT8) by introducing amino acid substitutions in the TOB1/TOB2 N-terminal region. This work demonstrated that disrupting the TOB1/TOB2 interaction with Caf1a (CNOT7)/Caf1b (CNOT8) caused TOB1 and TOB2 to lose anti-proliferative activity. Furthermore, Ezziddine et al (2012) demonstrated that preventing TOB1 phosphorylation by replacing target serine residues for alanine did not alter the TOB1/TOB2 anti-proliferative activity.

4.2 Expression and anti-proliferative activity of BTG/TOB proteins in MCF-7 cells

To identify which of the six BTG/TOB genes regulate cell-cycle progression in MCF-7 cells, we first analysed expression data previously obtained using the Affymetrix GeneChip platform (Mittal et al., 2011). The analysis identified significant levels of BTG1, BTG2, TOB1 and TOB2 mRNAs, and indicated no expression of BTG3 and BTG4 (Figure 4.2A). TOB1 was the most expressed gene and significantly higher than all other BTG/TOB members. BTG1 expression was approximately four fold lower than that of TOB1. BTG2 demonstrated the lowest expression level compared TOB1, TOB2 and BTG1. The relative expression of the BTG/TOB genes was confirmed through RT-qPCR analysis using GAPDH or 36B4 as reference genes (Figure 4.2B, 4.2C). This suggests that TOB1 is the most highly expressed of the six BTG/TOB proteins, BTG2 is the least expressed and BTG3 and BTG4 are not transcribed at all.

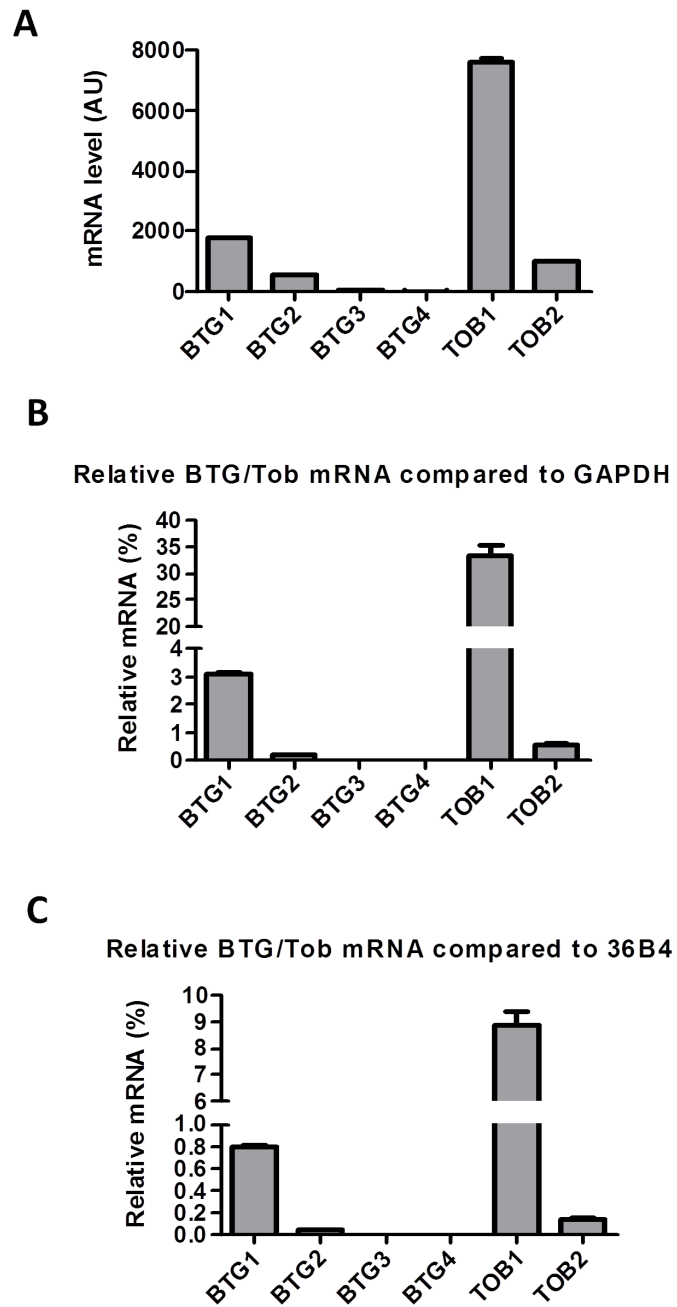


Figure 4.2 Expression of BTG/TOB family members in MCF-7 cells. (A) Expression of BTG/TOB family members in MCF-7 cells. Data was obtained by genome-wide expression data using microarrays. (B) RT-qPCR analysis indicating the relative level of BTG/TOB mRNA compared to GAPDH in MCF-7 cells. (C) RT-qPCR analysis indicating the relative level of BTG/TOB mRNA compared to 36B4 in MCF-7 cells.

To ascertain whether the expressed members of the BTG/TOB family regulate cell proliferation of MCF-7 cells, siRNA knockdown was performed. MCF-7 cells were treated with specific siRNA duplexes targeting BTG1, BTG2, TOB1 or TOB2, or non-targeting control siRNA. After 48 h, and the number of cells in S-phase was determined through the addition of a thymidine analogue (EdU) to the culture medium for 2 h before its detection via coupling of Alexa Fluor 488 azide using 'click' chemistry. Knockdown of BTG1, BTG2, TOB1 and TOB2 was confirmed through RT-qPCR analysis with GAPDH as a reference gene (Figure 4.3A). For all tested BTG/TOB mRNAs, the relative mRNA abundance decreased by >75% after siRNA treatment. Interestingly, siRNA knockdown of BTG1 and TOB2 did not affect the number of cells in S-phase compared to cells treated with non-targeting siRNA. By contrast, siRNA-mediated knockdown of BTG2 and TOB1 caused a significant increase in the number of cells in S-phase (Figure 4.3B). This is surprising because the expression of BTG1 and TOB2 are significantly higher than that of BTG2. Another surprise was the lack of a significant difference in the number of cells in S-phase after BTG2 knockdown compared to TOB1 knockdown, because the expression of TOB1 is approximately 8-fold higher than that of BTG2 (Figure 4.2A).

In summary, Affymetrix GeneChip expression data and RT-qPCR analysis demonstrates that BTG1, BTG2, TOB1 and TOB2 are expressed in MCF-7 cells. However, only two BTG2 and TOB1 affect cell proliferation rates. The fact that BTG2 knockdown causes a comparable increase in the number of cells in S-phase as compared to knockdown of the highly expressed TOB1 suggests that the effect on cell proliferation of BTG2 and TOB1 is independent of gene expression level.

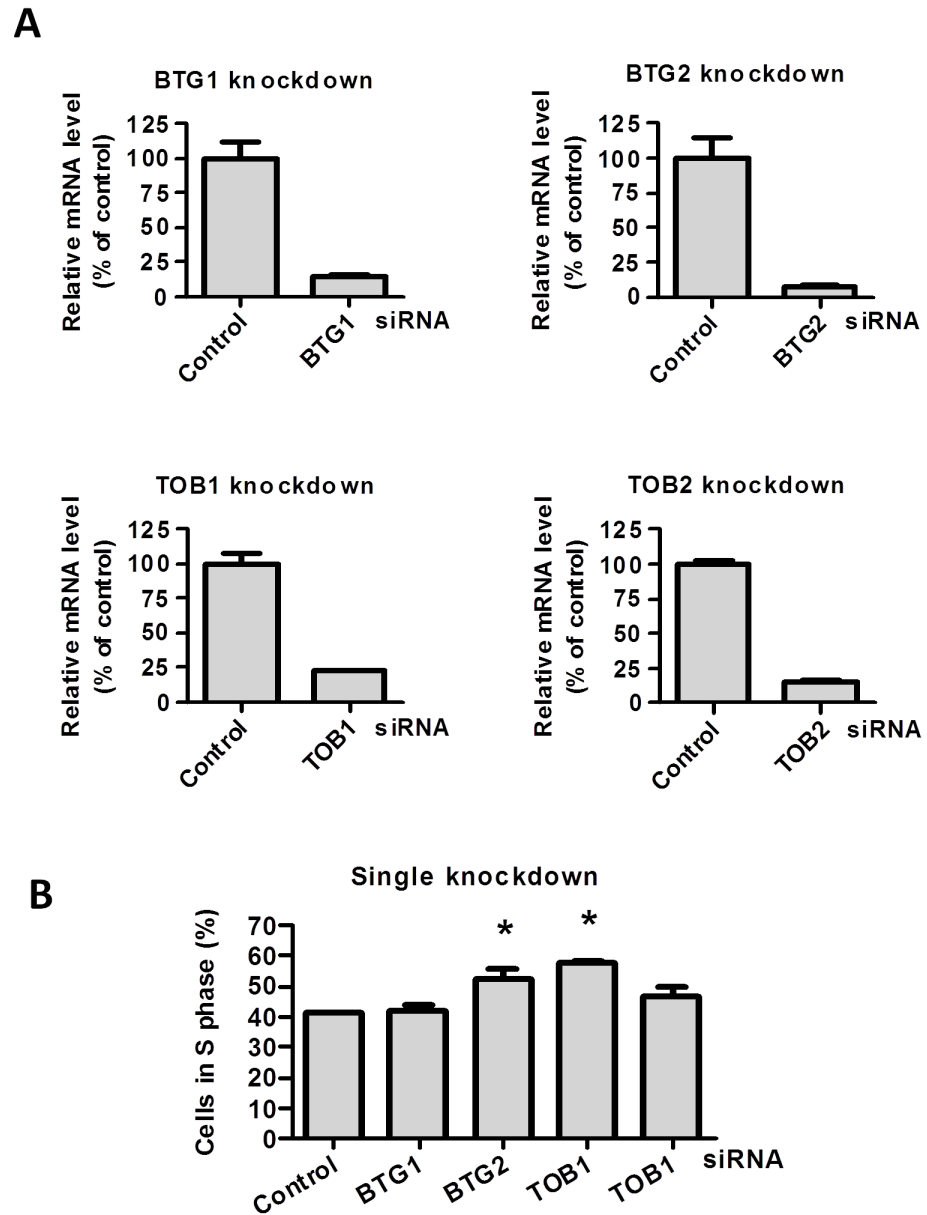


Figure 4.3 Increased cell proliferation following siRNA-mediated knockdown of BTG/TOB components. (A) RT-qPCR analysis to confirm knockdown of BTG1, BTG2, TOB1 and TOB2 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment, total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. (B) Knockdown of BTG2 and TOB1 causes an increase in the number of cells in S phase. Cells in S-phase were detected by pulse labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

4.3 BTG2 and TOB1 display limited redundancy

To exclude that functional redundancy masked the involvement of BTG1 and TOB2 in the regulation of MCF-7 cell proliferation, dual knockdowns were performed for all binary combinations of expressed BTG/TOB family members.

To investigate redundancy between BTG1 and BTG2, MCF-7 cells were transfected with a mixture of siRNA duplexes targeting BTG1 and non-targeting siRNA, BTG2 and non-targeting siRNA, or a pool of siRNAs targeting both BTG1 and BTG2, respectively. Combined knockdown of BTG1 and BTG2 was confirmed by RT-qPCR analysis with GAPDH as a reference gene (Figure 4.4A, 4.4B). The knockdown efficiency was similar for the single and dual knockdown of BTG1 and BTG2. As expected, single knockdown of BTG1 had no effect on cell cycle progression into S-phase, whereas knockdown of BTG2 caused an increase of cells in S-phase. The combined knockdown of BTG1 and BTG2 did not cause a further increase of the number of cells in S-phase as compared to knockdown of BTG2 (Figure 4.4C). This suggests that there is no functional redundancy between BTG1 and BTG2 in MCF-7 cells.

A similar approach was used to investigate possible redundancy between BTG1 and TOB1. Combined knockdown of BTG1 and TOB1 was confirmed through RT-qPCR analysis with GAPDH as a reference gene (Figure 4.5A, 4.5B). In this case, the knockdown efficiency of BTG1 and TOB1 was reduced upon combined knockdown compared to MCF-7 cells treated with the single siRNA. As expected, knockdown of BTG1 had no effect on progression into S-phase, while knockdown of TOB1 caused an increase in the number of cells in S-phase. Importantly, the combined knockdown of BTG1 and TOB1 did not cause a further increase of the number of cells in S-phase as compared to knockdown of TOB1 (Figure 4.5C). This indicates that there is no redundancy between TOB1 and BTG1. Interestingly, the reduced knockdown efficiency of TOB1 when combined with BTG1 did not affect the number of cell in S-phase.

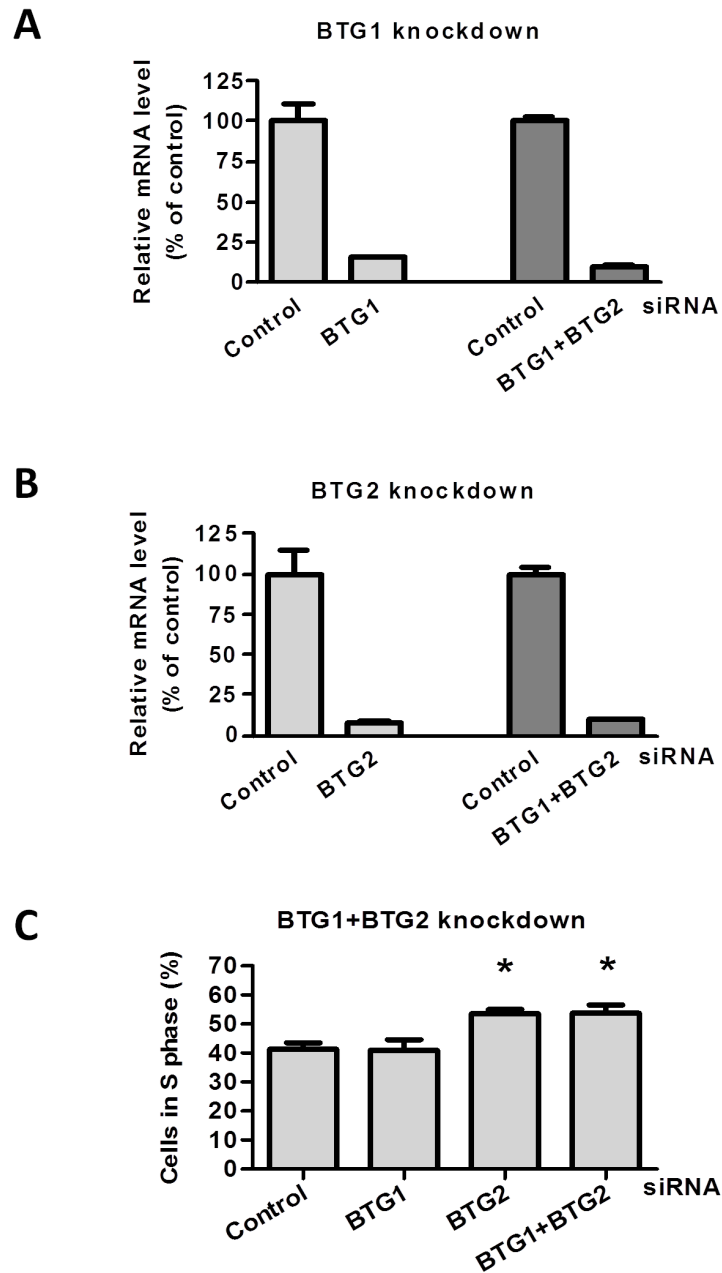


Figure 4.4 Combined knockdown of BTG1 and BTG2 does not further increase cell proliferation. (A, B) RT-qPCR analysis to confirm knockdown of BTG1 and BTG2 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. **(C)** Combined knockdown of BTG1 and BTG2 did not cause a further increase in the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

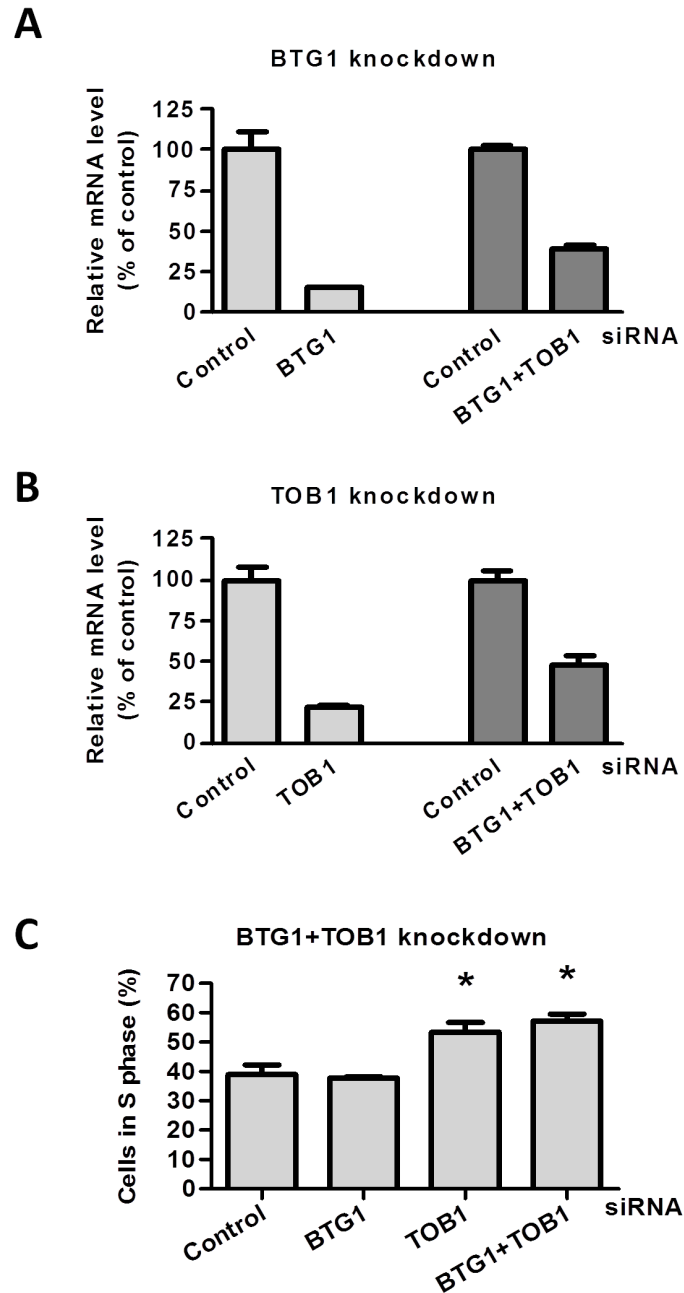


Figure 4.5 Combined knockdown of BTG1 and TOB1 does not further increase cell proliferation. (A, B) RT-qPCR analysis to confirm knockdown of BTG1 and TOB1 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. **(C)** Combined knockdown of BTG1 and TOB1 did not cause a further increase in the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

Combined knockdown of BTG1 and TOB2 was confirmed through RT-qPCR analysis with GAPDH as a reference gene (Figure 4.6A, 4.6B). The knockdown efficiency was similar for the single and dual knockdown of BTG1 and TOB2. Combined knockdown of BTG1 and TOB2 had no effect on the numbers of cells progressing into S-phase demonstrating that there is no redundancy between the proteins (Figure 4.6C).

Similarly, no effect on cell proliferation was observed when knockdown of TOB2 was combined with knockdown of BTG2, or TOB1 (Figure 4.7C, 4.8C). Again, (combined) knockdown of TOB2 and BTG2 or TOB1 was confirmed by RT-qPCR analysis with GAPDH as a reference gene (Figure 4.7A, 4.7B, 4.8A 4.8B).

In summary, combined knockdown of BTG1 or TOB2 with the remaining expressed BTG/TOB genes, BTG1, BTG2, TOB1 and TOB2 does not cause a further increase in cell proliferation when compared to the single knockdown of BTG2 or TOB1. This suggests that BTG1 and TOB2 do not influence cell cycle progression in MCF-7 cells.

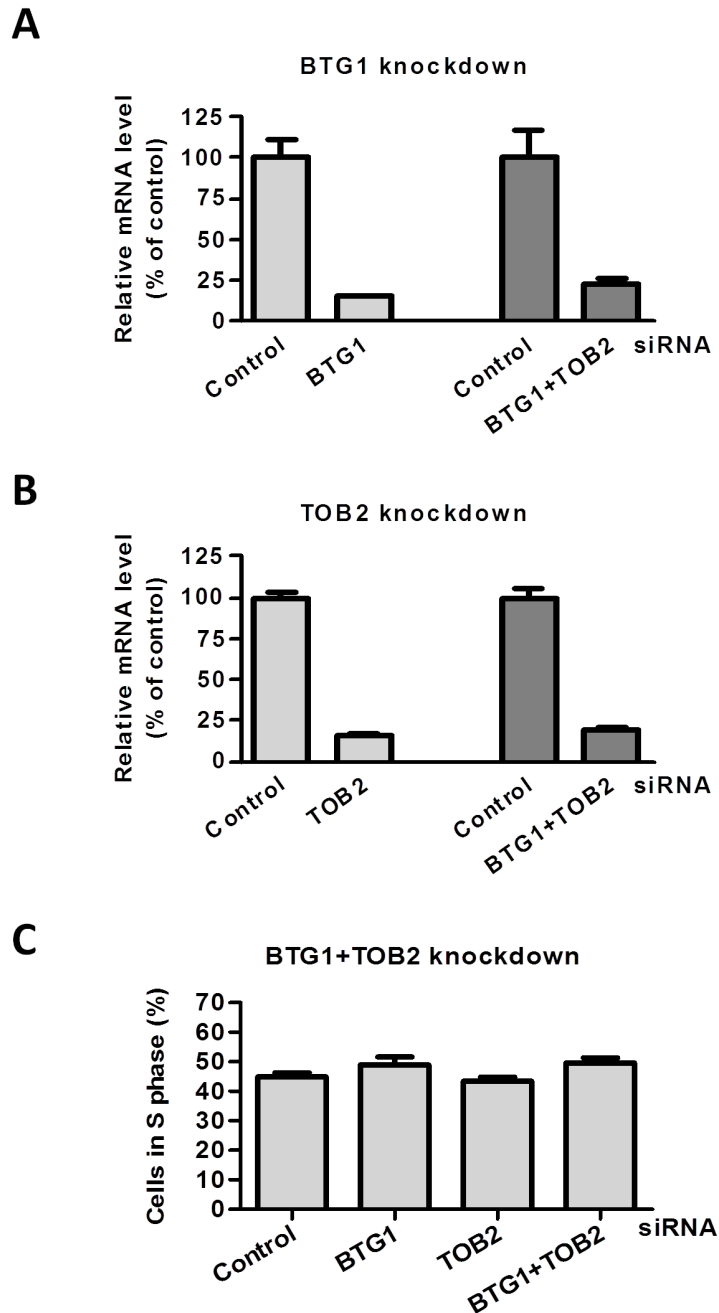


Figure 4.6 Combined knockdown of BTG1 and TOB2 does not further increase cell proliferation. (A, B) RT-qPCR analysis to confirm knockdown of BTG1 and TOB2 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. **(C)** Combined knockdown of BTG1 and TOB2 did not cause a further increase in the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

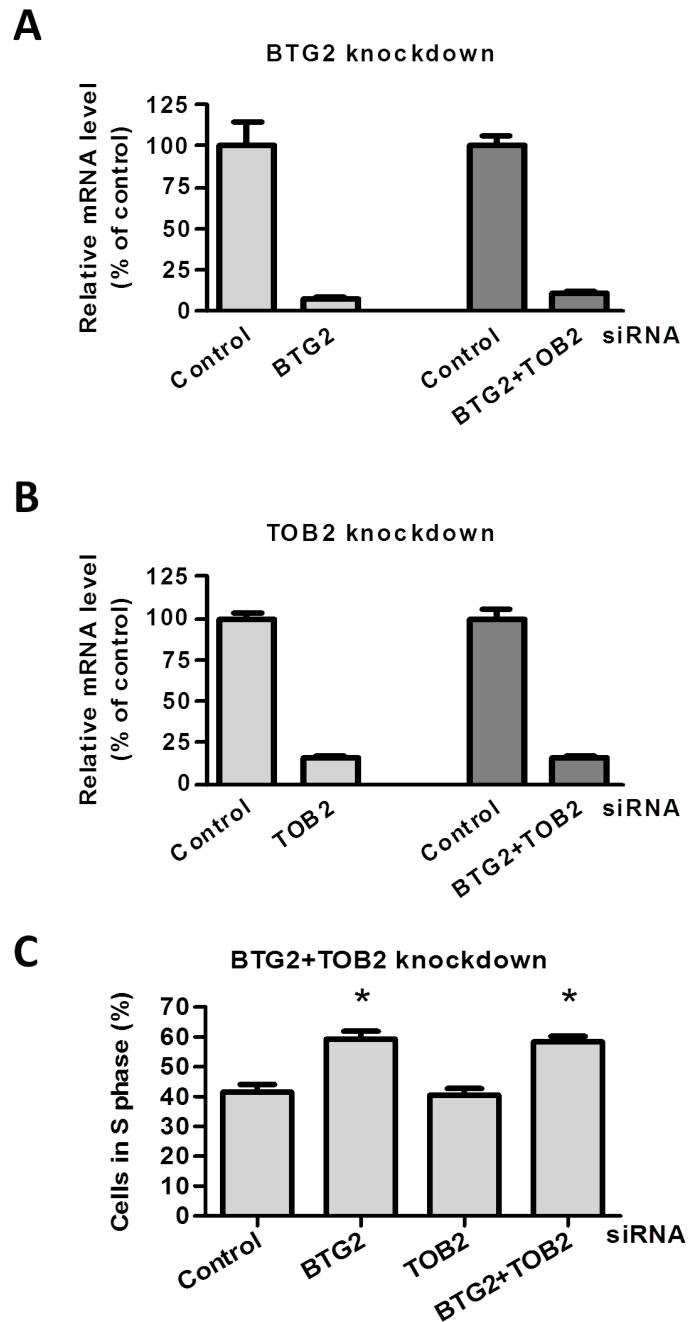


Figure 4.7 Combined knockdown of BTG2 and TOB2 does not further increase cell proliferation. (A, B) RT-qPCR analysis to confirm knockdown of BTG2 and TOB2 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. (C) Combined knockdown of BTG2 and TOB2 did not cause a further increase in the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

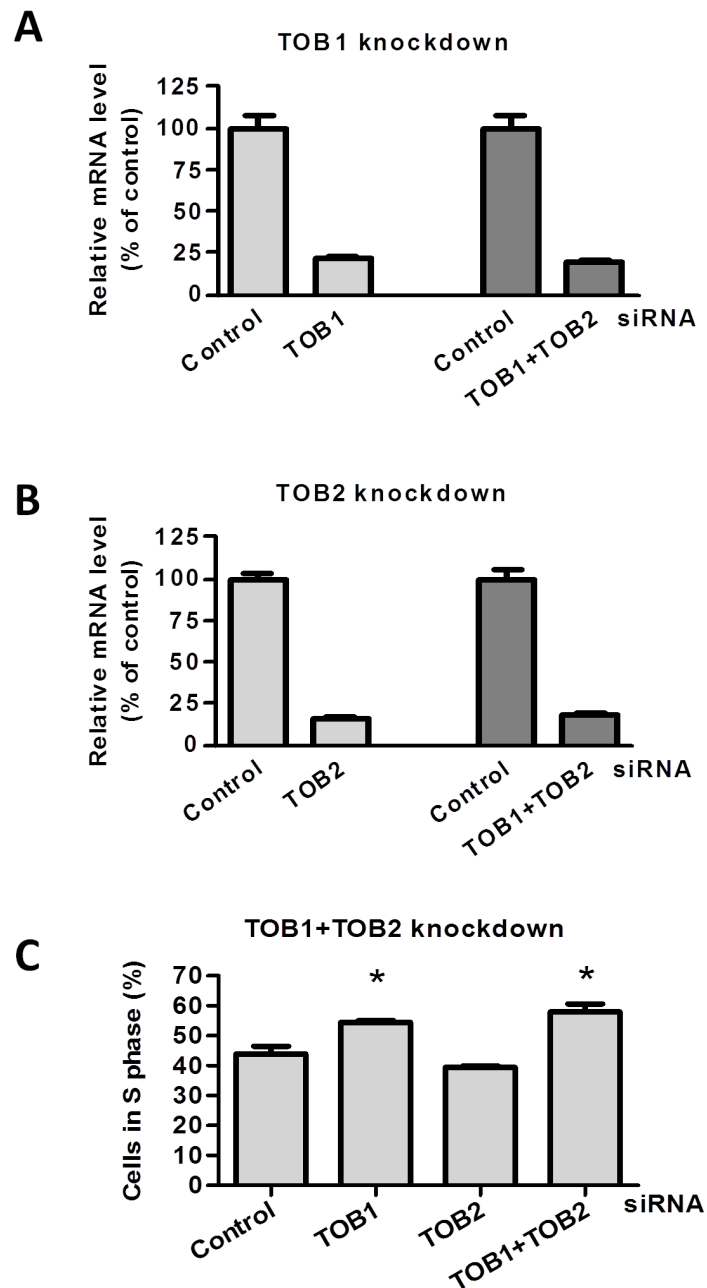


Figure 4.8 Combined knockdown of TOB1 and TOB2 does not further increase cell proliferation. (A, B) RT-qPCR analysis to confirm knockdown of TOB1 and TOB2 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. (C) Combined knockdown of TOB1 and TOB2 did not cause a further increase in the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA).

By contrast, the percentage of cells in S-phase increased significantly upon combined knockdown of BTG2 and TOB1 in comparison to single knockdown of BTG2 or TOB1 (Figure 4.9C). As before, combined knockdown of BTG2 and TOB1 was confirmed through RT-qPCR analysis with GAPDH as a reference gene. The knockdown efficiency was comparable for single and dual knockdown of BTG2 and TOB1 (Figure 4.9A, 4.9B). Interestingly, the increase in S-phase cells caused by the combined knockdown of BTG2 and TOB1 is lower than would be expected for a simple additive effect suggesting that BTG2 and TOB1 may share overlapping functions.

In summary, four of the six BTG/TOB proteins are expressed in MCF-7 cells. However, of these only two, BTG2 and TOB1, are involved in the regulation of MCF-7 cell proliferation. The effect of BTG2 and TOB1 may be partially overlapping, however, combined knockdown does cause a further increase in cell proliferation indicating BTG2 and TOB1 may poses some unique functions.

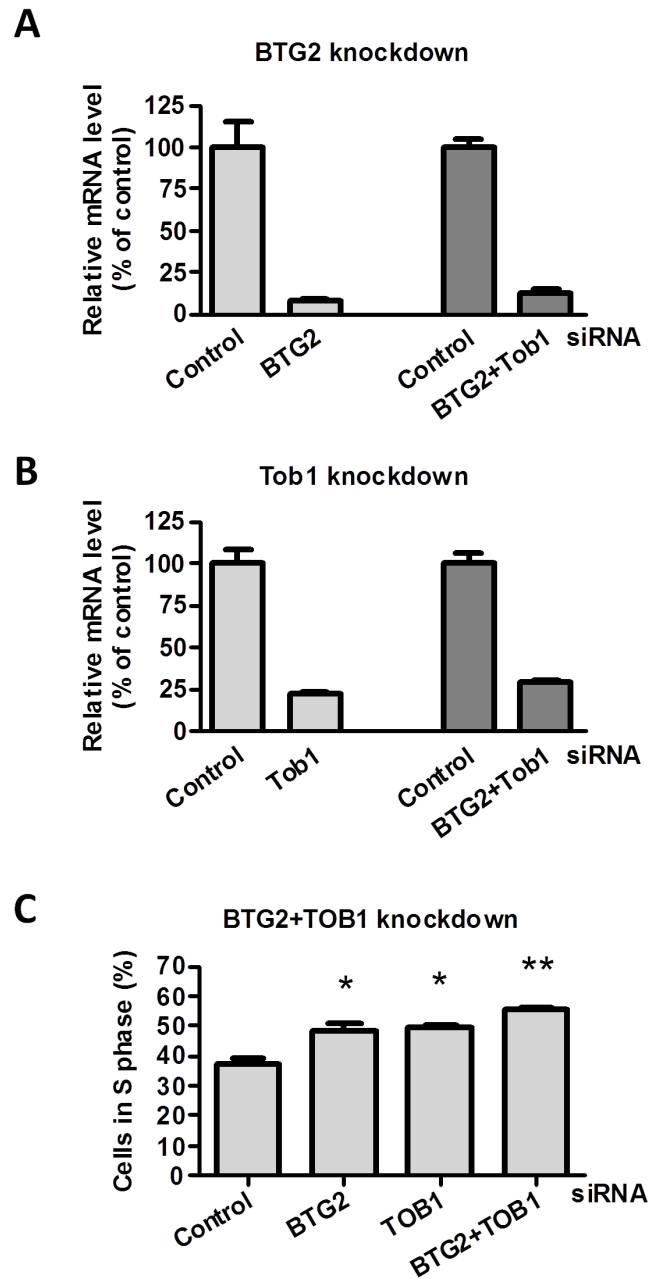


Figure 4.9 Increased cell proliferation upon combined knockdown of BTG2 and TOB1. (A, B) RT-qPCR analysis to confirm knockdown of BTG2 and TOB1 in MCF-7 cells after siRNA transfection. 48 h after siRNA treatment total RNA was isolated and subjected to RT-qPCR analysis using GAPDH as a reference. (C) Combined knockdown of BTG2 and TOB1 further increased the number of cells in S-phase. Cells in S-phase were detected by pulse-labelling using the nucleoside analogue EdU 48 h after siRNA transfection. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to non-targeting control siRNA). ** $p < 0.05$ (compared to BTG2 or TOB1 knockdown).

4.4 Generation of mammalian and HA-BTG2 and HA-TOB1 expression plasmids

To further investigate the role of BTG2 and TOB1 in the regulation of cell proliferation in MCF-7 cells, mammalian expression plasmids containing cDNAs encoding HA-tagged BTG2 and HA-TOB1 were generated. To introduce the HA tag, the BTG2 cDNA was removed from plasmid pcDNA4-TO-Flag-BTG2 using the BamHI and EcoRV restriction sites. The BTG2 cDNA was PCR amplified to include a BamHI restriction site followed by a sequence encoding the HA epitope 5' of the ATG start codon and an XhoI restriction site 3' of the stop codon. This PCR fragment was ligated into the SmaI restriction site of the pBluescript II KS(+) cloning vector. The pBluescript-HA-BTG2 plasmid was digested with BamHI and EcoRV to release the BTG2 cDNA, which was ligated into pcDNA4-TO-Flag via the BamHI and EcoRV restriction sites (Figure 4.10A).

To introduce the TOB1 cDNA into plasmid pCMV5-HA, TOB1 was amplified by PCR to include XhoI restriction sites 5' of the ATG start codon and 3' of the stop codon. This PCR fragment was ligated into the SmaI restriction site of the pBluescript II KS(+) cloning vector. The pBluescript-TOB1 plasmid was digested with XhoI to release the TOB1 cDNA, which was ligated into pCMV5-HA digested with SalI (Figure 4.10B). To introduce the desired point mutations resulting in amino acid changes W103A of BTG2 and W93A of TOB1, DNA oligos were designed containing the relevant modified codons and used for site directed mutagenesis. Plasmids containing the desired mutations were verified by DNA sequencing.

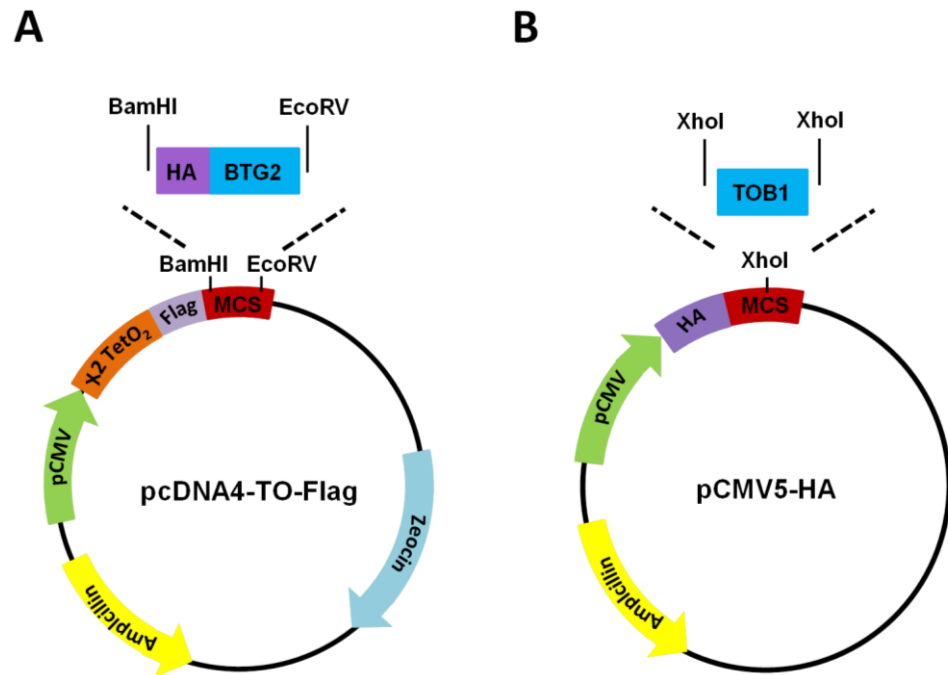


Figure 4.10 Generation of HA-BTG2 and HA-TOB1 expression vectors. (A) Construction of plasmid pcDNA4-TO-Flag-HA-BTG2. The BTG2 cDNA was removed from pcDNA4-TO-Flag-BTG2 using the BamHI and EcoRV restriction digestion. The BTG2 cDNA was PCR amplified to include the BamHI restriction site followed by a sequence encoding the HA epitope 5' of the AUG start codon. The reverse primer included an XhoI restriction site 3' of the stop codon. This PCR fragment was ligated into cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-HA-BTG2. The HA-BTG2 cDNA was excised with BamHI and EcoRV and subcloned into plasmid pcDNA4-TO-Flag. **(B) Construction of plasmid pCMV5-HA-TOB1.** The human TOB1 cDNA was amplified by reverse transcriptase PCR using total RNA isolated from MCF-7 cells as a template. Primers included XhoI restriction sites 5' of the ATG start codon and 3' of the stop codon. This PCR fragment was ligated into cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-TOB1. The TOB1 cDNA was excised with XhoI and subcloned into plasmid pCMV5-HA using the SmaI restriction site in the polylinker.

4.5 Generation of MCF-7 cell lines containing inducible BTG2 cDNA expression cassettes

To study the effect of the BTG2-Caf1a (CNOT7)/Caf1b (CNOT8) interaction on cell proliferation in MCF-7 cells, stable cell lines were generated that allow inducible expression of BTG2 and BTG2 W103A. Inducible stable cell lines are advantageous over transient transfection, because plasmid expression is limited to 24-72 h during transient transfection.

To generate stable cell lines with inducible BTG2 expression, MCF-7 TR cells were used. MCF-7 TR cells were generated by transfection of MCF-7 cells with plasmid pcDNA6/TR that expresses the Tet repressor protein (Invitrogen). The BTG2 cDNA was introduced through plasmid pcDNA4-TO-Flag-HA-BTG2. The Tet repressor acts to inhibit expression of plasmid pcDNA4-TO in the absence of doxycycline due the binding of the Tet repressor onto the Tet operator (TO) sequences located upstream of the BTG2 cDNA. Repression is relieved by the addition of doxycycline (a tetracycline derivative) to the culture medium, which binds to the Tet repressor thereby interfering with its ability to bind Tet operator sequences (Figure 4.11A).

Thus, MCF-7 TR cells were transfected with pcDNA4-TO, pcDNA4-TO-Flag-HA-BTG2, pcDNA4-TO-Flag-HA-BTG2 H53A or pcDNA4-TO-Flag-HA-BTG2 W103A. After selection with zeocin, clones with stably integrated expression cassettes were expanded in 24-well plates. Once sufficient cell numbers were obtained, clonal cells were seeded into six well culture plates in medium containing doxycycline (1.0µg/ml) to induce cDNA expression. Total cell lysates were screened for BTG2 expression by western blot analysis (Figure 4.11B).

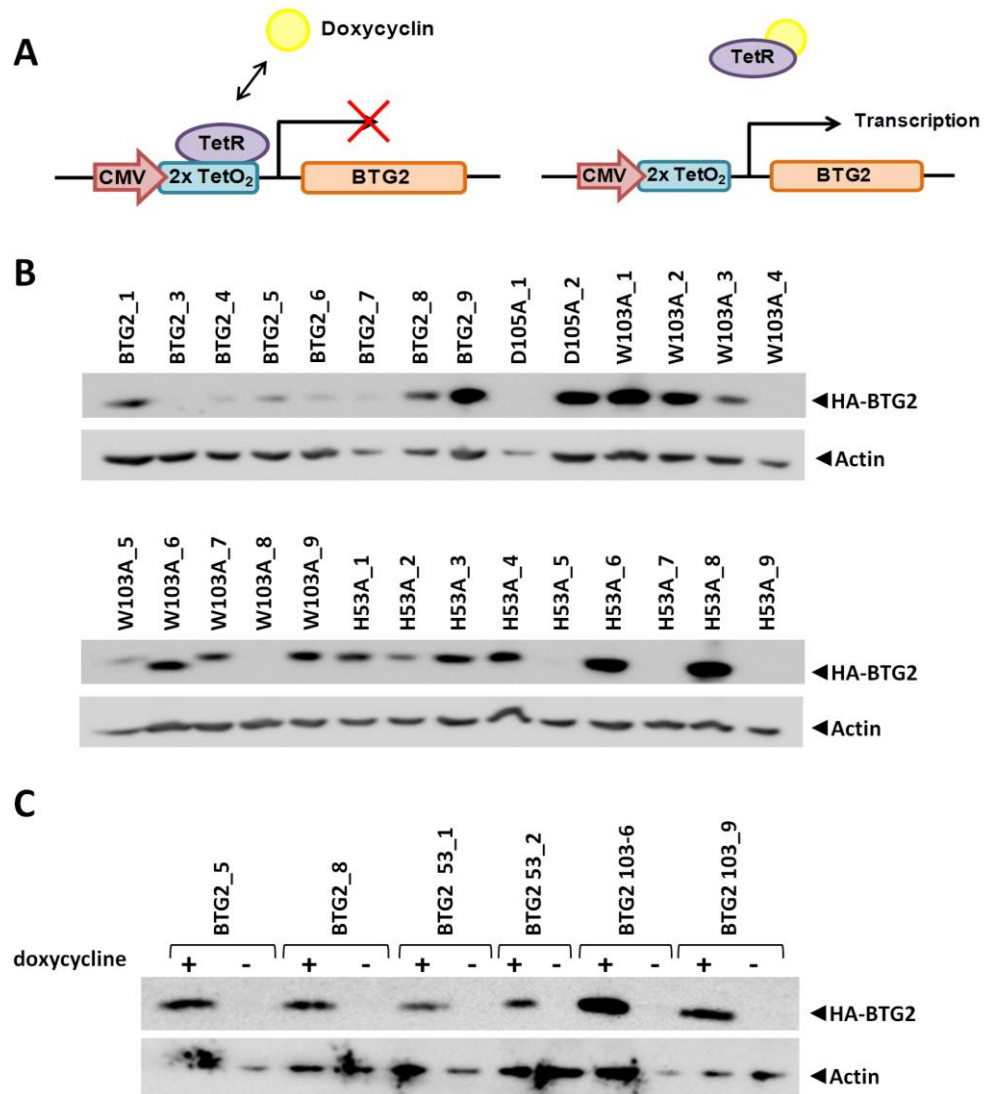


Figure 4.11 Screening for BTG2 expression in MCF-7 TR stable cell lines. (A) Schematic representing the inducible expression cassette of plasmid pcDNA4-TO-Flag-HA-BTG2. **(B)** Identification of MCF-7 TR clones with inducible expression of HA-BTG2, HA-BTG2 H53A, HA-BTG2 W103A or HA-BTG2 D105A. Expression of plasmid pcDNA4-TO containing the BTG2 cDNA was induced by the addition of doxycycline to the cell media. After 48 h total cell lysates were produced and subjected to western blot analysis. **(C)** MCF-7 TR cell lines expressing equal levels of HA-BTG2, HA-BTG2 H53A or HA-BTG2 W103A. MCF-7 TR BTG2 stable cell line clones were passaged twice in the absence of doxycycline before vector expression was induced again by the addition of doxycycline. After 48 h total cell lysates were produced and subject to western blot analysis.

As expected, the level of BTG2 varied across the clones as did the cell morphology and rate of proliferation. Clones displaying abnormal morphology or proliferation were discarded. The production of MCF-7 TR cells expressing BTG2 D105A was abandoned due to the limited number of positive clones and because of the successful expression of BTG2 H53A, the other partial Caf1a (CNOT7)/Caf1b (CNOT8) interaction mutant. From the remaining clones, two displaying relatively equal expression of BTG2, BTG2 H53A or BTG2 W103A were passaged twice in the absence of doxycycline before BTG2 expression was induced again. Total cell lysates were produced and subjected to western blot analysis to identify which clones stably expressed BTG2. The western blot shows that in the absence of doxycycline there is no background expression of BTG2 (Figure 4.11C). In addition, the clones showed relatively equal expression of BTG2 after doxycycline treatment apart from BTG2 W103A clone 6, which had a higher level of BTG2 expression. The clones taken for experimental validation were: BTG2 clone 5, BTG2 H53A clone 2 and BTG2 W103A clone 9.

4.6 The BTG2 anti-proliferative activity requires interactions with Caf1a (CNOT7)/Caf1b (CNOT8)

To investigate if the anti-proliferative activity of BTG2 is dependent upon interactions with Caf1a (CNOT7) and Caf1b (CNOT8), the rate of cell proliferation in the MCF-7 TR cell lines expressing BTG2 and BTG2 W103A were monitored. The MCF-7 TR stable cell lines pcDNA4-TO, pcDNA4-TO-Flag-HA-BTG2 and pcDNA4-TO-Flag-HA-BTG2 W103A were seeded and cultured in the presence or absence of doxycycline. The control cell line containing the empty expression vector pcDNA4-TO demonstrated that the addition of doxycycline to the cell media does not alter cell proliferation rates (Figure 4.12A). Expression of BTG2 caused a decrease in cell proliferation compared to the MCF-7 TR cells not expressing BTG2. A significant reduction

in total cell population number is evident on day six, seven and eight (Figure 4.12A). By day eight there is >25% fewer cells compared to the cell population not expressing BTG2 (Figure 4.12B). By contrast, expression of BTG2 W103A did not cause a reduction in the total number of cells compared to the cell population cultured in the absence of doxycycline (Figure 4.12A, 4.12B). To confirm the importance of BTG2 - Caf1a (CNOT7)/Caf1b (CNOT8) interaction for the BTG2 anti-proliferative effect, the percentage of cells in S-phase was also determined as a second measure of cell proliferation. MCF-7 cells containing stably integrated expression vectors pcDNA4-TO, pcDNA4-TO-Flag-HA-BTG2 and pcDNA4-TO-Flag-HA-BTG2 W103A were seeded before vector expression was induced by the addition of doxycycline. Control cells were cultured in parallel in the absence of doxycycline. The thymidine analogue EdU was added to the MCF-7 cell media for two hours to label cells in S-phase. The control cell line expressing plasmid pcDNA4-TO demonstrated that the addition of doxycycline to the cell media did not alter the number of cells entering S-phase (Figure 4.12C). After EdU incubation approximately 40% of nuclei incorporated the thymidine analogue in the pcDNA4-TO expressing control cell line. BTG2 expression caused a significant reduction in the number of cells in S-phase compared to the control cell population cultured in the absence of doxycycline. By contrast, expression of BTG2 W103A did not alter the number of cells in S-phase (Figure 4.12C). Western blot analysis of the MCF-7 TR stable cell lines cells harvested on day eight of the proliferation assay demonstrated that BTG2 expression is maintained (Figure 4.12D).

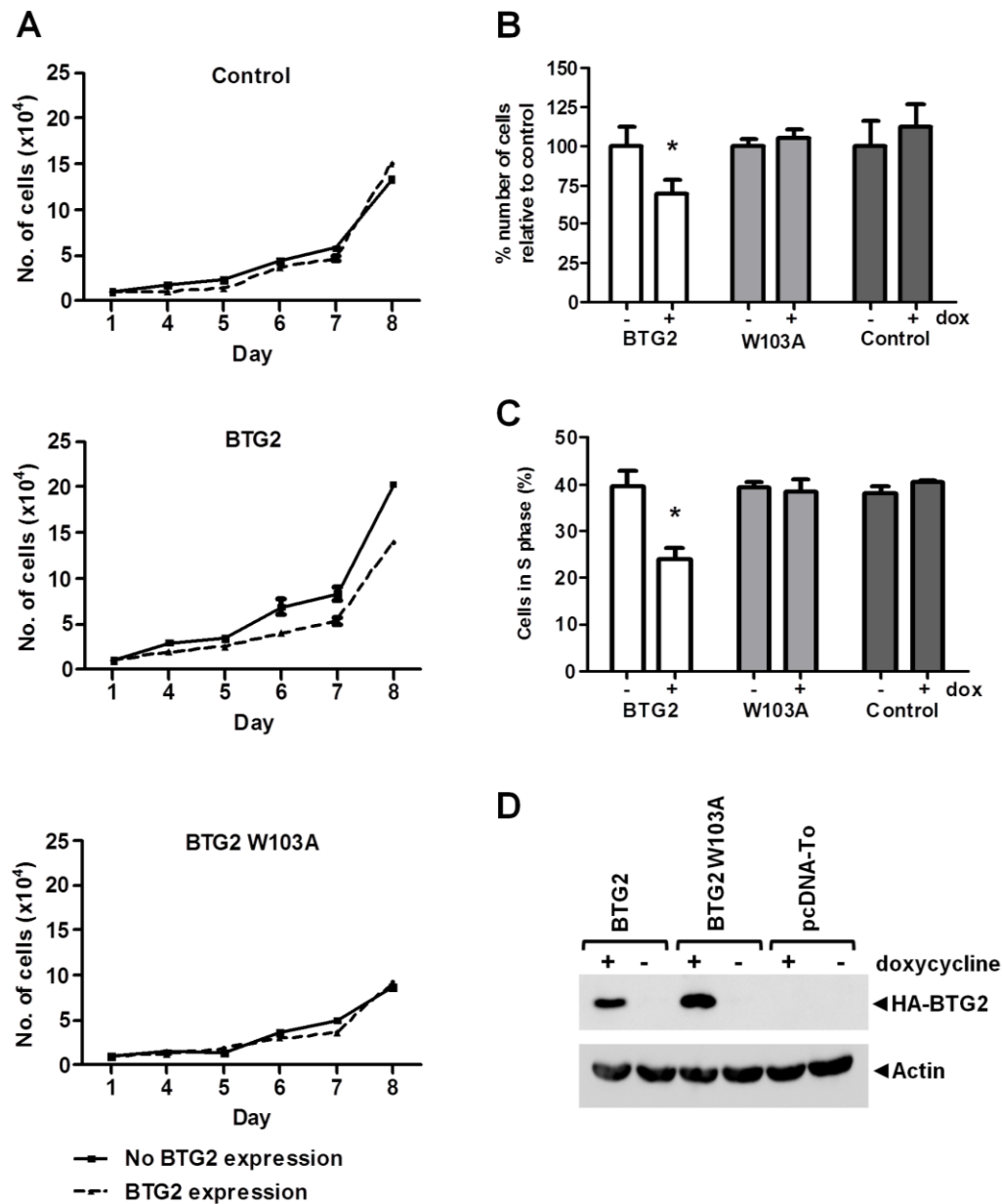


Figure 4.12 Interactions with the Caf1a (CNOT7)/Caf1b (CNOT8) deadenylase enzymes is required for the anti-proliferative activity of BTG2. (A) Reduced proliferation upon BTG2 expression. MCF-7 TR BTG2 stable cell lines were seeded at 100,000 cells per well. Expression of BTG2, BTG2 W103A or empty vector was induced by addition of doxycycline. From day four until day eight, cells were trypsinised and counted in triplicate using a haemocytometer at 24 h intervals. **(B)** Total number of cells after expression of pcDNA4-TO (control), BTG2 or BTG2 W103A for eight days. A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to control cells not treated with doxycycline). **(C)** Expression of BTG2 but

not BTG2 W103A causes a reduction in the number of cells in S phase. MCF-7 TR cell lines were seeded onto glass cover slips and expression of pcDNA4-TO, pcDNA4-TO-BTG2 or pcDNA4-TO-BTG2W103A induced by the addition of doxycycline. 48 h after induction, cells were incubated with EdU (thymidine analogue) A Student's t-test was used to calculate p-values. * $p < 0.05$ (compared to control cells not treated with doxycycline). **(D)** Similar levels of doxycycline-dependent expression of HA-BTG2 and HA-BTG2 W103A. Total cell lysates produced 8 days after induction of doxycycline-dependent expression and subject to western blot analysis using anti-HA, actin used as loading control.

The effect on cell proliferation of BTG2 H53A was also tested. This amino acid substitution produces a partial disruption in the Caf1a (CNOT7) interaction (Chapter 3). BTG2 H53A was capable of reducing cell proliferation rates to the same extent as wild type BTG2. This suggests that even a weak interaction with Caf1a (CNOT7) is sufficient for BTG2 to function as an anti-proliferative protein.

Taken together, the data demonstrates that expression of BTG2 reduces cell proliferation by preventing cell cycle progression. Moreover, cell proliferation is dependent upon interactions between BTG2 and the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8), because the reduction of cells in S-phase is not seen upon expression of BTG2 W103A.

4.7 Effect of BTG2 expression on the expression of G1/S-phase regulatory proteins

To identify whether BTG2 expression affects the cellular concentration or expression of specific G1/S-phase regulatory proteins, RT-qPCR and western blot analysis were performed. The proteins investigated were Cyclin D1, Cyclin D3, CDK4, CDK6, p21, p27, p15 and p16. Protein lysates were extracted from the MCF-7 TR pcDNA4-TO and pcDNA4-TO-Flag-HA-BTG2 stable cell lines after vector expression was induced for 72 h. The western blot analysis demonstrated the inducible expression of HA-BTG (Figure 4.13A). Actin was used as a loading control. Protein levels of Cyclin D1, Cyclin D3, CDK4, CDK6, p21, p27, p15 did not change for the pcDNA4-TO stable cell line cultured in the presence or absence of doxycycline. In addition, the amount of protein for all the cell cycle proteins tested did not change upon BTG2 expression (Figure 4.13A). A western blot was also conducted for p16 but this protein was not detected.

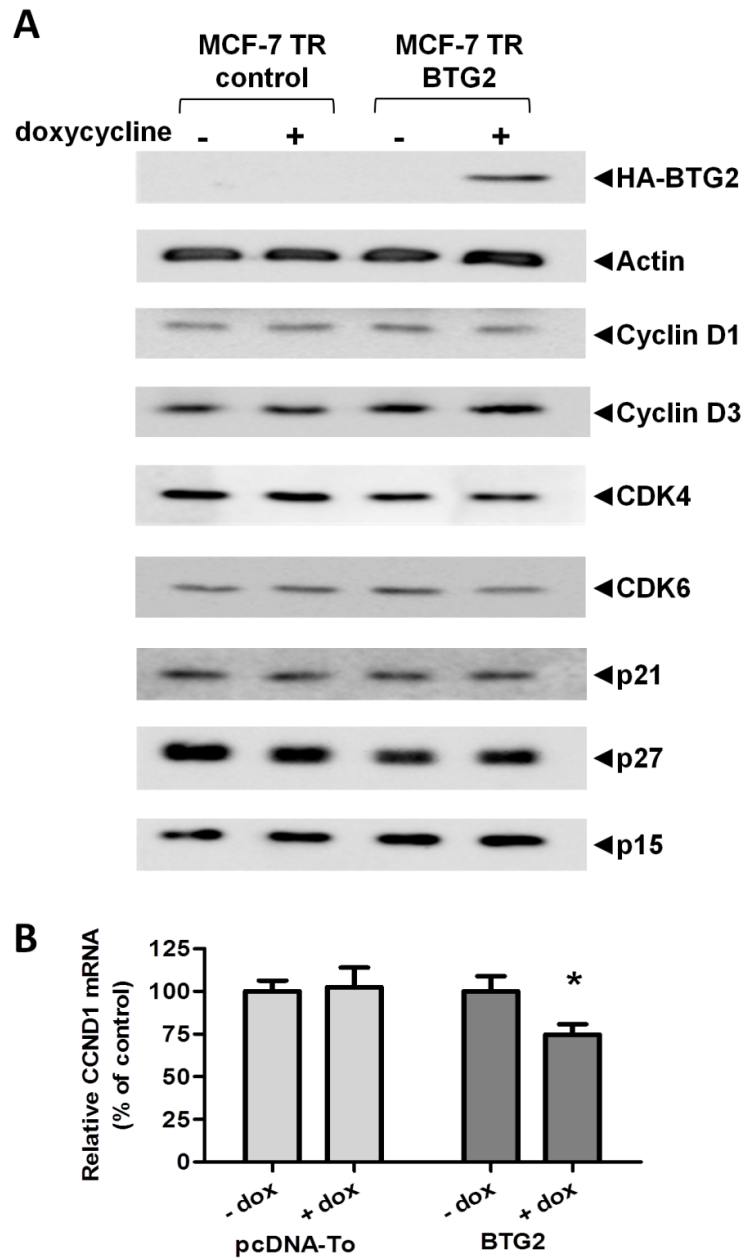


Figure 4.13 BTG2 expression causes a reduction in CCND1 (Cyclin D1) mRNA but does not affect the level of cell cycle regulatory proteins. (A) Analysis of cell cycle regulatory proteins by western blotting. BTG2 expression was induced in MCF-7 TR stable cell lines by the addition of doxycycline. 72 h after induction total cell lysates were produced and subject to western blot analysis. Actin was used as an equal loading control. **(B)** CCND1 (Cyclin D1) mRNA levels are reduced after BTG2 expression. BTG2 expression was induced in MCF-7 TR stable cell lines through the addition of doxycycline. After 48 h, total RNA was isolated and CCND1 (Cyclin D1) mRNA levels determined through RT-qPCR using GAPDH as a reference gene. Experiments carried out in triplicate. A Student's t-test was used to calculate significance-values, * $p=0.08$ (compared to control cells not treated with doxycycline).

To investigate if BTG2 is causing a transcriptional down-regulation of CCND1 (Cyclin D1), total RNA was extracted from the MCF-7 TR stable cells lines after BTG2 (or control vector) expression and subjected to RT-qPCR analysis using GAPDH as a reference gene. The analysis indicated a small reduction in CCND1 (Cyclin D1) mRNA (p-value 0.1) however, this is not statically significant and may be due to chance (Figure 4.13B).

Together the western blot and RT-qPCR data produce an inconclusive view of the pathway used by BTG2 to regulate cell cycle progression. However, the RT-qPCR analysis indicates down regulation of Cyclin D1 may be a factor which is consistent with the current published literature (Donato et al., 2007; Guardavaccaro et al., 2000; Suzuki et al., 2002).

4.8 BTG2 and TOB1 require interactions with Caf1a (CNOT7)/Caf1b (CNOT8) to reduce cell proliferation.

In addition to the use of cell lines containing stably integrated expression cassettes, transient transfections were also used to determine the effect of BTG2/TOB1 on cell proliferation. To this end, several transfection reagents were tested to identify which one combined optimal transfection efficiency with minimal effects on cell viability and proliferation. Using Genejuice (Merck), a transfection efficiency of 70% was routinely achieved (Appendix.1).

As expected, expression of BTG2, but not of BTG2 W103A, reduced the number of cells in S-phase as compared to cells transfected with empty vector (Figure 4.14A). Western blot analysis confirmed similar levels of HA-BTG2 and HA-BTG2 W103A expression (Figure 4.14B). Amino acid Trp-93 of TOB1 corresponds to Trp-103 of BTG2. As was observed for BTG2, expression of TOB1, but not of TOB1 W93A, resulted in a reduced percentage of cells in S-phase as compared to empty vector-transfected cells (Figure 4.15A).

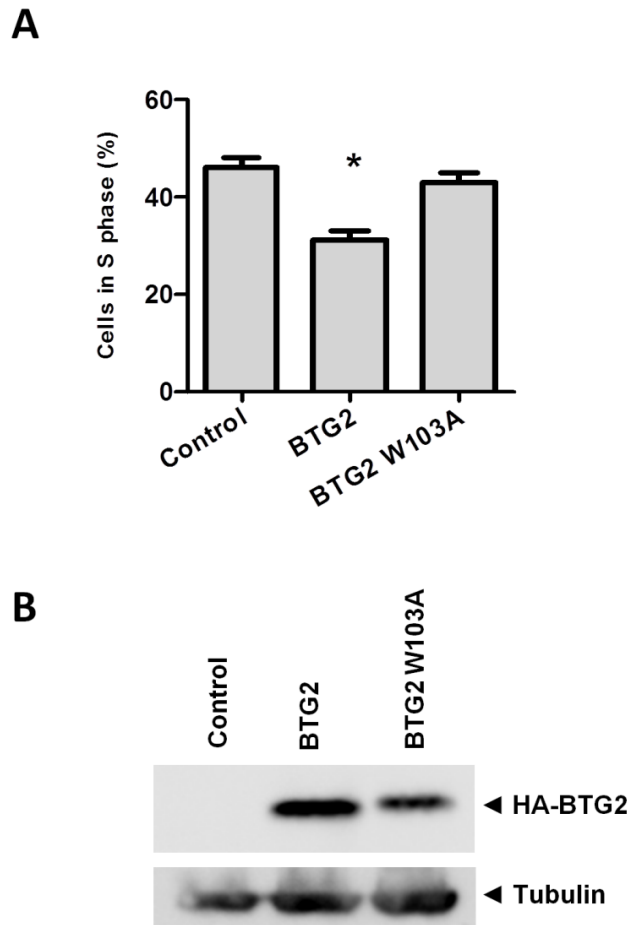
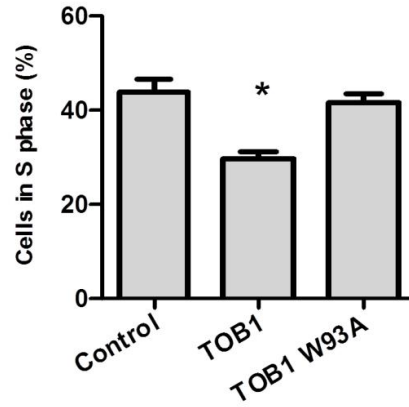


Figure 4.14 Transient expression of BTG2 but not BTG2 W103A causes a reduction in the number of cells in S-phase. (A) MCF-7 cells were seeded onto glass cover slips before transfection with pCMV5-HA-BTG2, pCMV5-HA-BTG2 W103A, or empty vector. After 48 h, cells were incubated with the thymidine analogue EdU and detected by fluorescence microscope. All samples were tested in biological triplicate and the number of nuclei was counted for three images from each slide. A Student's t-test was used to calculate significance values. * $p < 0.05$ (compared to control cells transfected with pCMV5-HA). **(B)** Western blot analysis indicating similar expression of HA-BTG2 and HA-BTG2 W103A. Total cell lysates produced 48 h after transfection and subject to western blot with anti-HA, tubulin used as a loading control.

A



B

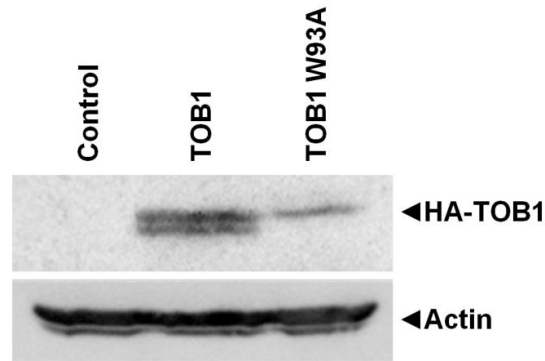


Figure 4.15 Transient expression of TOB1 but not TOB1 W93A causes a reduction in the number of cells in S-phase. (A) MCF-7 cells were seeded onto glass cover slips before transfection with pCMV5-HA-TOB1, pCMV5-HA-TOB1 W93A, or empty vector. After 48 h, cells were incubated with the thymidine analogue EdU and detected by fluorescence microscope. All samples were tested in biological triplicate and the number of nuclei were counted for three images from each slide. A Student's t-test was used to calculate significance values. * $p < 0.05$ compared to control cells transfected with pCMV5-HA. **(B)** Western blot analysis indicating similar expression of HA-BTG2 and HA-BTG2 W103A. Total cell lysates produced 48 h after transfection and subject to western blot with anti-HA, actin used as a loading control.

This result demonstrates that TOB1 also requires an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) in order to maintain its anti-proliferative activity in MCF-7 cells. Western blot analysis confirmed the expression of HA-TOB1 and HA-TOB1 W93A (Figure 4.15B). Interestingly, however, while TOB1 is represented as a double band on the western blot, TOB1 W93A migrates as a single band. Because TOB1 contains three C-terminal phosphorylation sites located on serine residues Ser-152, Ser-154 and Ser-164 (Suzuki et al., 2002), this raised the possibility that the phosphorylation status of TOB1 W93A is different from the wild type protein.

4.9 The anti-proliferative activity of BTG2 and TOB1 is not affected by post-translational phosphorylation

Both BTG2 and TOB1 are phosphorylated at serine residues located outside the BTG domain. Substitution of these amino acids with alanine residues prevents their inactivation and results in increased anti-proliferative activity (Hong et al., 2005; Suzuki et al., 2002). Western blot analysis of TOB1 indicated that it is subject to post-translation modifications which may affect its anti-proliferative activity. Therefore, we reasoned that the substitution of the serine residues combined with the alterations W103A of BTG2 or W93A of TOB1 might reveal weak residual anti-proliferative activity of BTG2 and TOB1 that is independent of the interaction with the Caf1a and Caf1b deadenylase enzymes.

To investigate if TOB1 is subject to phosphorylation that affects its anti-proliferative activity, the three serine residues subject to phosphorylation were substituted for alanine. The desired point mutations were introduced into pCMV5-HA-TOB1 via site directed mutagenesis. Correct clones were identified through DNA sequencing. MCF-7 cells were transfected with pCMV5-HA, pCMV5-HA-TOB1 or pCMV5-HA-TOB1 S-A (serine to alanine mutations) and after 48 h cells in S-phase were labelled by EdU. As seen previously, the expression of TOB1 reduced the number of cells in S-phase

compared to the pCMV5-HA transfected control cells. The expression of TOB1 S-A reduced the number of cells in S-phase to the same extent as wild type TOB1, suggesting that the phosphorylation status of TOB1 does not affect its anti-proliferative activity in MCF-7 cells (Figure 4.16A). Western blot analysis confirmed the expression of HA-TOB1 and HA-TOB1 S-A (Figure 4.16B). As expected, TOB1 S-A is represented as a single band on the western blot that corresponded to the lower band of wild type TOB1 indicating that TOB1 S-A is not phosphorylated.

To determine if TOB1 phosphorylation is masking any anti-proliferative activity of TOB1 W93A the numbers of cells in S phase were determined after TOB1 S-A W93A expression. MCF-7 cells were transfected with the appropriate plasmid before cells in S-phase were labelled by EdU as describe previously. Expression of TOB1 S-A reduced the number of cells in S-phase compared to the pCMV5-HA transfected control cells. Interestingly, expression of TOB1 S-A W93A did not cause any reduction in the number of cells in S-phase demonstrating that phosphorylation is not affecting TOB1 activity. This confirms that the loss of anti-proliferative activity witnessed in TOB1 W93A is caused by preventing the TOB1-Caf1a (CNOT7/Caf1b (CNOT8) interaction. Western blot analysis confirmed the expression of the HA-TOB1S-A and HA-TOB1 W93A S-A (Figure 4.16D).

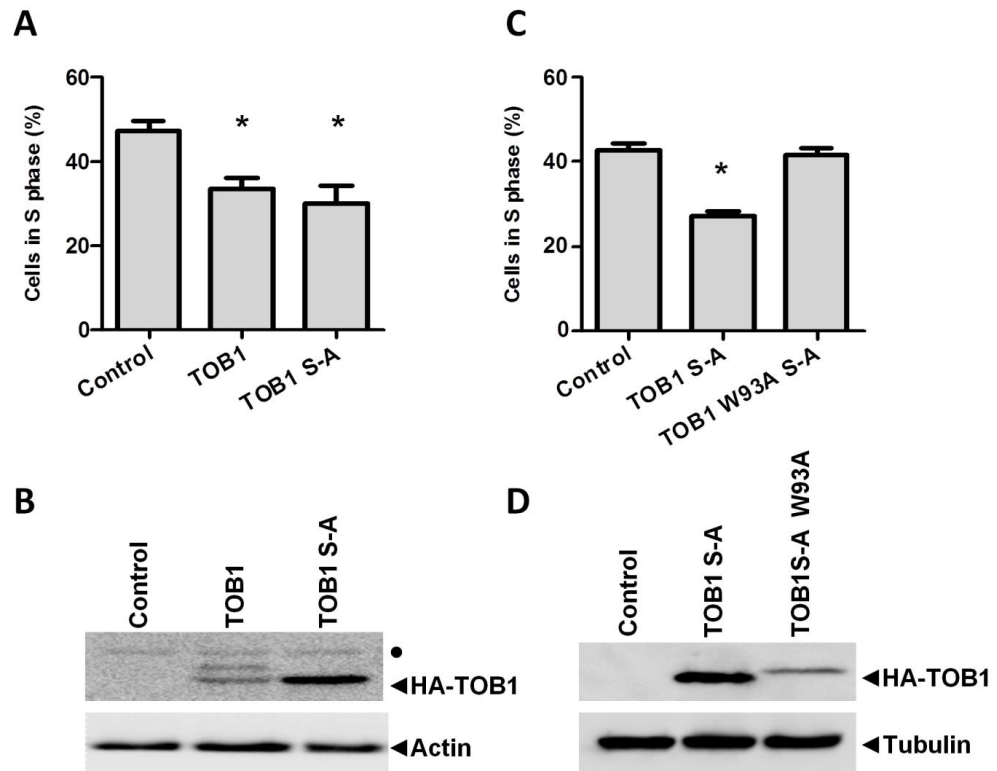


Figure 4.16 The phosphorylation status of TOB1 does not affect its anti-proliferative activity in MCF-7 cells. **(A)** MCF-7 cells were seeded onto glass cover slips and transfected with pCMV5-HA, pCMV5-HA-TOB1 or pCMV5-HA-TOB1 S-A. 48 h after transfection cells were incubated with the thymidine analogue EdU and detected after incubation with Alexa Fluor 488 azide. Cells were counter stained with Hoechst 33342 to allow total cells to be counted on the LSM fluorescence microscope. Samples were tested in biological triplicate; the number of nuclei were counted for three images from each slide. Students t-test used to calculate statistical significance, * indicates a p value of < 0.05 compared to control cells transfected with pCMV5-HA. **(B)** Western blot analysis to show the expression of HA-TOB1 and HA-TOB1 S-A. Total cell lysates produced 48 h after transfection and subject to western blot with anti-HA, • indicated none specific band, actin used as a loading control. **(C)** MCF-7 cells were seeded onto cover slips before being transfected with pCMV5-HA, pCMV5-HA-TOB1 S-A or pCMV5-HA-TOB1 W93A S-A. Cell were treated with EdU as previously described in (A), * indicates a p value of < 0.05 compared to control cells transfected with pCMV5-HA. **(D)** Western blot analysis to show the expression of HA-TOB1 S-A and HA-TOB1 W93A S-A. Western blot conducted as in (B).

BTG2 contains two phosphorylation sites at Ser-147 and Ser-149 within its C-terminal region. To determine if the phosphorylation status of BTG2 or BTG2 W103A is affecting their anti-proliferative activity Ser-147 and Ser-149 were replaced with alanine using site-directed mutagenesis. MCF-7 cells were transfected with pCMV5-HA, pCMV5-HA-BTG2, pCMV5-HA-BTG2S-A, pCMV5-HA-BTG2 W103A and pCMV5-HA-BTG2 W103A S-A and after 48 h EdU was added for two hours to label all cells in S-phase. Expression of BTG2 S-A reduced the number of cells entering S-phase however, this reduction was not significantly higher than seen for wild type BTG2 (Figure 4.17A). By contrast, no effect on S-phase was observed upon expression of BTG2 S-A W103A as compared to empty vector transfected cells. As seen with TOB1 W93A, the phosphorylation status of BTG2 W103A does not cause its loss of anti-proliferative activity. Western blot analysis confirmed the expression of the HA-BTG2, HA-BTG2S-A, HA-BTG2 W103A and HA-BTG2 W103A S-A (Figure 4.17B). Together it can be concluded that the amino acid substitutions W103A and W93A of BTG2 and TOB1, respectively, do not result in increased inactivation by phosphorylation. Therefore, the anti-proliferative activity of BTG2 and TOB1 is mediated via interactions with the Caf1a and Caf1b deadenylase enzymes.

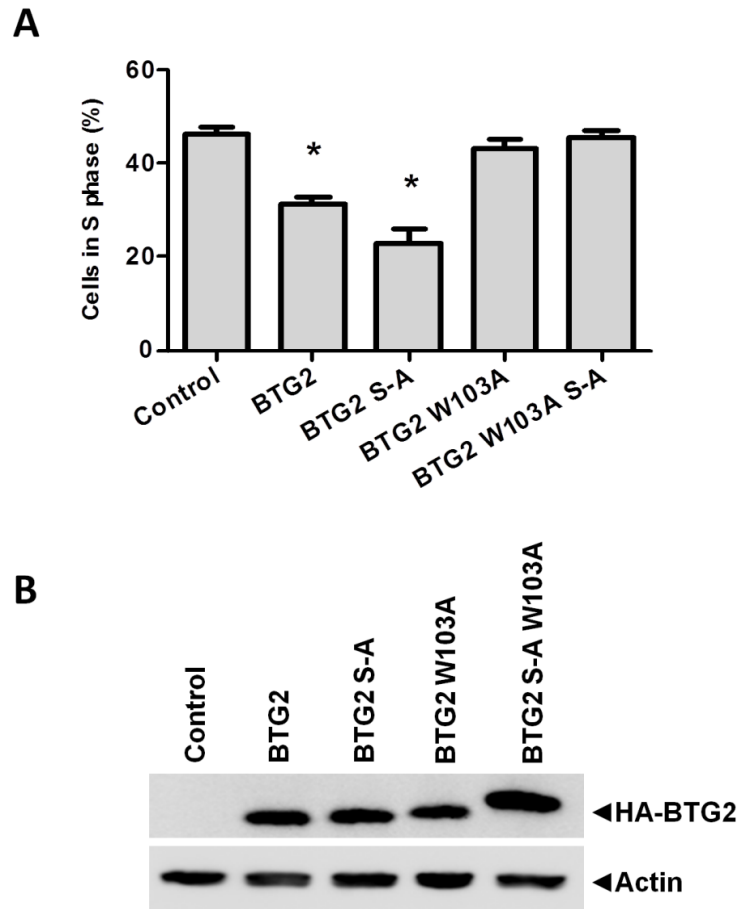


Figure 4.17 The phosphorylation status of BTG2 does not affect its anti-proliferative activity in MCF-7 cells. (A) MCF-7 cells were seeded onto glass cover slips before being transfected with pCMV5-HA, pCMV5-HA-BTG2, pCMV5-HA-BTG2 S-A, pCMV5-HA-BTG2 W103A or pCMV5-HA-BTG2 W103A S-A. 48 h after transfection cells were incubated with the thymidine analogue EdU and detected after incubation with Alexa Fluor 488 azide. Cells were counter stained with Hoechst 33342 to allow total cells to be counted on the LSM fluorescence microscope. All samples were tested in biological triplicate and the number of nuclei were counted for three images from each slide. Students t-test used to calculate statistical significance, * indicates a p value of < 0.05 compared to control cells transfected with pCMV5-HA. **(B)** Western blot analysis to show the expression of HA-BTG2, HA-BTG2 S-A, HA-BTG2 W103A and HA-BTG2 W103A S-A. Total cell lysates produced 48 h after transfection and subject to western blot with anti-HA, tubulin used as a loading control.

4.10 Discussion

Of the six BTG/TOB proteins, only four are expressed in MCF-7 cells: TOB1, TOB2, BTG1 and BTG2. Surprisingly, the anti-proliferative activity does not correlate with their relative mRNA level. While the expression of BTG2 was significantly lower than that of TOB1, knockdown of BTG2 or TOB1 resulted in a similar increase of cell proliferation. Furthermore, despite displaying significantly higher mRNA levels compared to BTG2, neither knockdown of BTG1 nor TOB2 affected cell proliferation. This is surprising because BTG1 and BTG2 share a sequence similarity of 70%, the same observation can be made for TOB1 and TOB2 who share a sequence similarity of 73%. This high level of sequence conservation suggests a level of functional redundancy between the pairs of BTG/TOB proteins. It would be interesting to establish the relative protein levels of the expressed BTG/TOB proteins in MCF-7 cells, because the presence of mRNA does not directly indicate the presence of a function protein. To this end several antibodies were tested to determine if endogenous BTG2 or TOB1 could be detected in MCF-7 cells via western blot analysis. None of the antibodies screened were able to detected endogenous or over expressed BTG2 or TOB1, (Appendix.2). Further analysis to characterise the 3' UTR of the BTG/TOB mRNAs may provide new insights into the translational regulation of these genes which may account for the differences between the gene expression microarray data and effect on cell proliferation. Furthermore, to date we do not know whether BTG1 and TOB2 possess critical somatic mutations in the MCF-7 cells abrogating their activity.

Dual knockdown of the BTG/TOB proteins demonstrated that there is no functional redundancy masking the possible roles of BTG1 and TOB2. By contrast, combined knockdown of BTG2 and TOB1 resulted in a further increase in cell proliferation. An interesting progression for this work would be to establish whether BTG2 and TOB1 regulate the mRNA abundance of the same genes by using microarray expression profiling. This would establish whether BTG2 and TOB1 influence cell proliferation via regulating the

abundance and translational efficiency of the same set of genes or whether they target different mechanisms.

The interaction between BTG/TOB proteins and the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8) is well established (Ikematsu et al., 1999; Prevot et al., 2001; Rouault et al., 1998; Winkler, 2010; Yoshida et al., 2001). However, the importance of the interactions in the anti-proliferative activity of BTG/TOB proteins was unclear. Disrupting the TOB1-Caf1a (CNOT7) interaction through an amino acid substitution in Caf1a (CNOT7) prevented the anti-proliferative effect witnessed after TOB1 over-expression (Horiuchi et al., 2009). Conversely disrupting the BTG2-Caf1a (CNOT7) interaction again through amino acid substitution in Caf1a (CNOT7) did not significantly alter cell proliferation rates (Aslam et al., 2009). These divergent conclusions were obtained by preventing the BTG/TOB interaction with Caf1a (CNOT7)/Caf1b (CNOT8) through amino acid substitutions in Caf1a (CNOT7)/Caf1b (CNOT8). Here I used the amino acid substitution W103A in BTG2 and W93A in TOB1, which disrupts the BTG2/TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction to investigate the anti-proliferative effect. Expression of BTG2 or TOB1 in MCF-7 cells inhibited cell proliferation, which was not observed upon expression of BTG2 W103A and TOB1 W93A. Based on these results, it can be concluded that the anti-proliferative activity of BTG2 and TOB1 requires interactions with the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylases. This conclusion is consistent with that of Ezzeddine et al (2012), who independently arrived at the same conclusion during the course of this work. Furthermore, to verify this conclusion cell proliferation was monitored after BTG2/TOB1 expression in U2OS cells, the cell line used in the Ezzeddine et al (2012) study. Here, BTG2 and TOB1 also required an interaction with Caf1a (CNOT7) and Caf1b (CNOT8) to reduce the rate of cell proliferation, analogous to the published result by Ezzeddine et al (2012) (Appendix.3).

During the course of this study, western blot analysis of TOB1 indicated that it may be subject to post-translation modifications due to its double band

pattern on western blot. The activity of BTG2 and TOB1 is inhibited by the phosphorylation of serine residues located in their C-terminal domain (Hong et al., 2005; Suzuki et al., 2001). The phosphorylation of TOB1 has been shown to affect TOB1 activity in lung and thyroid cancer patient samples where hyper-phosphorylated TOB1 heightens cancer progression. (Ito et al., 2005; Iwanaga et al., 2003; Yoshida et al., 2003a). To date there has been no published data demonstrating that phosphorylation of TOB1 or BTG2 alters anti-proliferative activity or cancer progression in breast carcinomas. Western blot analysis during this study suggested that a significant fraction of TOB1 is phosphorylated in MCF-7 cells. Furthermore, introduction of the W93A amino acid substitution appears to increase TOB1 phosphorylation as seen through the western blot analysis. However, replacing residues Ser-152, Ser-154 and Ser-164 of TOB1 for alanine, which prevents phosphorylation, did not result in increased anti-proliferative activity. Additionally, expression of TOB1 S-A W93A did not reveal any weak residual anti-proliferative activity indicating the phosphorylation status of TOB1 is not affecting its anti-proliferative activity. Expression of BTG2 S147A, S149A resulted in an decrease in cell proliferation comparable to wild type BTG2. In agreement with TOB1, BTG2 S-A W103A did not display an anti-proliferative activity. It can therefore be concluded that inactivation by phosphorylation is not the main mechanism of inactivation of BTG/TOB activity in MCF-7 cells. The reduction in anti-proliferative activity demonstrated by TOB1 W93A and BTG2 W103A is as a result of disrupting the Caf1a (CNOT7)/Caf1b (CNOT78) interaction. This conclusion is shared with Eddeddine et al (2012) who demonstrated that phosphorylation of TOB1 and TOB2 does not affect anti-proliferative activity in U2OS cells.

Additional experiments were conducted to determine if the sub-cellular localisation of BTG2 changed due to the W103A amino acid substitution. Both BTG2 and TOB1 have been shown to have increased anti-proliferative effects when localised to the cytoplasm (Ito et al., 2005; Iwanaga et al., 2003; Kawakubo et al., 2006; Kawamura-Tsuzuku et al., 2004; Maekawa et al.,

2002; Yoshida et al., 2003a). To this end immunofluorescence was conducted. However, due to poor staining intensity the localisation of BTG2 and BTG2 W103A was inconclusive (Appendix.4)

To date evidence has indicated that expression of BTG2 leads to a down-regulation of CCND1 (Cyclin D1) transcription a key component of G1 to S-phase cell cycle progression (Donato et al., 2007; Guardavaccaro et al., 2000). Likewise, prevention of TOB1 expression leads to an increase in CCND1 (Cyclin D1) transcription to drive cell proliferation (Suzuki et al., 2002). To further investigate which cell cycle regulatory proteins are affected by BTG2 or TOB1 expression a series of western blots and RT-qPCR analysis was performed to monitor cellular protein and mRNA levels. Western blot analysis did not identify any significant changes in the protein level of the cell cycle regulatory factors tested. However, this may be caused by low turnover rates of the proteins or due to antibody insensitivity. By contrast, RT-qPCR analysis suggested a small decrease in CCND1 (Cyclin D1) mRNA levels in MCF-7 cells after BTG2 expression. RT-qPCR analysis was conducted on MCF-7 cells transiently transfected with HA-BTG2 and HA-TOB1 however, a significant reduction in CCND1 (Cyclin D1) mRNA was only seen after HA-TOB1 expression (Appendix.5). Together this data suggests that BTG2 and TOB1 do not have a dramatic effect on the abundance of cell cycle regulatory proteins tested. Conversely, it maybe that BTG2 and TOB1 are regulating cell cycle progression via other signalling pathways not tested here. It would be interesting to identify which mRNAs are targeted by BTG2 and TOB1 to gain a deeper understanding as to how their expression brings about a reduction in cell proliferation.

Chapter 5

Regulation of mRNA abundance and translation by BTG/TOB proteins

Chapter 5 – Regulation of mRNA abundance and translation by BTG/TOB proteins

5.1 Introduction

The six BTG/TOB proteins are characterised by a conserved N-terminal BTG domain which binds to the Caf1a (CNOT7)/Caf1b (CNOT8) deadenylases and confers anti-proliferative activity. By examining the crystal structures of TOB1–Caf1a (CNOT7) and the related DEDD-type deadenylase PARN bound to RNA, it can be predicted that TOB1 binds away from the Caf1a (CNOT7) active site (Winkler, 2010). This suggests that the binding of TOB1 may not interfere with the deadenylase activity of Caf1a (CNOT7) and Caf1b (CNOT8). Furthermore, co-immunoprecipitation assays conducted have demonstrated that BTG2 not only co-precipitates with Caf1a (CNOT7) but also CNOT1 and CNOT3 (Aslam et al., 2009). CNOT1 functions as the scaffold protein for the Ccr4-Not complex to which Caf1a (CNOT7)/Caf1b (CNOT8) bind directly (Basquin et al., 2012; Lau et al., 2009; Sandler et al., 2011). The remaining EEP type deadenylases Ccr4a (CNOT6) and Ccr4b (CNOT6L) interact with the Ccr4-Not complex via Caf1a (CNOT7) and Caf1b (CNOT8) (Collart and Panasenko, 2012).

The divergent C-terminal domains of the BTG/TOB family have the ability to mediate further protein-protein interactions possibly conferring specific protein function. TOB1 interacts with PABP through a C-terminal PAM2 motif suggesting the TOB1 can bind to the poly(A) tail of mRNA (Ezzeddine et al., 2007; Funakoshi et al., 2007; Okochi et al., 2005). TOB1 can simultaneously bind to PABP and Caf1a (CNOT7) indicating a role for TOB1 as an adaptor protein mediating the recruitment of the Ccr4-Not complex to mRNAs (Ezzeddine et al., 2007).

TOB1 may not only be recruited to poly(A) tails through PABP but can also interact with CPEB3 a sequence-specific RNA-binding protein in conjunction with Caf1a (CNOT7)/Caf1b (CNOT8). Cytoplasmic Polyadenylation Element-

Binding protein 3 (CPEB3) recognises a U-rich RNA sequence via two RRM domains that also bind the C-terminal region of TOB1 (Hosoda et al., 2011). CPEB3 associated deadenylation and mRNA decay is dependent upon the recruitment of TOB1 and Caf1a (CNOT7)/Caf1b (CNOT8). This provides evidence that TOB1 is functioning as an adaptor protein, recruiting the Ccr4-Not deadenylases to mRNA through both sequence specific (CEBP3) and general (PABP) routes. TOB1 is also implicated in general deadenylation coupled to termination of translation. Deadenylation mediated by eRF3 is catalysed by the Ccr4-Not deadenylases Caf1a (CNOT7)/Caf1b (CNOT8), Ccr4a (CNOT6)/Ccr4b (CNOT6L) and Pan2-Pan3 deadenylases. Interestingly, competitive interactions between the PAM2 motifs of eRF3, Pan3, and TOB1 are implicated in the subsequent activation of Pan2-Pan3 and Ccr4-Not-mediated deadenylation (Funakoshi et al., 2007).

One unresolved question is the link between the BTG/TOB anti-proliferative activity and their role in mRNA translation and degradation. During the course of this work Ezzeddine et al (2012) highlighted this link for TOB1 and TOB2 which require an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) to induce a proliferation defect. Furthermore, when TOB1 and TOB2 are tethered to a reporter mRNA 3' UTR they require an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) to reduce reporter activity and stimulate mRNA degradation. To date this link between anti-proliferative activity and regulation of mRNA translation/abundance for other members of the BTG/TOB family has not been established. Here I will investigate the effect BTG2 and TOB1 have on translation and mRNA degradation when recruited to mRNA using the RNA tethering assay. BTG2 and TOB1 are fused to a λ N peptide that binds to a specific RNA hairpin loop box B motif present in the 3'UTR of a renilla luciferase mRNA. This luciferase construct can be used to monitor mRNA stability or translation efficiency when BTG2 and TOB1 are bound to it.

5.2 Generation of vectors for the RNA tethering assay

The pCI λ N RNA tethering vector fuses a desired cDNA to an N-terminal λ N epitope and V5 tag. The HA epitope was introduced to aid western blotting detection. The λ N peptide functions to tether a desired protein to a corresponding RNA hair pin loop present in a luciferase reporter mRNA (plasmid pRL-5boxB).

To construct cDNA fusions with the RNA binding peptide λ N, the BTG2 cDNA was PCR amplified to include an XhoI restriction site and the HA epitope 5' of the AUG start codon and an XhoI restriction site 3' of the termination codon. This PCR fragment was ligated into cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-HA-BTG2. Plasmid pBluescript-HA-BTG2 was digested with XhoI to release the HA-BTG2 cDNA and subcloned into plasmid pCI λ N via the XhoI restriction site (Figure 5.1A). The TOB1 cDNA was PCR amplified to include an XhoI restriction site and the HA epitope 5' of the AUG start codon and a XbaI restriction site 3' of the termination codon. This PCR fragment was ligated into the cloning plasmid pBluescript as described previously to generate pBluescript-HA-TOB1. Plasmid pBluescript-HA-TOB1 was digested with XhoI and XbaI to release the HA-TOB1 cDNA and subcloned into plasmid pCI λ N via the XhoI and XbaI restriction sites (Figure 5.1B). All plasmids were confirmed by DNA sequencing.

To introduce the desired point mutations, DNA oligos were designed containing the relevant modified codons and used for site directed mutagenesis. The desired clones were identified through DNA sequencing. The amino acid substitutions introduced to BTG2 were W103A that disrupts the BTG2-Caf1a (CNOT7)/Caf1b (CNOT8) interaction and S147A and S149A that prevent post-translational phosphorylation. The equivalent amino acid substitutions W93A and S152A, S154A, S164A were introduced in TOB1.

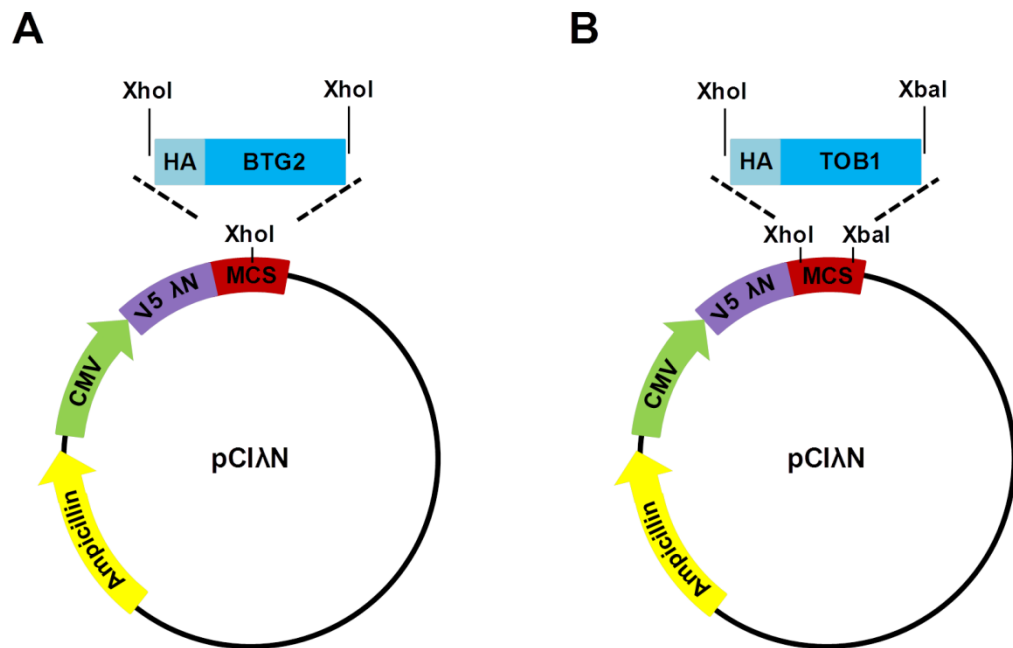


Figure 5.1 Generation of λ N-HA-BTG2 and λ N-HA-TOB1 expression vectors. (A) The HA-BTG2 cDNA was generated by PCR amplification to include the XhoI restriction site 5' of the ATG start codon and 3' of the stop codon. This PCR fragment was ligated into cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-HA-BTG2. The HA-BTG2 cDNA was excised with XhoI and subcloned into plasmid pCIAN. **(B)** The HA-TOB1 cDNA was generated by PCR amplification to include the XhoI restriction site 5' of the ATG start codon and the XbaI restriction site 3' of the stop codon. This PCR fragment was ligated into cloning vector pBluescript II KS(+) via the SmaI restriction site to generate pBluescript-HA-TOB1. The TOB1 cDNA was excised with XhoI and XbaI and subcloned into plasmid pCIAN.

5.3 Tethering BTG2 or TOB1 to the 3' UTR of a reporter mRNA causes reduced activity dependent on interactions with Caf1a (CNOT7)/Caf1b (CNOT8)

RNA tethering assays were carried out to determine if the presence of BTG2 on the 3'UTR of a reporter mRNA alters its activity and to ascertain if any changes are dependent on the Caf1a (CNOT7)/Caf1b (CNOT8) interactions. Thus, MCF-7 cells were co-transfected with the reporter pRL-5boxB and plasmids expressing fusions of the λ N peptide with HA-BTG2, HA-BTG2W103A, HA-BTG2S-A or HA-BTG2W103AS-A. After transfection, total protein was extracted and luciferase activity determined (Figure 5.2A).

Tethering of λ N-HA-BTG2 to the luciferase mRNA caused a reduction in luciferase activity, which was not observed in cells expressing the λ N peptide only. By contrast, expression of λ N-HA-BTG2 W103A to the luciferase mRNA did not cause a reduction in luciferase activity (Figure 5.2B). This indicates that the reduction in luciferase protein is dependent upon the interaction between BTG2 and the deadenylases Caf1a (CNOT7)/Caf1b (CNOT8). Tethering of BTG2 S-A to the luciferase mRNA also caused a reduction in luciferase activity comparable to wild type BTG2, which was not observed with BTG2W 103A S-A (Figure 5.2B). This indicates that the phosphorylation status of BTG2 does not alter its activity when tethered to a reporter mRNA.

To confirm that reduced luciferase activity was caused by tethering of BTG2 to the 3' UTR, we used reporter plasmid pRL-TK that does not contain the BoxB motifs. Expression of λ N-HA-BTG2, λ N-HA-BTG2 W103A, λ N-HA-BTG2 S-A or λ N-HA-BTG2 W103A S-A caused a modest reduction in luciferase activity compared to cells transfected with empty vector (Figure 5.2C). Moreover, there was no significant difference between the samples expressing the various λ N fusion proteins indicating that tethering is indeed responsible for reduced luciferase activity when a reporter containing BoxB sequences was used. Western blot analysis was conducted on protein lysates to confirm expression of BTG2 (Figure 5.2D).

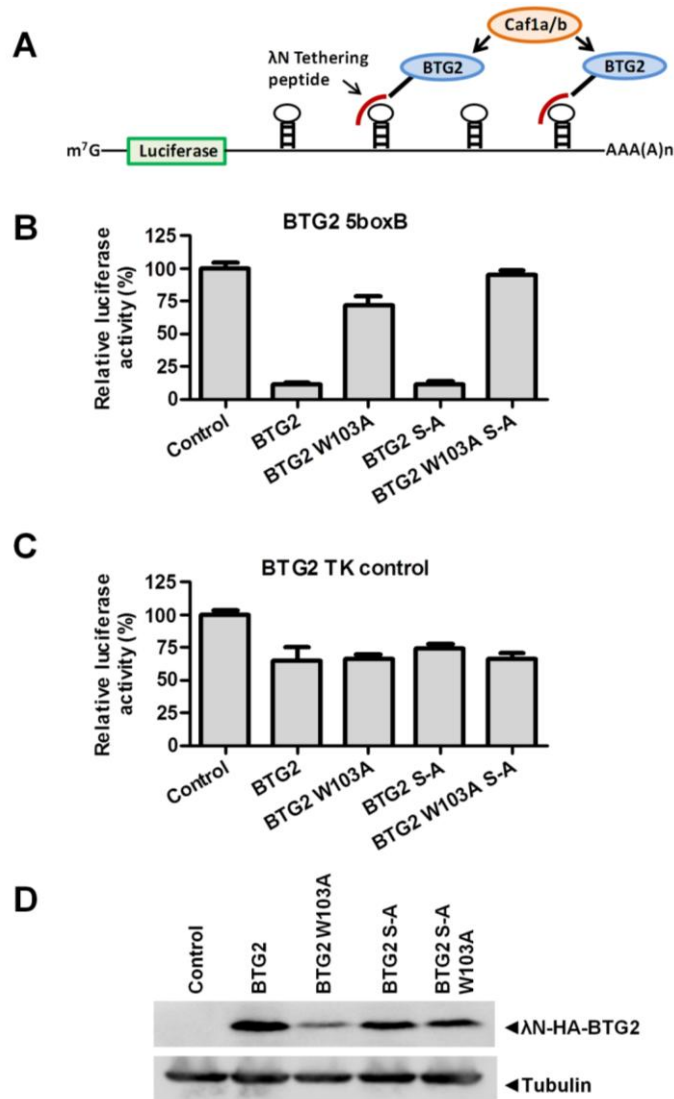


Figure 5.2 BTG2 requires interactions with Caf1a (CNOT7)/Caf1b (CNOT8) to reduce reporter activity upon 3' UTR tethering. (A) Schematic representation of the RNA tethering assay. (B) Decreased reporter activity upon RNA tethering of λN-HA-BTG2 or λN-HA-BTG2 S→A, but not of λN-HA-BTG2 W103A or λN-HA-BTG2 W103A S→A. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated BTG2 cDNA, and the luciferase reporter plasmid pRL-5BoxB. Luciferase activity was measured 24 h after transfection. (C) No specific effect of λN-HA-BTG2, λN-HA-BTG2 W103A, λN-HA-BTG2 S→A and λN-HA-BTG2 W103A S→A expression on the activity of a Luciferase reporter lacking BoxB sequences. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated BTG2 cDNA, and the luciferase reporter plasmid pRL-TK. Luciferase activity was measured 24 h after transfection. (D) Western blot showing the expression for λN-HA-BTG2, λN-HA-BTG2 W103A, λN-HA-BTG2 S→A and λN-HA-BTG2 W103A S→A. λN-fusion proteins were detected using anti-HA antibodies. Tubulin was used as a loading control.

Similarly, the influence of TOB1 on reporter activity was investigated using a tethering assay (Figure 5.3A). As was observed with BTG2, expression of λ N-HA-TOB1 reduced reporter activity, which was not observed upon expression of λ N-HA-TOB1 W93A (Figure 5.3B). The phosphorylation status of TOB1 did not affect reporter activity (Figure 5.3B). The observed effects were specific, because no effect of λ N-HA-TOB1 fusion proteins was measured on reporter activity in the absence of BoxB motifs (Figure 5.3C). Western blot analysis was conducted on the protein lysates to confirm expression of the TOB1 plasmids (Figure 5.3D).

This data demonstrates that binding of BTG2 or TOB1 to the 3'UTR of a luciferase mRNA reduces its reporter activity. This is not dependent upon the phosphorylation status of BTG2 or TOB1. However, tethering BTG2 W103A or TOB1 W93A to the luciferase mRNA 3'UTR does not result in reduced luciferase activity implying that the reduced activity is due to an interaction with Caf1a (CNOT7) and Caf1b (CNOT8).

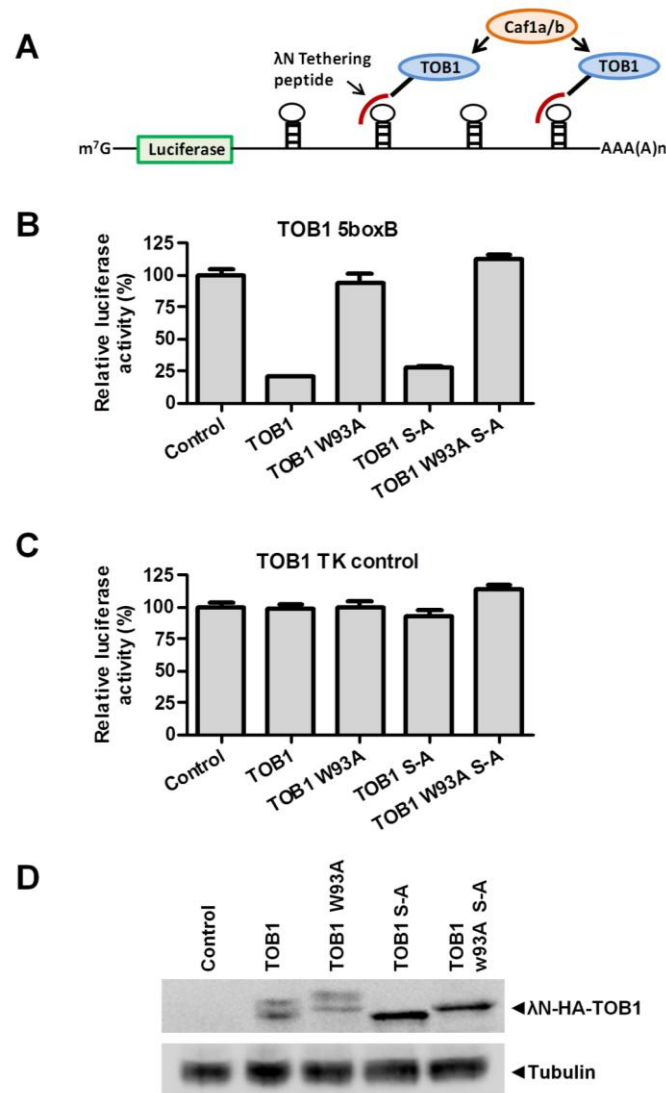


Figure 5.3 TOB1 requires interactions with Caf1a (CNOT7)/Caf1b (CNOT8) to reduce reporter activity upon 3' UTR tethering. (A) Schematic representation of the RNA tethering assay. (B) Decreased reporter activity upon RNA tethering of λN-HA-TOB1 or λN-HA-TOB1 S→A, but not of λN-HA-TOB1 W93A or λN-HA-TOB1 W93A S→A. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated TOB1 cDNA, and the luciferase reporter plasmid pRL-5BoxB. Luciferase activity was measured 24 h after transfection. (C) No specific effect of λN-HA-TOB1, λN-HA-TOB1 W93A, λN-HA-TOB12 S→A and λN-HA-TOB1 W93A S→A expression on the activity of a Luciferase reporter lacking BoxB sequences. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated TOB1 cDNA, and the luciferase reporter plasmid pRL-TK. Luciferase activity was measured 24 h after transfection. (D) Western blot showing the expression for λN-HA-TOB1, λN-HA-TOB1 W93A, λN-HA-TOB1 S→A and λN-HA-TOB1 W93A S→A. λN-fusion proteins were detected using anti-HA antibodies. Tubulin was used as a loading control.

5.4 Recruitment of BTG2 and TOB1 to an mRNA leads to mRNA degradation via Caf1a (CNOT7) and Caf1b (CNOT8)

To determine whether reduced reporter activity upon tethering of BTG2 or TOB1 was caused by mRNA degradation, or due to decreased translation, the RNA tethering assay was performed in combination with reverse-transcriptase qPCR analysis (Figure 5.4A). MCF-7 cells were co-transfected with plasmids pRL-5BoxB and pCI λ N, pCI λ N-HA-BTG2, pCI λ N-HA-BTG2W103A, pCI λ N-HA-TOB1 or pCI λ N-HA-TOB1 W93A before total RNA was extracted. Relative luciferase mRNA levels were obtained and normalisation to GAPDH.

Tethering of BTG2 to the luciferase reporter mRNA caused a significant decrease in the amount of luciferase mRNA compared to tethering of the λ N peptide alone (Figure 5.4B). As expected, tethering of BTG2 W103A did not cause a significant decrease in luciferase mRNA compared to tethering of the λ N peptide. Similar results were obtained when TOB1 tethering was carried out. Tethering of TOB1 to the luciferase mRNA caused a significant decrease in the amount of luciferase mRNA compared to tethering of the λ N peptide alone, which was not observed in the presence of TOB1 W93A (Figure 5.4C). These results indicate that tethering of BTG2 or TOB1 to the luciferase mRNA promotes its degradation, which is dependent upon an interaction with Caf1a (CNOT7)/Caf1b (CNOT8). Interestingly, tethering of BTG2 and TOB1 to the luciferase reporter mRNA has a greater effect on reporter activity compared to mRNA levels. These differences may be caused by a difference in signal to noise ratios between the luciferase assay and RT-qPCR analysis. However, it may also be the case that tethering of BTG2 and TOB1 to a mRNA has a greater effect on translation efficiency due to shortening of the poly(A) tail than on mRNA degradation.

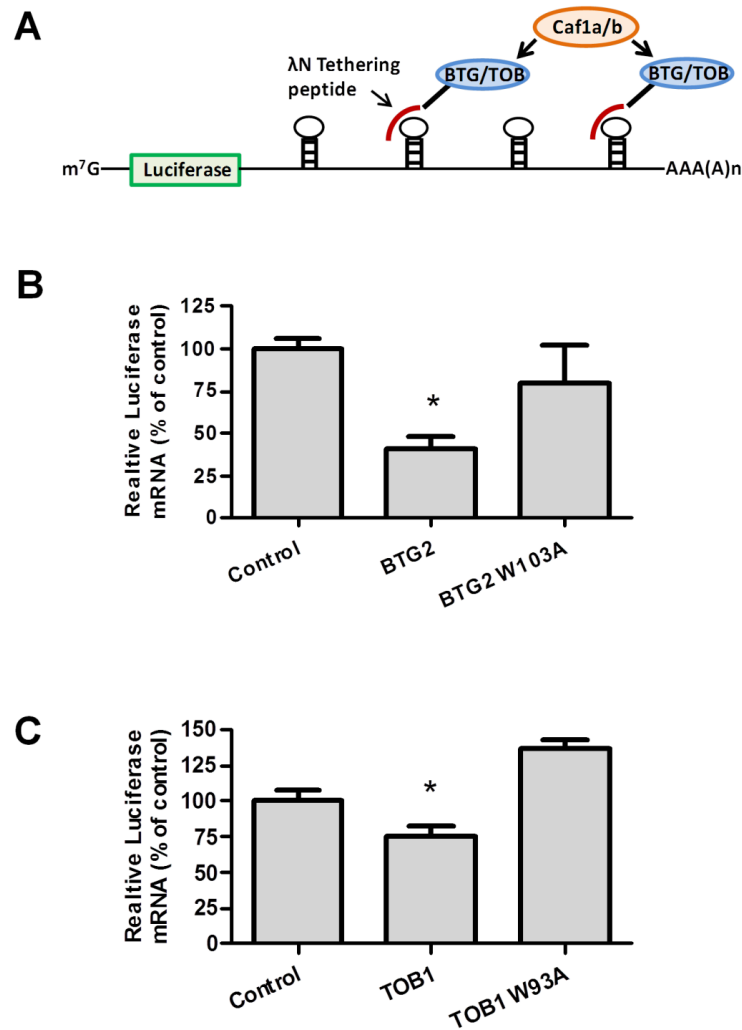


Figure 5.4 BTG2 and TOB1 require an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) to reduced mRNA abundance of a tethered mRNA. (A) Schematic representation of the RNA tethering assay. **(B)** Decreased mRNA abundance upon RNA tethering of λN-HA-BTG2 but not of λN- HA-BTG2 W103A. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated BTG2 cDNA, and the luciferase reporter plasmid pRL-5Box5. Total RNA was isolated after 48 h and subject to RT-qPCR analysis using GAPDH as a reference gene. **(C)** Decreased mRNA abundance upon RNA tethering of λN-HA-TOB1 but not of λN- HA-TOB1 W93A. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated BTG2 cDNA, and the luciferase reporter plasmid pRL-5BoxB. Total RNA was isolated after 48 h and subject to RT-qPCR analysis using GAPDH as a reference gene.

5.5 Identification of Ccr4-Not components required for reduced reporter activity caused by tethering of BTG2 or TOB1

The above results show that BTG2 and TOB1 require an interaction with the deadenylase Caf1a (CNOT7) or Caf1b (CNOT8) to reduce reporter activity as established using 3' UTR tethering assays. To identify which other members of the Ccr4-Not complex are involved in the repression of reporter activity, the RNA tethering assay was conducted after siRNA knockdown of Ccr4-Not components.

To confirm that Caf1a (CNOT7) and Caf1b (CNOT8) are required for reduced luciferase activity, knockdown of Caf1a (CNOT7) and Caf1b (CNOT8) was carried out before the RNA tethering assay was conducted. MCF-7 cells were transfected with siRNA against Caf1a (CNOT7) and Caf1b (CNOT8) or non-targeting siRNA. After 48 h the cells were transfected with the reporter plasmid pRL-5BoxB and pCI λ N, pCI λ N-HA-BTG2, or pCI λ N-HA-TOB1 before total protein was extracted and used for a luciferase assay (Figure 5.5A).

As expected, tethering of BTG2 or TOB1 to the 3' UTR of the luciferase reporter mRNA in the presence of non-targeting siRNA caused a reduction in luciferase activity indicating that the siRNA transfection does not affect the BTG2 or TOB1 activity. However, upon expression of λ N-HA-BTG2 or λ N-HA-TOB1 after combined knockdown of Caf1a (CNOT7) and Caf1b (CNOT8), the repression of luciferase activity was partially reversed (Figure 5.5B). Western blot analysis confirmed the expression of pCI λ N-HA-BTG2 and pCI λ N-HA-TOB in Caf1a (CNOT7) and Caf1b (CNOT8) knockdown cells (Figure 5.5C). Western blot and RT-qPCR analysis confirmed the knockdown of Caf1a (CNOT7) and Caf1b (CNOT8) (Figure 5.5C and 5.5D). These results are consistent with the finding that tethering of BTG2 or TOB1 requires interactions with the Caf1a (CNOT7)/Caf1b (CNOT8) enzymes to reduce reporter activity as presented in Figure 5.2 and 5.3.

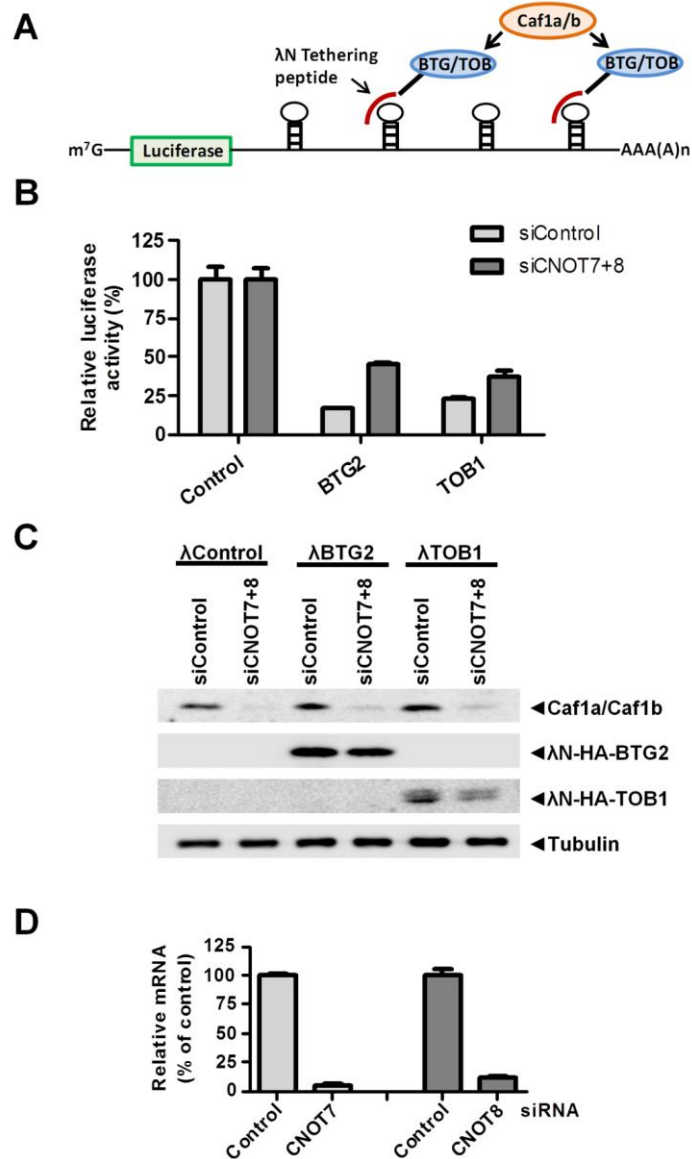


Figure 5.5 Combined knockdown of the deadenylases Caf1a (CNOT7)/Caf1b (CNOT8) partially rescue repression of reporter activity upon tethering of BTG2 or TOB1. (A) Schematic representation of the RNA tethering assay. (B) Combined knockdown of the Caf1a (CNOT7)/Caf1b (CNOT8) partially rescue repression of reporter activity upon tethering of BTG2 or TOB1. MCF-7 cells were transfected with siRNA targeting CNOT7 and CNOT8 or control non-targeting siRNA. After 48 h cells were transfected with plasmids pCI λ N-HA, pCI λ N-HA-BTG2, pCI λ N-HA-TOB1 and the luciferase reporter pRL-5boxB. Luciferase activity was measured 24 h after transfection. (C) Western blots to confirm siRNA knockdown of Caf1a (CNOT7)/Caf1b (CNOT8) and expression of λ N-HA-BTG2 and λ N-HA-TOB1 (D) Reverse transcriptase-qPCR analysis to confirm knockdown efficiency of CNOT7 and CNOT8. Total RNA was isolated 72 h after siRNA treatment and subjected to RT-qPCR analysis using GAPDH as a reference gene.

To determine if the deadenylases Ccr4a (CNOT6) and Ccr4b (CNOT6L) are also required for the reduced reporter activity upon tethering BTG2 and TOB1 to a luciferase mRNA, the RNA tethering assay was conducted after combined Ccr4a (CNOT6)/Ccr4b (CNOT6L) knockdown. MCF-7 cells were first transfected with siRNA targeting Ccr4a (CNOT6) and Ccr4b (CNOT6L) or non-targeting siRNA. After 48 h the cells were transfected with the pRL-5BoxB reporter and expression plasmids pCI λ N, pCI λ N-HA-BTG2, or pCI λ N-HA-TOB1 before total protein was extracted and used for a luciferase assay.

Upon combined knockdown of Ccr4a (CNOT6) and Ccr4b (CNOT6L), no change in luciferase activity was observed in the presence of λ N-BTG2 or λ N-TOB1 as compared to non-targeting siRNA treated cells (Figure 5.6B). Western blot analysis confirmed the expression of pCI λ N-HA-BTG2 and pCI λ N-HA-TOB1 in MCF-7 cells (Figure 5.6C). In addition, RT-qPCR analysis confirmed the knockdown of Ccr4a (CNOT6) and Ccr4b (CNOT6L) mRNA (Figure 5.6D). This result suggests that Ccr4a (CNOT6) and Ccr4b (CNOT6L) do not affect BTG2 or TOB1 activity when recruited to the 3' UTR.

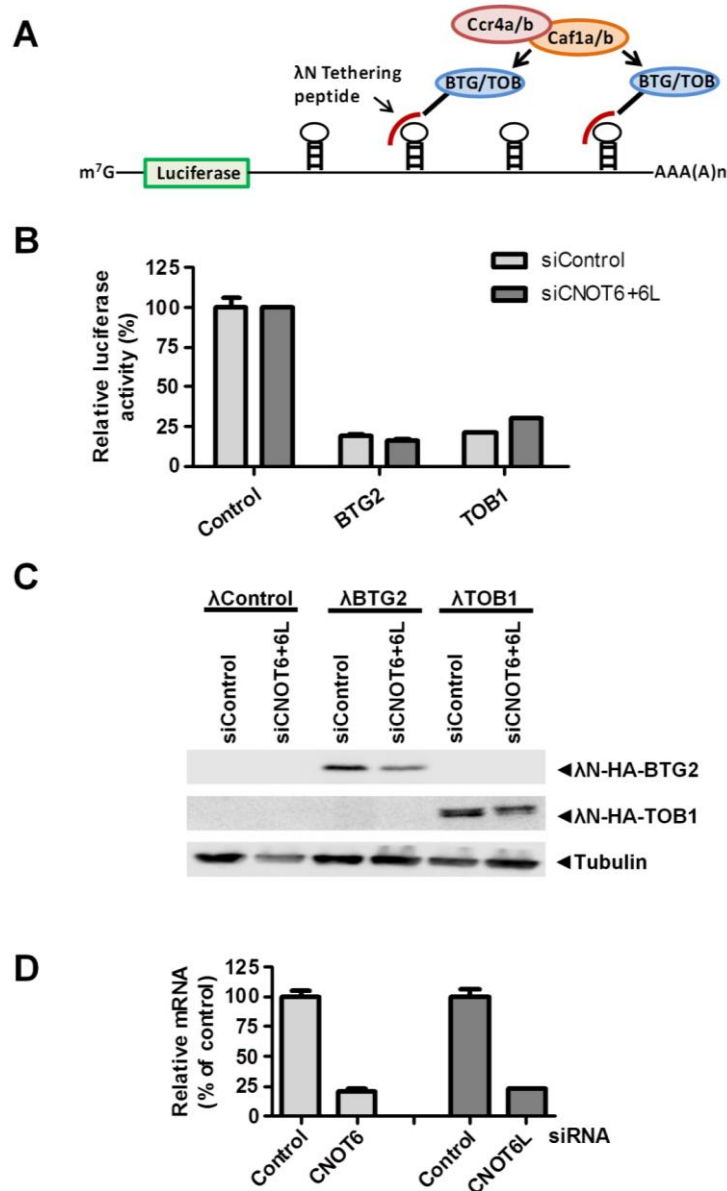


Figure 5.6 Combined knockdown of the deadenylases Ccr4a (CNOT6)/Ccr4b (CNOT6L) does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. (A) Schematic representation of the RNA tethering assay. (B) Combined knockdown of the Ccr4a (CNOT6)/Ccr4b (CNOT6L) does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. MCF-7 cells were transfected with siRNA targeting CNOT6 and CNOT6L or control non-targeting siRNA. After 48 h cells were transfected with plasmids pCI λ N-HA, pCI λ N-HA-BTG2, pCI λ N-HA-TOB1 and the luciferase reporter pRL-5boxB. Luciferase activity was measured 24 h after transfection. (C) Western blots to confirm expression of λ N-HA-BTG2 and λ N-HA-TOB1. (D) Reverse transcriptase-qPCR analysis to confirm knockdown efficiency of CNOT6 and CNOT6L. Total RNA was isolated 72 h after siRNA treatment and subjected to RT-qPCR analysis using GAPDH as a reference gene.

To determine if the Ccr4-Not components CNOT1 and CNOT3 are also required for the reduced reporter activity upon tethering BTG2 and TOB1 to a luciferase mRNA, CNOT1 or CNOT3 knockdown was combined with the RNA tethering assay. MCF-7 cells were transfected with siRNA targeting CNOT1, CNOT3 or non-targeting siRNA followed by transfection of pRL-5boxB and pCI λ N, pCI λ N-HA-BTG2, or pCI λ N-HA-TOB1 (Figure 5.7A, 5.8A).

Tethering of λ N-BTG2 or λ N-TOB1 to the luciferase reporter caused a reduction in luciferase activity in the presence of non-targeting siRNA, which was also observed upon knockdown of CNOT1 (Figure 5.7B). Western blot analysis confirmed the expression of pCI λ N-HA-BTG2 and pCI λ N-HA-TOB1 in MCF-7 cells after knockdown (Figure 5.7C). In addition, knockdown of CNOT1 was confirmed by western blotting and RT-qPCR analysis (Figure 5.7D).

Upon knockdown of CNOT3, luciferase activity caused by tethering of λ N-BTG2 or λ N-TOB1 was reduced to a similar extent as observed in cells treated with non-targeting siRNA (Figure 5.8B). Western blot analysis confirms the expression of pCI λ N-HA-BTG2 and pCI λ N-HA-TOB1 in MCF-7 cells after knockdown (Figure 5.8C). Knockdown of CNOT3 was confirmed by western blotting and RT-qPCR analysis (Figure 5.8D).

Together these experiments confirm that the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8) are required for the reduced luciferase activity witnessed upon tethering of λ N-BTG2 or λ N-TOB1 to the luciferase mRNA. However, other members of the Ccr4-Not complex do not appear to be required.

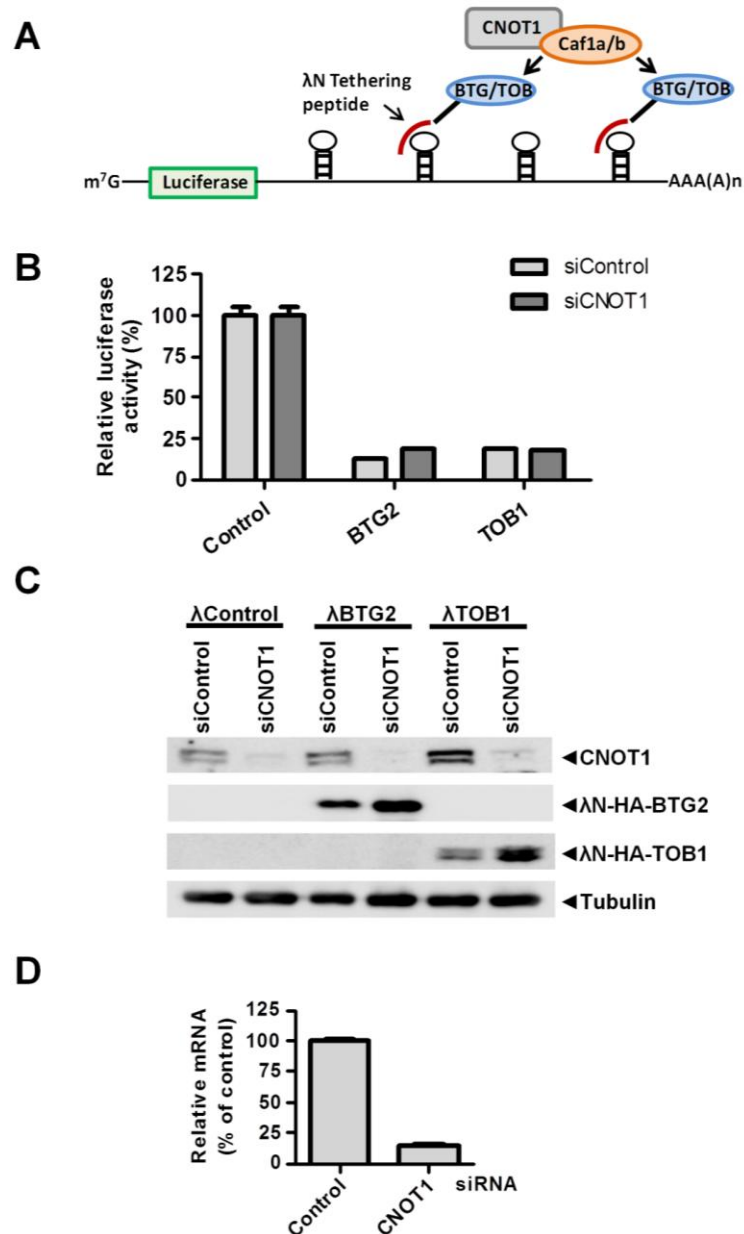


Figure 5.7 Combined knockdown of the deadenylase CNOT1 does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. (A) Schematic representation of the RNA tethering assay. (B) Knockdown of the CNOT1 does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. MCF-7 cells were transfected with siRNA targeting CNOT1 or control non-targeting siRNA. After 48 h cells were transfected with plasmids pCI λ N-HA, pCI λ N-HA-BTG2, pCI λ N-HA-TOB1 and the luciferase reporter pRL-5boxB. Luciferase activity was measured 24 h after transfection. (C) Western blots to confirm siRNA knockdown of CNOT1 and expression of λ N-HA-BTG2 and λ N-HA-TOB1 (D) Reverse transcriptase-qPCR analysis to confirm knockdown efficiency of CNOT1. Total RNA was isolated 72 h after siRNA treatment and subjected to RT-qPCR analysis using GAPDH as a reference gene.

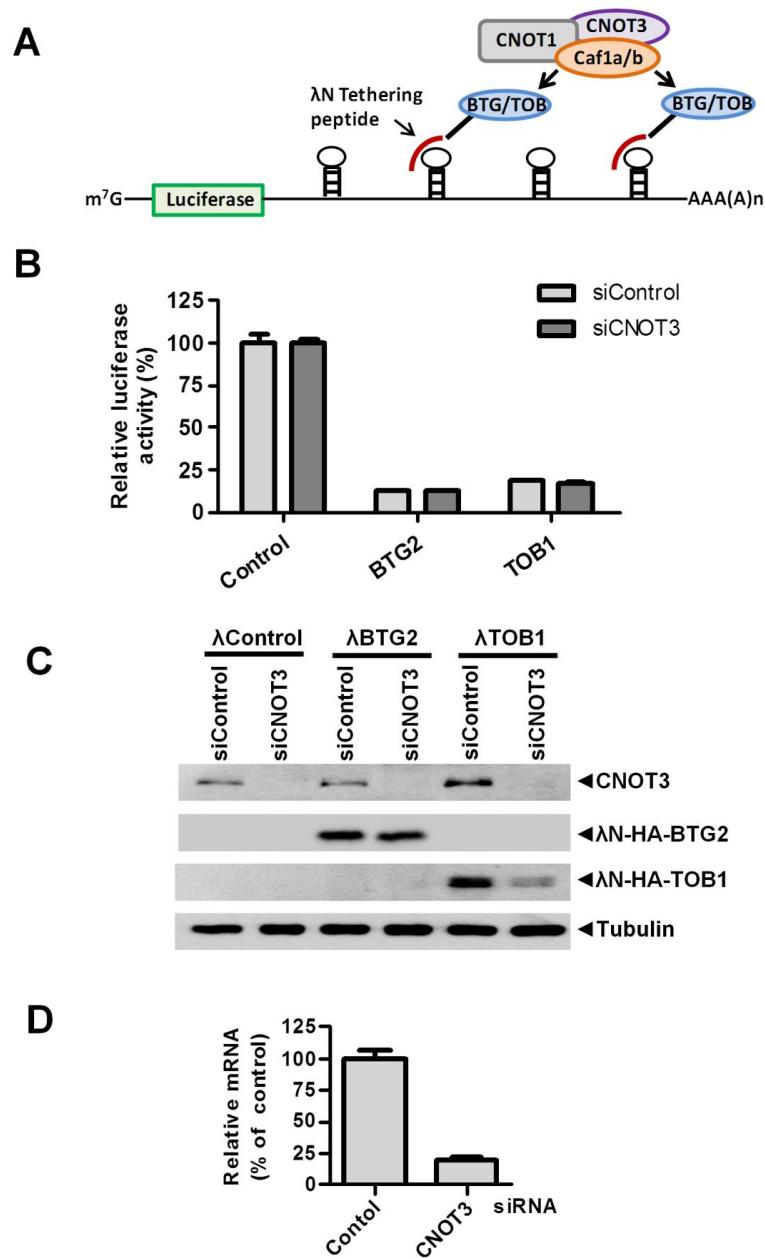


Figure 5.8 Combined knockdown of the deadenylase CNOT3 does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. (A) Schematic representation of the RNA tethering assay. (B) Knockdown of the CNOT3 does not affect the repression of reporter activity upon tethering of BTG2 or TOB1. MCF-7 cells were transfected with siRNA targeting CNOT3 or control non-targeting siRNA. After 48 h cells were transfected with plasmids pCI λ N-HA, pCI λ N-HA-BTG2, pCI λ N-HA-TOB1 and the luciferase reporter pRL-5boxB. Luciferase activity was measured 24 h after transfection. (C) Western blots to confirm siRNA knockdown of CNOT3 and expression of λ N-HA-BTG2 and λ N-HA-TOB1 (D) Reverse transcriptase-qPCR analysis to confirm knockdown efficiency of CNOT3. Total RNA was isolated 72 h after siRNA treatment and subjected to RT-qPCR analysis using GAPDH as a reference gene.

5.6 Discussion

The anti-proliferative activity of BTG2 and TOB1 is dependent upon their interaction with the deadenylases Caf1a (CNOT7) and Caf1b (CNOT8) (Chapter 4) (Ezzeddine et al., 2012). One possible mechanism through which BTG2 and TOB1 may slow cell proliferation is to alter gene expression by stimulating mRNA deadenylation and degradation. Tob1 can be recruited to the 3' UTR of an mRNA via interactions with poly(A)-binding proteins during termination of translation, or via sequence-specific RNA-binding proteins. In both cases TOB1 recruitment reduced mRNA stability and leads to mRNA degradation (Funakoshi et al., 2007; Hosoda et al., 2011). During the course of this work the artificial recruitment of TOB1 and TOB2 was shown to promote deadenylation via interactions with the Ccr4-Not deadenylases Caf1a (CNOT7) and Caf1b (CNOT8) (Ezzeddine et al., 2012). Observations here are consistent with the published data demonstrating that tethering TOB1 to an reporter mRNA 3' UTR leads to reduced reporter activity and mRNA degradation. Further to this, BTG2 was also observed to reduced the reporter activity when tethered to a reporter mRNA and simulate mRNA degradation. This result is consistent with the current hypothesis that BTG/TOB proteins function as recruitment factors of the Ccr4-Not deadenylase (Ezzeddine et al., 2007; Ezzeddine et al., 2012; Hosoda et al., 2011). What remains unclear is how BTG2 may be recruited to the 3' UTR region of mRNAs. Whereas TOB1 and TOB2 have large C-terminal regions that mediate interactions with poly(A)-binding proteins and CPEB3, BTG2 and the closely related BTG1 protein only have a short C-terminal region (Ezzeddine et al., 2007; Funakoshi et al., 2007; Hosoda et al., 2011). It is likely there are further proteins that interact with the BTG/TOB proteins to mediate their interaction with mRNA that have yet to be discovered.

A further area of ambiguity is which catalytic subunit contributes to deadenylation of mRNA upon recruitment by BTG/TOB proteins. The Ccr4a (CNOT6)/Ccr4b (CNOT6L) EEP type deadenylases interact with the Ccr4-Not complex through Caf1a (CNOT7)/Caf1b (CNOT8), the DEDD deadenylases. Both the DEDD/Caf1 and EEP/Ccr4-type subunits are active deadenylase enzymes *in vitro*. However, despite the fact that these proteins directly interact with each other, they regulate the abundance of different sets of mRNAs (Aslam et al., 2009; Mittal et al., 2011). Here we have demonstrated that when BTG2 and TOB1 are bound to a reporter mRNA they require an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) to reduced reporter activity. During the course of this work Ezzeddine et al 2012 also demonstrated that tethering TOB1/TOB2 to mRNA caused increased deadenylation which was abolished when the TOB1/TOB2 interaction with Caf1a (CNOT7)/Caf1b (CNOT8) was prevented. Interestingly, here, siRNA knockdown demonstrates that the catalytic subunit required for reduced reporter activity is Caf1a (CNOT7)/Caf1b (CNOT8). This suggests that despite the interaction between Caf1a (CNOT7)/Caf1b (CNOT8) and Ccr4a (CNOT6)/Ccr4b (CNOT6L) the latter EEP type deadenylases are not necessarily contributing towards the reduced reporter activity and mRNA level. Previous work showed that BTG2 co-precipitates with the Ccr4-Not components CNOT1 and CNOT3 in addition to Caf1a (CNOT7) (Aslam et al., 2009). Through siRNA knockdown of CNOT1 and CNOT3, it was here demonstrated that these non-catalytic subunits play no role in the reduced reporter activity and mRNA degradation following BTG2/TOB1 tethering to a reporter mRNA. However, this does not specify whether Caf1a (CNOT7)/Caf1b (CNOT8) are recruited alone or in complex with other Ccr4-Not components via BTG2 and TOB1. Immunoprecipitation experiments could prove interesting and provide further information as to the components recruited to mRNA 3' UTR as a consequence of BTG/TOB tethering.

In summary, the artificial recruitment of BTG2 and TOB1 to the 3'UTR of a luciferase mRNA leads to reduced reporter activity and mRNA levels. The effects on reporter mRNA degradation are dependent upon the interactions with the Caf1a (CNOT7)/Caf1b (CNOT8) deadenylase enzymes, but do not appear to require the EPP-type deadenylase subunits Ccr4a (CNOT6)/Ccr4b (CNOT6L), or the non-catalytic Ccr4-Not components CNOT1 and CNOT3.

Chapter 6

Concluding Remarks

6. Concluding Remarks

The aim of this work was to determine whether the anti-proliferative activity of the BTG/TOB proteins is dependent upon their interaction with the Ccr4-Not deadenylases Caf1a (CNOT7) and Caf1b (CNOT8). Furthermore, to determine if the BTG/TOB proteins could act as recruitment factors to direct Caf1a (CNOT7) and Caf1b (CNOT8) to mRNAs thereby reducing translational efficiency or inducing mRNA degradation. To achieve this, the BTG2-Caf1a (CNOT7) interaction was characterised to identify amino acid residues in BTG2 that are required for binding to Caf1a (CNOT7)/Caf1b (CNOT8). Through substituting these amino acids for alanine the interaction between BTG2 and TOB1 with Caf1a (CNOT7)/Caf1b (CNOT8) was disrupted. Using these BTG2 and TOB1 constructs the importance of the BTG/TOB-Caf1a (CNOT7)/Caf1b (CNOT8) interaction on cell proliferation was investigated via several methods. Additionally, utilising BTG2/TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction mutants the ability of the BTG/TOB proteins to recruit the Ccr4-Not deadenylases to target mRNA was assessed.

6.1 BTG/TOB interactions with the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylases enzymes.

The BTG/TOB proteins contain a conserved N-terminal domain that mediates protein-protein interaction with the Ccr4-Not deadenylases Caf1a (CNOT7)/Caf1b (CNOT8). The interaction has been mapped to the structurally conserved BTG domain as illustrated by the crystal structure of TOB1 in complex with Caf1a (CNOT7) (Horiuchi et al., 2009). Previous work had identified a number of amino acid residues in Caf1a (CNOT7) and Caf1b (CNOT8) that are required for their interaction with BTG2 (Aslam et al., 2009). Using available structural information and multiple sequence alignment of BTG/TOB proteins, potential amino acids required for the interaction with Caf1a (CNOT7) and Caf1b (CNOT8) were identified.

Experiments in Chapter 3 identified three amino acids in BTG2 and TOB1 that are required for the interactions with Caf1a (CNOT7) and Caf1b (CNOT8). These amino acids were Tyr-65, Asp-75 and Trp-103 of BTG2 and the corresponding residues Phe-55, Asp-65 and Trp-93 of TOB1. On the basis of the interaction analysis amino acid Trp-103 of BTG2 and Trp-93 of TOB1 were selected to disrupt the BTG2/TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction. From analysis of the crystal structure of BTG2/TOB1 in complex with Caf1a (CNOT7) the amino acid may form direct interactions with the aliphatic portion of the Lys-203 side chain of Caf1a (CNOT7).

6.2 The BTG/TOB anti-proliferative activity is mediated through interactions with Caf1a (CNOT7) and Caf1b (CNOT8)

Four BTG/TOB proteins are expressed in MCF-7 cells, however, their relative mRNA levels do not correlate with their anti-proliferative activities. The expression of TOB1 was significantly higher than BTG2 expression. However, upon knockdown cell proliferation increased by the same margin. Furthermore, despite significantly higher expression of BTG1 and TOB2 compared to BTG2, neither had an effect on cell proliferation. Interestingly, the combined knockdown of BTG2 and TOB1 increased cell proliferation further compared to single knockdown of BTG2 or TOB1.

It remained unresolved as to whether the BTG/TOB anti-proliferative activity was dependent upon interactions with the Ccr4-Not deadenylases Caf1a (CNOT7) and Caf1b (CNOT8). Divergent conclusions have been drawn based on mutagenesis studies where amino acids were substituted in the BTG/TOB and Caf1a/Caf1b proteins (Aslam et al., 2009; Horiuchi et al., 2009). This study showed that expression of BTG2 and TOB1 caused a reduction in cell proliferation of MCF-7 cells. By contrast expression of BTG2 W103A and TOB1 W93A does not affect cell proliferation. From this data, it was concluded that the BTG2/TOB1 anti-proliferative activity is dependent upon

an interaction with Caf1a (CNOT7)/Caf1b (CNOT8). This conclusion is consistent with recent work by Ezzeddine et al (2012) who independently determined that TOB1/TOB2 requires interactions with Caf1a (CNOT7)/Caf1b (CNOT8) for their anti-proliferative activities.

The over-expression of BTG/TOB proteins has been linked to the reduction in CCND1 (Cyclin D1) expression. Stimulating changes in CCND1 (Cyclin D1) maybe the cell cycle pathway targeted by the BTG/TOB proteins to reduced cell proliferation by stalling cells in G1. To assess this, a series of western blots and RT-qPCR analysis was conducted to identify any changes in protein of mRNA levels of the key components of the G1 to S cell cycle progression pathway. A significant reduction was seen in the relative level of CCND1 (Cyclin D1) mRNA after BTG2 expression indicating that BTG2 expression is affecting CCND1 (Cyclin D1) expression. However, whether this is a direct effect of BTG2 expression is unknown as the observed reduction in CCND1 (Cyclin D1) expression maybe an indirect consequence of reduced cell proliferation.

6.3 Regulation of the BTG/TOB anti-proliferative activity via phosphorylation

The anti-proliferative activity of BTG2 and TOB1 is regulated by the phosphorylation of serine residues located in the proteins C-terminal region (Hong et al., 2005; Suzuki et al., 2002). During the course of this work, western blot analysis identified that TOB1 may be subject to phosphorylation, which is affected by the W93A amino acid substitution. Interestingly, the expression of BTG2 S→A and TOB1 S→A did not increase cell proliferation to a greater extent than wild type BTG2 or TOB1. Therefore, it was concluded that phosphorylation of BTG2 and TOB1 does not affect the activity of BTG2/TOB1 in MCF-7 cells. This conclusion is shared with recent

work by Ezzeddine et al 2012 who also demonstrated that phosphorylation of TOB1 and TOB2 did not alter their anti-proliferative activity.

6.4 Recruitment of BTG/TOB proteins to the 3' UTR reduces translation and mRNA abundance

The BTG/TOB divergent C-terminal domain mediates further protein-protein interactions. For instance, TOB1 can interact with PABPC1 and CPEB3, two RNA binding proteins (Ezzeddine et al., 2007; Funakoshi et al., 2007; Hosoda et al., 2011; Okochi et al., 2005; Suzuki et al., 2002). These interactions allow for the recruitment of TOB1 to the 3'UTR of mRNA which subsequently leads to reduced mRNA stability. Work conducted here demonstrated that tethering of BTG2 and TOB1 to an mRNA 3' UTR leads to reduced reporter activity and mRNA degradation and is consistent with currently published data (Ezzeddine et al., 2012). An interesting progression of this work would be to identify the mechanism through which BTG2 interacts with mRNA because unlike TOB1 is cannot bind to PABP or CPEB3.

Tethering of BTG2 W103A and TOB1 W93A to mRNA did not affect reporter activity or mRNA abundance indicating the interaction with Caf1a (CNOT7)/Caf1b (CNOT8) was required for this activity. However, it remained unclear as to whether other subunits of the Ccr4-Not complex were required for the effects observed upon tethering BTG2 to TOB1 to a reporter mRNA. This study indicates that Caf1a (CNOT7)/Caf1b (CNOT8) are the deadenylases required to reduce reporter activity and that Ccr4a (CNOT6)/Ccr4b (CNOT6L) are not necessary. Furthermore, the Ccr4-Not components CNOT1 and CNOT3, which have been shown to co-precipitate with BTG2 (Aslam et al., 2009) were all not required for reduced reporter activity upon tethering BTG2 and TOB2 to an mRNA 3' UTR.

In summary, this work demonstrated that the BTG2 and TOB1 require an interaction with Caf1a (CNOT7) and Caf1b (CNOT8) to be anti-proliferative.

Furthermore, tethering of BTG2 and TOB1 to an mRNA 3' UTR leads to mRNA degradation which is dependent upon Caf1a (CNOT7) and Caf1b (CNOT8). This builds on the currently published work to suggest that the BTG/TOB proteins function as mRNA recruitment factor for these deadenylases. It can therefore be hypothesised that the BTG/TOB proteins may regulate cell proliferation by targeting mRNAs for degradation to modulate gene expression. To date the endogenous target genes of the BTG/TOB protein have yet to be identified. Further investigation into this would be an exciting next step in the field of BTG/TOB research

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Appendix

Appendix

1. Optimisation of transfection reagents and S-phase labelling

Using MCF-7 TR BTG2 expressing stable cell lines it was demonstrated that BTG2 requires an interaction with Caf1a (CNOT7)/Caf1b (CNOT8) to reduce cell proliferation (Chapter 4). In addition, transient transfections were used to induce expression of BTG2, BTG2 W103A, TOB1 or TOB1 W93A to confirm the requirement of the BTG2/TOB1-Caf1a (CNOT7)/Caf1b (CNOT8) interaction for BTG/TOB anti-proliferative activity. Unlike with stable cell lines, not all cells in a transient transfected population will necessarily incorporate the BTG2/TOB1 expression plasmid. To this end, several transfection reagents were tested to identify which one combined optimal transfection efficiency with minimal effects on cell viability and proliferation. A number of transfection reagents were found to alter the rate of cell proliferation, without the presence of a BTG2/TOB1 containing plasmid. GeneJuice (Novagen) was identified as the transfection reagent with the slightest effect on cell proliferation. Other transfection reagents evaluated were JetPEI (Polyplus), Fugene HD (Roche), Nucleofector V (Lonza), Calcium phosphate.

To monitoring transfection efficiency, a control population of cells were transfected with a GFP containing plasmid in parallel to plasmid transfections containing the BTG2 or TOB1 cDNA (as shown in Chapter 4). The GFP transfected cells were used as a guide for transfection efficiency where numbers of cells expressing GFP were compared to total number of nuclei identified using Hoescht (Figure 1A). If the number of cells expressing GFP was below 50% the transfection efficiency was considered too low and the S-phase data not collected. Using Genejuice (Merck), a transfection efficiency of 70% was routinely achieved. To calculate total number of cells in S-phase the number of cells with EdU labelled nuclei were compared to total nuclei identified through Hoescht staining (Figure 1B).

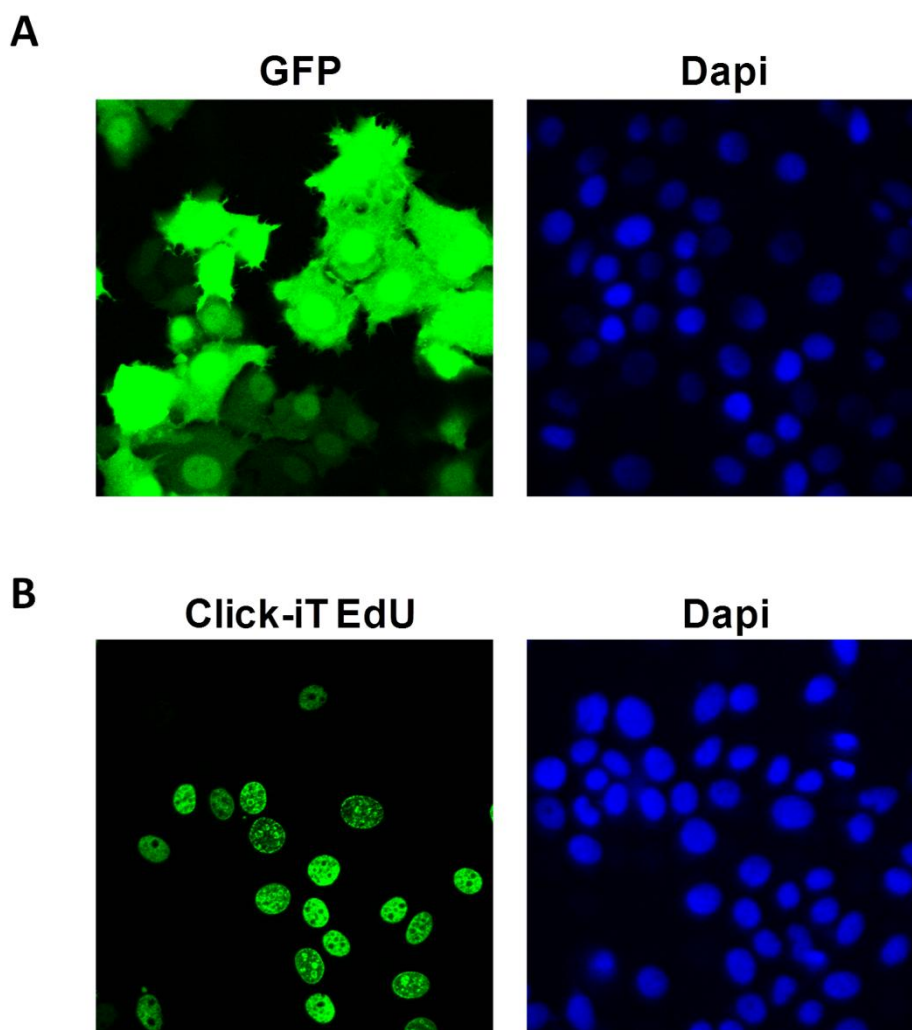


Figure 1. Immunofluorescence images of EdU-labelled MCF-7 cells. (A) To monitor transfection efficiency MCF-7 cells were seeded onto glass cover slips and transfected with a GFP plasmid. 48 h after transfection cells were fixed and cell nuclei stained with Hoescht. Cells transfected with the GFP plasmid were not incubated with the thymidine analogue EdU. **(B)** To monitor the number of cells in S-phase MCF-7 cells were seeded onto glass cover slips and transfected with appropriate plasmid pCMV5-HA-BTG2 or pCMV5-HA-TOB1. After 48 h cells were incubated with EdU. Cells with incorporated EdU were detected using conjugation of Alexa Fluor 488 azide, and nuclei were stained using Hoescht. All samples were tested in biological triplicate and the number of nuclei was counted for three images from each slide.

2. Antibody characterisation to detect endogenous BTG2 and TOB1 via western blot

To determine the relative protein levels of BTG2 and TOB1 in MCF-7 cells, western blot analysis was conducted using several commercially available antibodies. The MCF-7 TR stable cell lines pcDNA4-TO-Flag-HA-BTG2 and pcDNA4-TO-Flag-HA-BTG2 W103A were seeded in 6-well culture plates in the presence or absence of doxycycline. After 48 h total cell lysates were produced and subject to western blot analysis with anti-HA (Roche) or anti-BTG2 (Sigma AV33561). The western blot probed with anti-HA demonstrated that HA-BTG2 and HA-BTG2 W103A were expressed in the presence of doxycycline (Figure 2). However, HA-BTG2 or HA-BTG2 W103A expression was not detected on an equivalent western blot probed with anti-BTG2 (Sigma AV33561) (Figure 2). This indicates that anti-BTG2 (Sigma AV33561) was not able to detect BTG2 protein in MCF-7 cell lysates via western blot analysis.

To confirm that the HA epitope was not interfering with the western blot detection of BTG2 further experiments were conducted where un-tagged BTG2 was over expressed in MCF-7 cells. MCF-7 cells were seeded in 6-well culture plates and transfected with pCMV5-HA, pCMV5-HA-BTG2 and pBabe-puro-BTG2. After 48 h total cell lysates were produced and subject to western blot analysis with anti-HA (Roche), anti-BTG2 (Santa Cruz sc-33775), anti-BTG2 (Sigma AV33561) or anti-BTG2 (Gene-Tex 1082795). Western blot analysis using anti-HA confirmed the transfection and expression HA-BTG2. All BTG2 specific antibodies were unable to detect BTG2 or HA-BTG2 via western blot (Figure 3). Together, this data demonstrated that the three anti-BTG2 antibodies tested were all unable to detect BTG2 over-expression in MCF-7 cells.

To determine the endogenous expression of TOB1, one commercial antibody was tested. HEK293T cells were seeded in 6-well culture plates and transfected with pCMV5-HA, pCMV5-HA-BTG2 and pCMV5-HA-TOB1. After

48 h total cell lysates were produced and subject to western blot analysis with anti-HA (Roche) or anti-TOB1 (Santa Cruz sc-33192). Western blot analysis using anti-HA confirmed the transfection and expression HA-BTG2 and HA-TOB1 (Figure 4A). The anti-TOB1 (Santa Cruz sc-33192) antibody did not detect HA-TOB1 via western blot (Figure 4B). Surprisingly, this antibody did produce a band around 20kDa, the size of BTG2, in the pCMV5-HA-BTG2 transfected sample.

In summary, of the four antibodies tested none were able to detect endogenous or over-expressed BTG2 or TOB1. Further optimisation work is necessary to determine the optimal conditions for each antibody for the detection of BTG2 and TOB1. However it may be that the antibodies are not specific or sensitive enough to detect BTG2 and TOB1 via western blot.

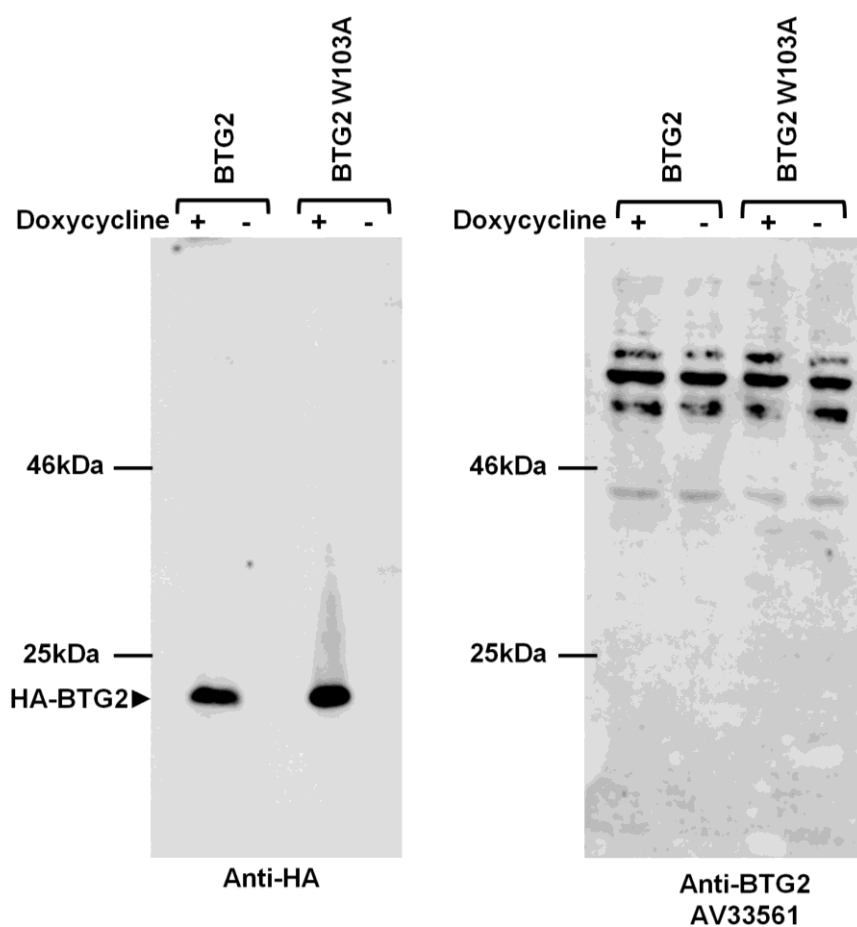


Figure 2. Sigma AV33561 anti-BTG2 antibody is unable to detect endogenous or over expressed BTG2. MCF-7 TR BTG2 and MCF-7 TR BTG2 W103A stable cell line cells were seeded in 6-well culture plates before doxycycline added to the media to induce HA-BTG2/HA-BTG2 W103A expression. After 48 h total cell lysates were produced and subjected to western blot analysis with anti-HA or anti-BTG2 (Sigma AV33561).

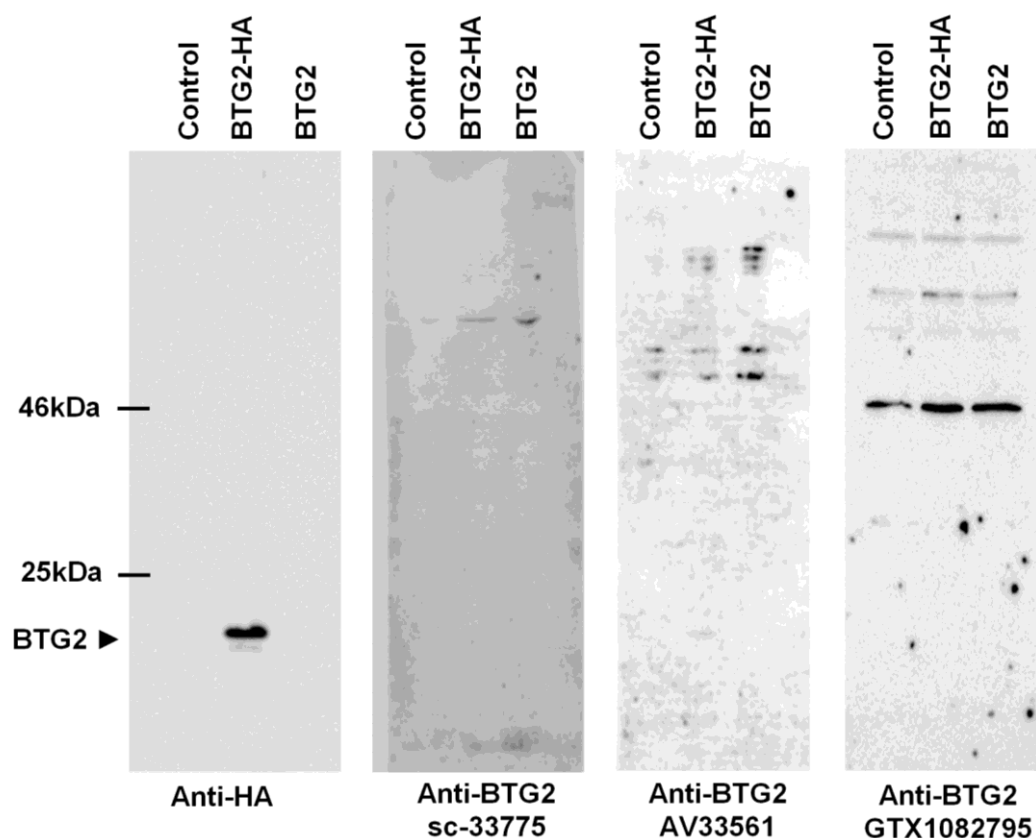


Figure 3. Commercial anti-BTG2 antibodies are unable to detect endogenous, overexpressed HA-BTG2 or overexpressed un-tagged BTG2. MCF-7 cells were seeded in 6-well culture plates and transfected with plasmids pCMV5-HA, pCMV5-HA-BTG2 or pBabe-puro-BTG2. After 24 h total cell lysates were produced and subjected to western blot analysis using anti-HA, Santa Cruz sc-33775, Sigma AV33561 and Gene-Tex 1082795.

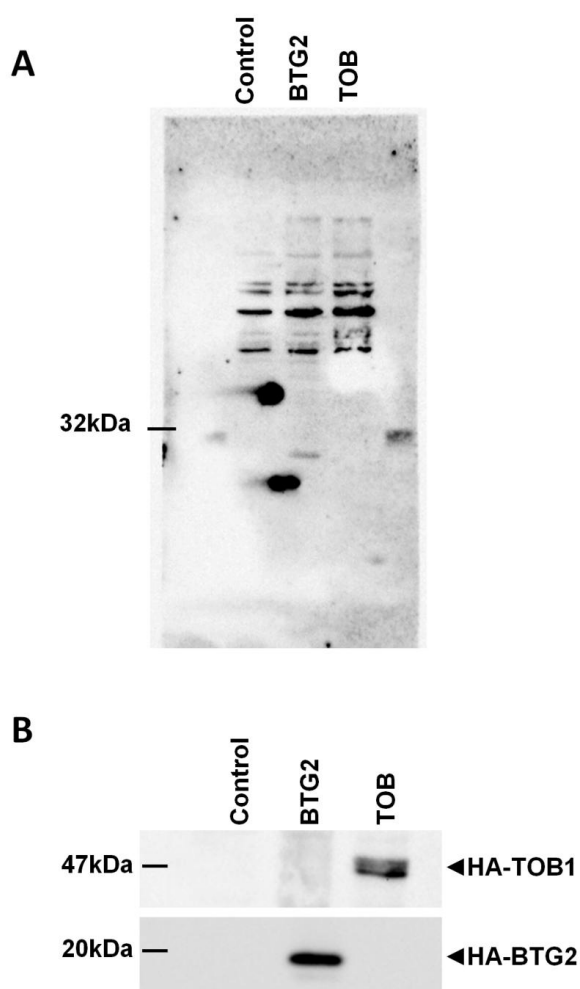


Figure 4. Santa Cruz anti-TOB1 antibody is unable to detect endogenous or over expressed HA-TOB1. HEK293T cells were seeded in 6-well culture plates and transfected with pCMV5-HA, pCMV5-HA-BTG2 or pCMV5-HA-TOB1. After 24 h total cell lysates were produced subject to western blot analysis **(A)** with anti-TOB1 (Santa Cruz sc-33192). **(B)** with anti-HA (Santa Cruz sc-33192). To detect HA-BTG2 membrane was exposed with chemiluminescence reagent for 2 min, for HA-TOB1 membrane was exposed with chemiluminescence reagent for 10min. HA-BTG2 expression was used to confirm successful plasmid transfection.

3. The anti-proliferative activities of BTG2 and TOB1 require interactions with Caf1a (CNOT7)/Caf1b (CNOT8) in U2OS cells.

We used U2OS cells to investigate if expression BTG2 and TOB1 can reduce the rate of cell proliferation in bone cancer cells, and to test if their anti-proliferative activity is dependent upon the Caf1a (CNOT7)/Caf1b (CNOT8) interactions in this cell type.

Expression of BTG2 significantly decreased the number of cells in S-phase compare to cells transfected with the empty vector pCMV5-HA. Expression of BTG2 W103A did not cause a reduction in the numbers of cells in S-phase (Figure 5A). This demonstrates that BTG2 expression reduces cell proliferation rates in U2OS cells and that the Caf1a (CNOT7)/Caf1b (CNOT8) interaction is required for the anti-proliferative activity. Expression of HA-BTG2 and HA-BTG2 W103A was confirmed via western blot analysis (Figure 5C). As expected the expression of TOB1 but not TOB1 W93A reduced the number of cells in S-phase as compared to cells transfected with empty vector (Figure 5B). Western blot analysis was unable to detect HA-TOB1 or HA-TOB1 W93A expression. However, there is a striking reduction of cells in S-phase after TOB1 expression comparable to results seen after BTG2 expression in U2OS cells. Furthermore, the results parallel those seen after TOB1 expression in MCF-7 cells (Chapter 4).

These results demonstrate that BTG2 and TOB1 not only reduced the rate of cell proliferation in MCF-7 cells but also in U2OS cells and that BTG2/TOB1 activity is dependent upon interactions with Caf1a (CNOT7)/Caf1b (CNOT8).

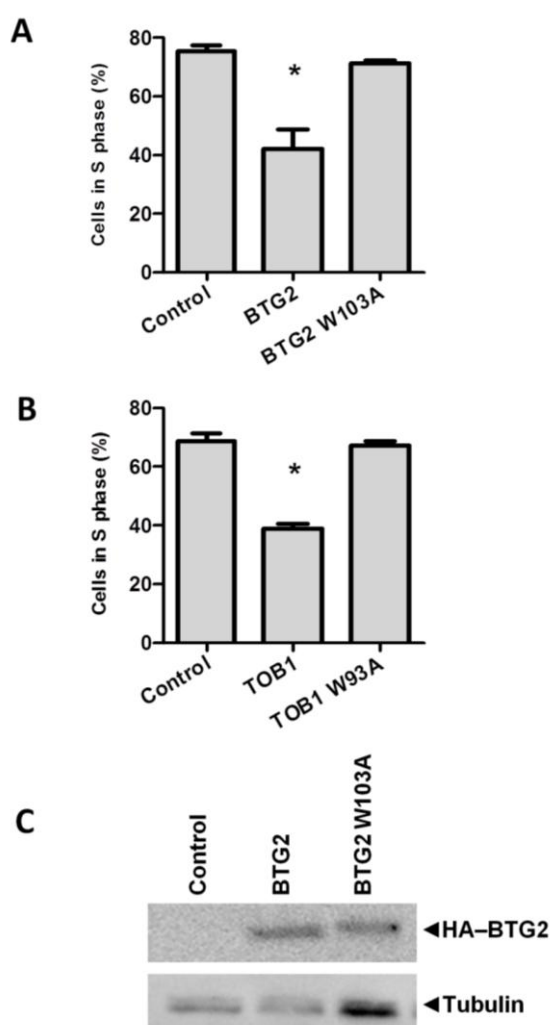


Figure 5. BTG2 and TOB1 require interactions with Caf1a (CNOT7)/Caf1b (CNOT8) for their anti-proliferative activities in U2OS cells. U2OS cells were seeded onto glass cover slips before being transfected with pCMV5-HA, pCMV5-HA-BTG2 or pCMV5-HA-BTG2 W103A. 48 h after transfection cells were incubated with the thymidine analogue EdU and detected after incubation with Alexa Fluor 488 azide. Cells were counter stained with Hoechst 33342 to allow total cells to be counted on the LSM fluorescence microscope. **(A)** Cells were transfected with pCMV5-HA, pCMV5-HA-BTG2 or pCMV5-HA-BTG2 W103A. Cells in S-phase were labelled with EdU and detected by fluorescence microscopy after conjugation with Alexa Fluor 488 azide. All samples were tested in biological triplicate and the number of nuclei were counted for three images from each slide. Students T test used to calculate statistical significance, * indicates a p value of < 0.05 compared to control cells transfected with pCMV5-HA. **(B)** Cells were transfected with pCMV5-HA, pCMV5-HA-TOB1 or pCMV5-HA-TOB1 W93A and analysed as described in (A). **(C)** Western blot analysis of U2OS cells transfected with pCMV5-HA, pCMV5-HA-BTG2 or pCMV5-HA-BTG2 W103A. Proteins were detected with anti-HA and anti-Tubulin.

4. Cellular localisation of BTG2 and BTG2 W103A

To determine if the localisation of BTG2 W103A is different to wild type BTG2, immunofluorescence was performed. The MCF-7 TR stable cell lines containing expression cassettes pcDNA4-TO, pcDNA4-TO-Flag-HA-BTG2 and pcDNA4-TO-Flag-HA-BTG2 W103A were seeded onto cover slips before expression was induced by the addition of doxycycline. Antibodies recognising the Flag epitope were used in combination with Alexa Fluor 594 conjugated secondary antibody to detect Flag-HA-BTG2 and Flag-HA-BTG2 W103A.

Immunofluorescence microscopy indicated that BTG2 is dispersed in a granular or pin point like pattern throughout the cytoplasm. The expression of BTG2 W103A appears to conform to the same pattern as wild type BTG2 (Figure 6). However, background staining from the pcDNA4-TO expressing stable cell line displays a similar pattern but with reduced staining intensity. Therefore, the cellular localisation of BTG2 and BTG2 W103A is inconclusive.

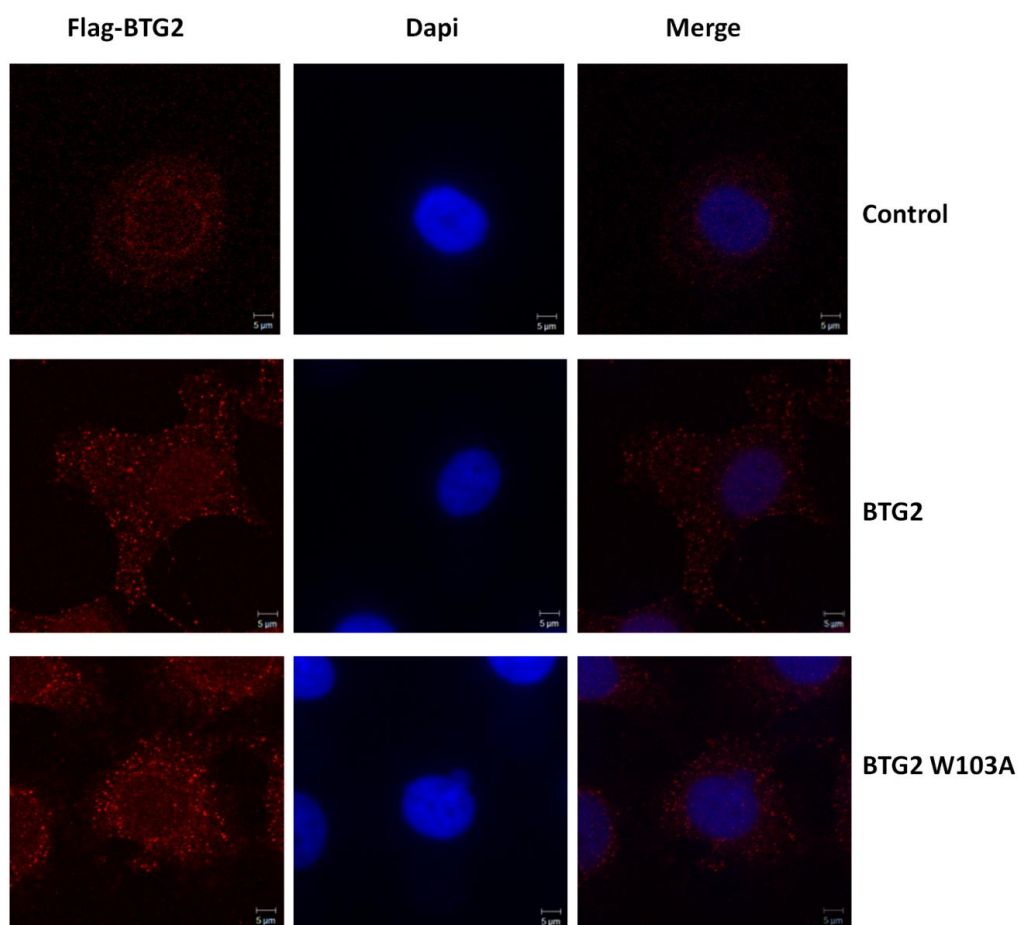


Figure 6. Cellular localisation of BTG2 and BTG2 W103A in MCF-7 cells. BTG2, BTG2 W103A or empty vector MCF-7 TR stable cell lines were seeded onto glass coverslips before vector expression was induced by the addition of doxycycline to the cell media. After 48 h cells were fixed and Flag-BTG2 or Flag-BTG2 W013A protein was detected using anti-Flag primary antibody and an anti-mouse alexa Fluor 594 secondary antibody. Cells were counter stained with Hoescht to identify the nuclei. Images shown are representative Z stack images.

5. Changes in CCND1 (Cyclin D1), p21 (Waf1/CIP1) and p27 (Kip1) expression in response to BTG2 and TOB1 overexpression

To identify if expression of CCND1 (Cyclin D1), p21 (Waf1/CIP1) and p27 (Kip1) is reduced after BTG2 or TOB1 expression, RT-qPCR analysis was conducted. MCF-7 cells were transfected with pCMV5-HA, pCMV5-HA-BTG2 or pCMV5-HA-TOB1 and total RNA was extracted after 48 h. CCND1 (Cyclin D1), p21 (Waf1/CIP1) and p27 (Kip1) mRNA levels were determined by RT-qPCR analysis using GAPDH as a control gene. Upon BTG2 expression, there was no significant change in the relative amount of CCND1 (Cyclin D1), p21 (Waf1/CIP1) or p27 (Kip1) mRNA (Figure 7A). The assay was repeated with RNA extracted after 72 h but this also showed no significant difference in the relative level of CCND1 (Cyclin D1), p21 (Waf1/CIP1) or p27 (Kip1) mRNA. After TOB1 expression there was a decrease in the relative amount of CCND1 (Cyclin D1) ($p < 0.1$). There was no significant change in the relative amount p21 (Waf1/CIP1) or p27 (Kip1) mRNA (Figure 7B). This demonstrates that over-expression of BTG2 and TOB1 causes no dramatic change in the mRNA levels of CCND1 (Cyclin D1), p21 (Waf1/CIP1) or p27 (Kip1) in MCF-7 cells.

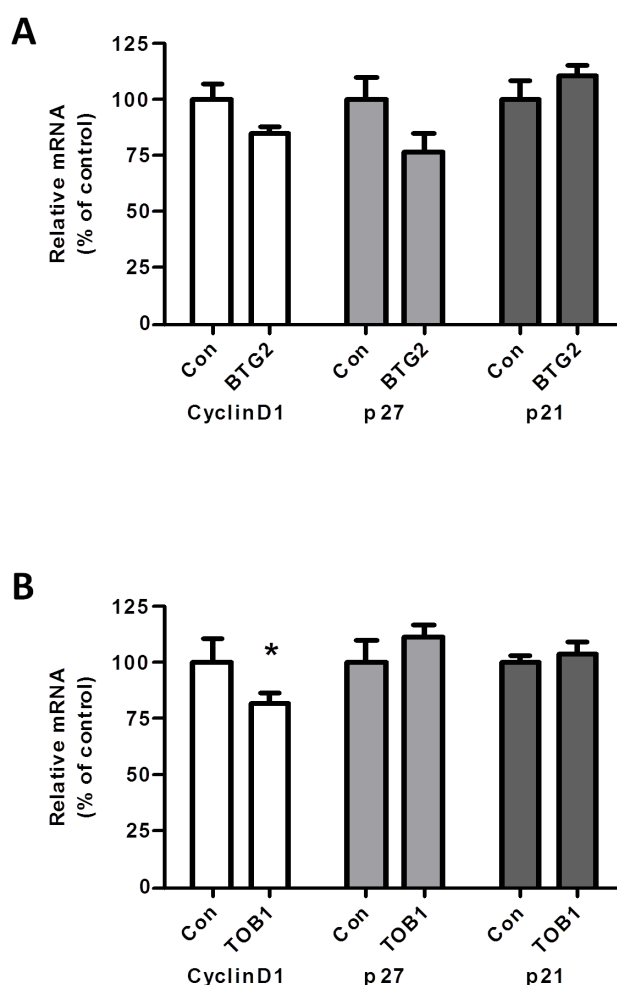


Figure 7. Transient expression of BTG2 and TOB1 do not strongly affect the level of CCND1 (Cyclin D1), p27 (Kip1) or 21 (Waf1/CIP1) mRNA in MCF-7 cells. (A) RT-qPCR to identify changes in the level of CCND1 (Cyclin D1), p27 (Kip1) and p21 (Waf1/CIP1) mRNA after BTG2 expression. MCF-7 cells were transfected with pCMV5-HA or pCMV5-HA-BTG2. After 48 h total RNA was isolated and mRNA levels determined through RT-qPCR using GAPDH as a reference gene. Experiments were carried out in duplicate and a Student T test was used to calculate statistical significance. **(B)** RT-qPCR to identify changes in the level of CCND1 (Cyclin D1), p27 (Kip1) and p21 (Waf1/CIP1) mRNA after TOB1 expression. MCF-7 cells were transfected with pCMV5-HA or pCMV5-HA-BTG2. After 48 h total RNA was isolated and mRNA levels determined through RT-qPCR using GAPDH as a reference gene. Experiments were carried out in duplicate and a Student T test was used to calculate statistical significance. * p value of 0.09 compared to cells transfected with empty vector.

6. Tethering Caf1a (CNOT7) to the 3' UTR of a reporter mRNA causes reduced activity which is dependent on deadenylase activity but not a BTG2 interaction

RNA tethering assays were conducted to determine if the Caf1a (CNOT7) deadenylase activity and interaction with BTG2 alter reporter activity when tethered to the 3' UTR of a reporter mRNA. Thus, MCF-7 cells were co-transfected with the reporter pRL-5boxB and plasmids expressing fusions of the λ N peptide with CNOT7, CNOT7 D40A (catalytic mutant), or CNOT7 E247A Y260A (BTG2 interaction mutant). After transfection, total protein was extracted and luciferase activity determined (Figure 8A).

Tethering of λ N-Caf1a (CNOT7) to the luciferase mRNA caused a reduction in luciferase activity, which was not observed in cells expressing the λ N peptide only. By contrast, tethering of λ N-Caf1a (CNOT7) D40A to the luciferase mRNA did not cause a reduction in luciferase activity (Figure 8B). This indicates that the reduction in luciferase protein is dependent upon the catalytic activity of the Caf1a (CNOT7) deadenylase. Tethering of Caf1a (CNOT7) E247A Y260A to the luciferase mRNA also caused a reduction in luciferase activity comparable to wild type Caf1a (CNOT7) (Figure 8B). This indicates that the interaction with BTG2 does not alter the Caf1a (CNOT7) activity when tethered to a reporter mRNA. To confirm that reduced luciferase activity was caused by tethering of Caf1a (CNOT7) to the 3' UTR, we used reporter plasmid pRL-TK that does not contain the BoxB motifs. Expression of λ N-Caf1a (CNOT7) caused a modest reduction in luciferase activity compared to cells transfected with empty vector (Figure 8C). There was no significant difference between the samples expressing λ N-Caf1a (CNOT7) D40A and λ N-Caf1a (CNOT7) E247A Y260A fusion proteins. Together this indicates that tethering of Caf1a fusion proteins is indeed responsible for reduced luciferase activity when a reporter containing BoxB sequences was used. Western blot analysis was conducted on protein lysates to confirm expression of Caf1a (CNOT7) (Figure 8D).

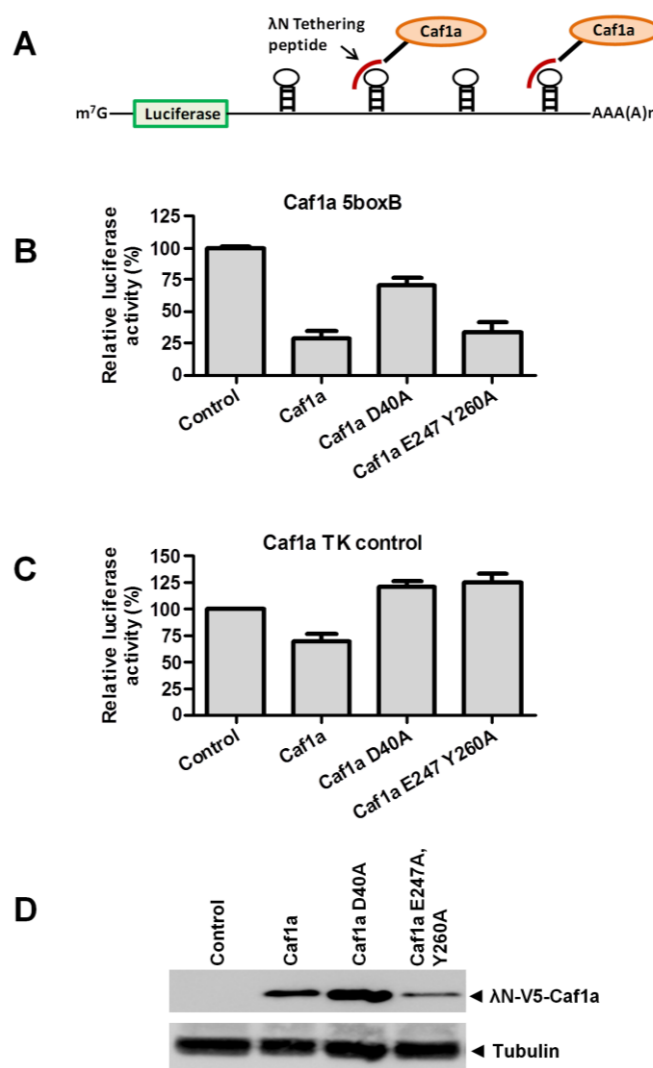


Figure 8. Caf1a (CNOT7) requires deadenylase activity but not an interaction with BTG2 to reduce reporter activity upon 3' UTR tethering. (A) Schematic representation of the RNA tethering assay. **(B)** Decreased reporter activity upon RNA tethering of λN-Caf1a (CNOT7) or λN-HA-Caf1a (CNOT7) E247A Y260A, but not of λN-HA-Caf1a (CNOT7) D40A. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated CNOT7 cDNA, and the luciferase reporter plasmid pRL-5BoxB. Luciferase activity was measured 24 h after transfection. **(C)** No specific effect of λN-Caf1a (CNOT7), λN-HA-Caf1a (CNOT7) D40A or λN-HA-Caf1a (CNOT7) E247A Y260A expression on the activity of a Luciferase reporter lacking BoxB sequences. MCF-7 cells were transfected with empty vector or plasmid pCIλN containing the indicated Caf1a (CNOT7) cDNA, and the luciferase reporter plasmid pRL-TK. Luciferase activity was measured 24 h after transfection. **(D)** Western blot showing the expression for λN-Caf1a (CNOT7), λN-HA-Caf1a (CNOT7) D40A or λN-HA-Caf1a (CNOT7) E247A Y260A. λN-fusion proteins were detected using anti-V5 antibodies. Tubulin was used as a loading control.