Molecular Analysis of Claspin Function In Vertebrate Cells

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B.Sc.(Hons)

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Abstract

Claspin is a large, acidic, DNA binding protein required for the ATR mediated activation of Chk1 in response to DNA replication stress. Upon checkpoint activation Claspin is phosphorylated which allows binding to Chk1. This binding in turn promotes the phosphorylation and activation of Chk1 by ATR. Although these findings have led to the designation of Claspin as a mediator protein in the activation of Chk1, the broader biological functions of Claspin have not been extensively explored at the cellular level, in part due to the lack of a genetically tractable system in which to study Claspin function.

The purpose of this study was to use reverse genetics in order to generate a Claspin knock-out cell line with which study Claspin function at the cellular level. This was to be achieved by exploiting the recombinogenic vertebrate DT40 cell line, a pre B-cell line of chicken origin. The chicken homologue of Claspin was successfully isolated and characterised. The chicken Claspin orthologue shares 56% identity with human Claspin and the overall domain structure of Claspin appears to be conserved based on sequence comparison. This information facilitated the construction of gene targeting vectors to disrupt Claspin expression in the DT40 cell line. One allele of Claspin was successfully targeted. Despite multiple attempts at disrupting the remaining allele homozygous Claspin knock-out cells were not obtained. This strongly indicates that Claspin is essential for the viability of DT40 cells. In order to circumvent this lethality, efforts were made to conditionally express Claspin in the hemizygous background to facilitate the generation of a conditional knock-out cell line. These efforts proved unsuccessful.

Other studies using a polyclonal antibody raised against Claspin investigated phosphorylation of the protein under various stress conditions. Data is presented which shows that phosphorylation of Claspin in response to replication stress is dependent on Chk1 kinase activity. However results from an in vitro kinase assay suggest that Chk1 itself does not directly phosphorylate Claspin. In addition it is also shown that Claspin is phosphorylated following inhibition of protein synthesis. Using specific kinase inhibitors against p38, JNK and mTOR, protein kinases known to be activated during protein synthesis inhibition, the

involvement of these kinases in mediating this response was ruled out. The significance of this observation is yet to be determined.

Overall the work presented here provides evidence that a conditional DT40 knock-out cell line will be a valuable tool in revealing novel functions of Claspin once a successful rescue of lethality is achieved.

Keywords: Claspin; Chk1; ATR; DT40 knock-out; Phosphorylation; Gene targeting; DNA Replication; Protein synthesis.

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Author's Declaration

I declare that I am the sole author of this thesis. The work presented here is my own, unless otherwise acknowledged. This thesis has not been submitted for consideration for another degree in this or any other university.

Abbreviations

ALV	Avian Leukosis Virus
Asp (D)	Aspartic Acid
Asp (N)	Asparagine
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Bim1	Bisindolylmaelimide I
BsdR	Blasticidin Resistance (conferred by Blasticidin – S – Deaminase)
cClaspin	Chicken Claspin
CDS	Coding DNA sequence
Chk1	Checkpoint Kinase 1
CHX	Cycloheximide
Claspin	Chk1 Large Associated Protein
Cys (C)	Cysteine
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
dsDNA	double stranded DNA
DTT	Dithiothreitol
E. coli	Escherichia coli
EDD	E3 identified by differential display (an E3 ubiquitin ligase)
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FACS	Fluorescence Activated Cell Sorting
Glu (Q)	Glutamine
Gly (G)	Glycine
hClaspin	Human Claspin
HisR	Histidinol Resistance (conferred by Histidinol Dehydrogenase)
HR	Homologous Recombination
HU	Hydroxyurea
IPTG	Isopropyl-1-thio-ß-D-galactopyranoside
Luc	Luciferase
Lys (K)	Lysine
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester

mESC	mouse Embryonic Stem cells
Met (M)	Methionine
MGMT	O-6-methylguanine-DNA methyltransferase
MMEJ	Microhomology Mediated End Joining
Mrc1	Mediator of Replication Checkpoint 1
mTOR	Mammalian target of Rapamycin
NeoR	Neomycin (G418) Resistance (conferred by Neomycin phospho-transferase
NHEJ	Non-homologous End Joining
Noc	Nocodazole
PCR	Polymerase Chain Reaction
Pfu	Plaque forming unit
PI	Propidium Iodide
PMSF	Phenylmethylsulphonyl fluoride
Pro (P)	Proline
PuroR	Puromycin Resistance (conferred by Puromycin Acetyltransferase)
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
Sc	Saccharomyces cerevisiae
SDS	Sodium Dodecylsulphate
Ser (S)	Serine
Sp	Schizosaccharomyces pombe
ssDNA	Single stranded DNA
TAP	Tandem Affinity Purification
TEMED	Tetramethylethylenediamine
TetO	Tetracycline Operator
Thr (T)	Threonine
TRE	Tetracycline responsive Element
tTA	tetracycline Transactivator
tTA_{AD}	Advanced TetracyclineTransactivator
UCN-01	7-Hydroxystaurosporine
USP	Ubiquitin Specific Protease
xClaspin	Xenopus Claspin
X-gal	5-Bromo-4-chloro-3-indoyl-ß-D-galactopyranoside
γ-IR	Gamma Ionising Irradiation

Chapter 1. Introduction

1.1. The DNA Damage response

Damaged lipids, proteins and RNA can be degraded and re-synthesised. However damage to DNA is more serious. The information required to re-synthesise RNA, proteins and indirectly lipids is contained within DNA and if this information gets corrupted, through the introduction of mutations for example, it can drastically affect the normal functioning of the cell. Therefore due to the critical importance of protecting DNA from mutation multiple pathways have evolved in order to prevent damage to the DNA or to sense when the DNA is damaged and then signal this to both the cell cycle machinery and to the DNA repair machinery. These processes are described below.

1.1.1. DNA Damage sensing and signalling

DNA damage signalling encompasses a complex set of co-ordinated events that occur upon damage to the DNA, replication stress and in response to shortened telomeres. Two kinases play a central role in this process; ATM and ATR (Abraham, 2001). Classically these kinases were thought to be activated following DNA double strand breaks and replication stress respectively, but it has now become clear that there is considerable cross talk between the two pathways as illustrated in Figure 1.1 (Cuadrado et al, 2006; Yoo et al, 2007; Yoo et al, 2009).

ATR is activated by tracts of single stranded DNA (ssDNA) such as are found at the replication fork if the DNA polymerase and the replicative helicase become uncoupled. ssDNA generated through the process of DNA repair can also lead to the activation of ATR. ssDNA rapidly becomes coated with the ssDNA binding protein RPA and this complex recruits the ATR kinase via its binding partner ATRIP (Ball et al, 2005; Namiki & Zou, 2006). Independently a complex with a similar structure to PCNA, the processivity factor for DNA replication, is loaded onto DNA by the Rad17-RFC complex. This complex is made up of the Rad9-Rad1-Hus1 proteins. Stimulation of ATR kinase activity is brought about by the recruitment of another factor called TopBP1. TopBP1 has been shown to contain a domain that greatly stimulates ATR kinase activity – the ATR activation domain (Kumagai et al, 2006). TopBP1 is recruited through binding to the Rad9 subunit

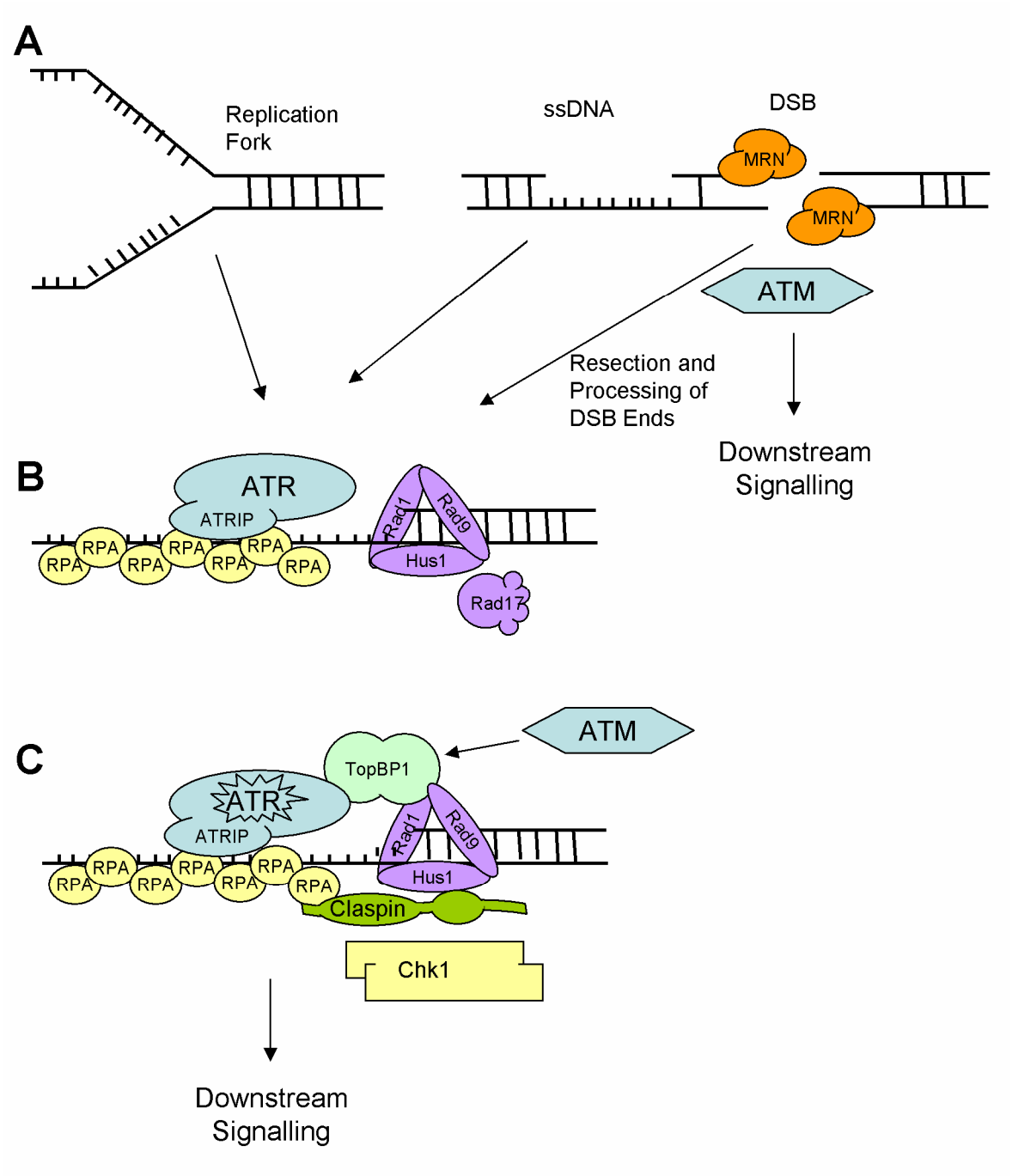


Figure 1.1 ATM/ATR Signalling
A) Single stranded DNA is generated at replication forks or through the process of DNA repair such as the resection of DNA DSBs. DNA DSB's can directly lead to the activation of ATM. B) RPA is recruited to this single stranded DNA which in turn leads to the recruitment of ATR via its ATRIP subunit. The Rad9-Rad1-Hus1 complex is loaded onto the DNA by the Rad17-RFC complex. C) TopBP1 which stimulates ATR kinase activity is recruited via the Rad9 subunit of the 9-1-1 complex. ATM stimulates ATR kinase activity by phosphorylating TopBP1 which promotes the ATR-TopBP1 interaction. Active ATR then phosphorylates its downstream targets.

of the 9-1-1 complex (Lee et al, 2007). TopBP1 is phosphorylated by ATR following replication stress and by ATM following DNA DSBs which further promotes the ATR-TopBP1 interaction leading to enhanced activation of ATR. The Nbs1 subunit of the MRN complex facilitates this ATM mediated phosphorylation of TopBP1 (Yoo et al, 2009). Once activated ATR then phosphorylates its target proteins on SQ/TQ sites in order to transmit the signal to the downstream effector proteins. An important target of ATR is the Chk1 protein kinase. ATR phosphorylates Chk1 which increases its kinase activity allowing it to phosphorylate its down stream targets in order to control cell cycle progression. Claspin functions in this process by binding to Chk1 and facilitating the phosphorylation of Chk1 by ATR (Chini & Chen, 2004; Cimprich & Cortez, 2008; Shiotani & Zou, 2009). (See sections 1.2 and 1.3 for the discussion on Chk1 activation and Claspin function respectively)

ATM is activated following DNA DSBs. The exact mechanism of ATM activation by DNA damage is not yet completely understood but it is thought to require the MRN complex, changes to the chromatin structure and post translational modifications including phosphorylation and acetlyation (Bakkenist & Kastan, 2003; Sun et al, 2007). One model proposes that ATM exists as an inactive homodimer which dissociates upon activation due to autophosphorylation at S1981 to release active ATM monomers (Bakkenist & Kastan, 2003). The validity of this model has been challenged however with the observation that mice bearing a mutation at the equivalent mATM site behave as WT mice with no defects in mATM activation (Daniel et al, 2008; Pellegrini et al, 2006). The down stream targets of ATM overlap significantly with those of ATR and influence the processes of cell cycle progression and DNA repair (Lavin & Kozlov, 2007).

In addition to the core signalling molecules described above other proteins, which have been termed mediators or adaptors, have been implicated in the DNA damage response. MDC1, 53BP1, MCPH1, Nbs1 and Brca1 each have functions in ensuring optimal signalling following DNA damage. All of these proteins contain BRCT domains which are phospho-peptide binding motifs. Other domains present in these proteins include FHA domains (in MDC1 – binds phospho-threonine peptides) and Tudor domains (53BP1 – binds methylated histones). One of the earliest signalling events to occur following DNA damage is the phosphorylation of the C-terminal tail of the variant Histone H2AX. This

serves as a molecular beacon to attract the adaptor proteins to the site of damage leading to a concentration of DNA damage signalling and repair proteins in nuclear foci (Mohammad & Yaffe, 2009).

1.1.2. Cell cycle checkpoints

1.1.2.1. Overview

Damaging DNA per se does not automatically result in a mutation. For example an undetected mis-paired base within a gene will only become a mutation once the cell passes undergoes DNA replication during S phase. In this instance one daughter cell will inherit the correct copy of the gene where as the other cell will get the mutated copy of the gene. Similarly gross changes at the chromosome level only become mutations once the cell has passed through mitosis. Such mutations can be chromosome rearrangements or even the loss of portions of or entire chromosomes. Given the vulnerability of both S phase and mitosis with regards to mutation of the genome it is not surprising that elaborate mechanisms are in place to either prevent entry into or halt the progression of these phases of the cell cycle.

1.1.2.2. G1/S

Before committing to DNA replication the cell must ensure that there is no damage present in its DNA. Cells damaged before entry into S phase display two responses – an immediate response through the inhibition of Cyclin dependent kinases and a later response due to the accumulation of Cdk inhibitors e.g. p21 induced through the activation of p53. ATM is activated in G1 phase of the cell cycle where it phosphorylates p53 leading to its stabilisation. p53 in turn induces the transcription of p21 which inhibits Cdk's and inhibits S phase. At the same time but with a quicker response ATM phosphorylates Chk2 which phosphorylates and inhibits Cdc25A also leading to an inhibition of Cyclin dependent kinases (Bartek & Lukas, 2001).

1.1.2.3. S phase

Checkpoint responses in S phase can be broadly divided into three different categories. Inhibition of DNA synthesis, for example through nucleotide pool imbalance or by direct damage to the replicating DNA, leads to the activation of

the Replication checkpoint which serves to protect the process of replication itself (Bartek et al, 2004). It does this by inhibiting the activation of late firing origins and stabilising stalled forks in order to facilitate the resumption of DNA synthesis when the conditions are once again favourable (Branzei & Foiani, 2009; Feijoo et al, 2001). The S-M checkpoint on the other hand prevents entry into mitosis in the presence of incompletely replicated DNA. Much less is known about what the molecular signal that activates the S-M checkpoint is, but it has been speculated to arise from viable replication forks. Chk1 plays a major role in orchestrating all of these responses (Broderick & Nasheuer, 2009; Kumagai et al, 1998; Zachos et al, 2005).

1.1.2.4. G2-M

Prevention from entry into mitosis in the presence of damaged DNA is carried out by the G2-M checkpoint. Through the activation of the ATM/ATR kinases the down-stream kinase Chk1 is phosphorylated and activated leading to inhibition of Cdk1-cyclin B. This is mediated through the phosphorylation and inhibition of the Cdc25 phosphatases which are required to activate Cdk1-Cyclin B to allow mitosis to proceed. Other kinases such as p38 have also been implicated in carrying out this function. A transcriptional programme regulated by p53 and Brca1 can also contribute to sustaining G2-M arrest (Kastan & Bartek, 2004).

1.1.2.5. Mitosis

Cells activate the Spindle Assembly checkpoint in order to prevent loss of chromosomes during mitosis. Until all chromosomes are attached to two microtubules from opposite poles of the cell an inhibitory signal preventing the activation of the Anaphase promoting complex (APC), which is required to progress through mitosis, is maintained. Once this signal is shut off the APC becomes active leading to the degradation of key proteins, such as Cyclin B and Separase, which facilitates mitotic exit (Tanaka & Hirota, 2009). Whether a pathway exists that responds to DNA damage during mitosis is still unclear (Mikhailov et al, 2002; Skoufias et al, 2004).

1.1.3. DNA repair

1.1.3.1. Overview

The repair of damaged DNA and the activation of cell cycle checkpoints following DNA damage are intimately linked and essentially interdependent (Lazzaro et al, 2009). Many of the signals that activate the cell cycle checkpoint machinery are generated through the repair of damaged DNA, but DNA repair is in turn stimulated and facilitated through the activation of checkpoint signalling (Goodarzi et al, 2008; Sorensen et al, 2005). Indeed many proteins involved in the DNA damage response have functions in both the repair of DNA and checkpoint signalling e.g. Brca1, MRN complex, RPA etc.

DNA damage can arise from both endogenous and exogenous factors. Within the cell errors in replication and damage from the by products of metabolism e.g. reactive oxygen species can lead to DNA structural aberration. External sources of damage include UV light, ionising irradiation and environmental toxins and mutagens. These types of damage can lead to a variety of different lesions that include chemical modification of individual nucleotides, inter and intrastrand crosslinks and single and double strand breaks.

To counteract the deleterious effects of damaged DNA multiple DNA repair pathways have evolved. Some damaged DNA can be directly repaired through the action of specialised enzymes e.g. MGMT (Verbeek et al, 2008). Other damage can be by-passed and repaired later through the action of by-pass polymerases (Guo et al, 2009). Other lesions if left un-repaired would have more drastic effects on the cell for example DNA strand breaks. A brief overview of some of the main details of these pathways follows.

1.1.3.2. Repair of DNA double strand breaks

DNA double strand breaks (DSBs) represent a particular difficulty for the cell. Unlike with other forms of damage an un-repaired DSB can lead to the loss of a large amount of genetic information and incorrectly repaired breaks can result in mutations which lead to disease (Hartlerode & Scully, 2009). Double strand breaks are repaired in two ways – either faithfully by the process of homologous recombination (HR) or through error-prone mechanisms such as non-homologous

end joining (NHEJ) or micro-homology mediated end joining (MMEJ). The proteins involved in these pathways (especially for HR and NHEJ) have been described and much of the mechanistic detail of how they function has been elucidated (Lieber, 2008; Sung & Klein, 2006).

Briefly the process of NHEJ simply results in the ligation of two broken DNA ends. The double strand breaks are recognised by the Ku70/80 heterodimer complex. This complex then serves to recruit the proteins necessary to mediate the repair of the broken end. DNA-PKcs in complex with an endonuclease Artemis binds to Ku. In addition DNA polymerases λ and μ are also recruited. The nuclease activity of the DNA-PKcs-Artemis is stimulated by binding to Ku and this together with the activity of the polymerases prepares the broken DNA end for ligation. The ligation step is carried out by the XLF/Xrcc4/DNA ligase IV complex which due to its flexible nature can re-join imperfect ends leading to the re-formation of the phosphodiester backbone (Downs & Jackson, 2004; Lieber, 2008). A similar but less characterised process called microhomology mediated end joining (MMEJ) requires some resection to reveal regions of microhomology that facilitate annealing of the broken ends and re-ligation of the break (McVey & Lee, 2008).

Whereas non-homologous end joining often leads to mutations at the ligation site the process of homologous recombination results in the high-fidelity repair of DNA DSBs breaks. This process occurs in S phase and in G2 phase once a sister chromatid, which serves as a template for repair, has been generated. In homologous recombination the DSB is recognised by the Mre11/Rad50/Nbs1 (MRN) complex. The exonuclease activity of the Mre11 subunit and probably the activity of other nucleases and factors (e.g. Exo1, CtIP) lead to the generation of single stranded resected DNA. Then, through the action of the RAD52 epistasis group of proteins, this stretch of ssDNA finds its homologous sequence in its sister chromatid and a joint DNA structure is formed which is resolved by the action of polymerases, helicases and nucleases to restore the original DNA sequence (San Filippo et al, 2008).

It must be remembered however that this process does not occur on free DNA but rather in the context of chromatin. Great advances in our understanding of the role played by chromatin marks in the repair of DNA DSBs have recently been

made. Phosphorylation, methylation and ubiquitination of chromatin all play a major role in orchestrating the recruitment of both DNA damage signalling and repair factors to the sites of DNA damage (Lisby & Rothstein, 2009).

1.1.3.3. Nucleotide/Base Excision and Mismatch Repair

Nucleotide and Base Excision repair are required to remove bulky lesions and damaged bases from the DNA respectively whereas mismatch repair serves to correct mismatched bases that arise from errors in replication or by mutagenic bases. Nucleotide excision repair recognises and repairs bulky lesions such as pyrimidine dimers and cyclobutane adducts. These lesions are recognised and processed by the Xeroderma pigmentosa family of proteins XPA-G and other factors including ERCC1, TFIIH and CSA/B proteins. Nucleotide excision repair can act globally (GG-NER) or coupled with transcription (TC-NER). In both cases the nucleotide lesion is recognised, the double strand helix is unwound and the damaged piece of DNA is removed. The gap is then filled in and religated (de Laat et al, 1999). Base excision repair removes damaged bases by the action of specific glycosylases that remove specific damaged bases e.g. UNG removes uracil from DNA and Ogg1 removes 8-oxoguanine lesions from DNA. The resulting AP (apurinic/apyrimidinic) site is recognised by AP endonuclease which nicks the DNA allowing removal of the damaged nucleotides and resynthesis and ligation by Polß and DNA Ligase I or DNA Ligase III/Xrcc1 (Fortini et al, 2003).

Mismatch repair corrects errors of replication if bases are incorrectly paired or if nucleotides are deleted or inserted. This process in vertebrate cells relies on the activity of the orthologues of the MutS and MutL found in bacteria. MutS recognises the mismatched DNA and recruits MutL. MutL then stimulates MutS to recruit the rest of the proteins involved in repairing the mismatch e.g. Exo1, DNA polymerase δ/ε and DNA Ligase 1 (Jiricny, 2006).

Overall these repair pathways serve to prevent single base pair mutations through out the genome. Loss of these pathways is said to lead to a mutator phenotype which can greatly increase genome instability, a process intimately linked with cancer (Loeb et al, 2008). Indeed mutations in some of the proteins involved in these pathways can lead to cancer predisposition syndromes. Furthermore defects in these repair pathways often lead to defects in DNA

damage signalling resulting in impaired signalling down the cell cycle checkpoint arm of the DNA damage response (Cejka et al, 2003; O'Brien & Brown, 2006).

1.2. Regulation of Chk1 Kinase Activity

Chk1 was first reported in the yeast *Schizosaccharomyces pombe* over 15 years ago (Walworth et al, 1993). Over subsequent years it has become clear that Chk1 plays a vital role in the response to DNA damage. It was discovered that spChk1 could phosphorylate and activate the kinase spWee1 which in turn phosphorylates and inactivates the mitotic CDK in *pombe*, spCdc2 (O'Connell et al, 1997; Rhind et al, 1997). Simultaneously it inactivates the spCdc25 phosphatase, which is required to remove the inhibitory phosphorylation on spCdc2 (Furnari et al, 1997). In combination, this leads to an inhibition of the spCdc2 kinase and a halt in cell cycle progression. This same pathway appears to be conserved in vertebrates with vertebrate Chk1 also playing the major role in the G_2-M checkpoint through the maintenance of the inhibitory phosphorylation on Cdk1 (Lee et al, 2001; Peng et al, 1997; Sanchez et al, 1997). In contrast with the situation in yeast however it is vertebrate Chk1 and not Chk2 (orthologue of spCds1) that is activated during replication stress and required to mediate S phase checkpoint responses (Feijoo et al, 2001; Zachos et al, 2005). As for the G2-M checkpoint the S phase checkpoint may also be enforced through the inhibition of Cyclin dependent kinases via the inhibition of Cdc25 (Mailand et al, 2000). However it also appears that intrinsic signals, distinct from checkpoint signalling, may also exist during S phase which prevent replicating cells from entering mitosis (Oehler, 2008; Rao & Johnson, 1970). The validity of this model has also been challenged recently due to the observation that Cdk2 (the S phase Cdk) activity actually increases in S phase following DNA damage (Bourke et al, 2009).

Given the importance of Chk1 in the response to DNA damage it is therefore not surprising that the regulation of this kinase might be complex. Purified full-length Chk1 from insect cells possesses intrinsic kinase activity (Jackson et al, 2000) and the kinase domain of human Chk1 is in a relatively open conformation, potentially not requiring any additional modifications to adopt the structure of an active kinase (Chen et al, 2000). However in the cellular context, the kinase activity is repressed. This repression is mediated by C-terminus of the protein,

as the kinase domain alone displays higher intrinsic kinase activity than the full length protein in vitro. It is of note however that C-terminal truncations that remove the entire regulatory domain inactivate biological functions implicating the C-terminus in having both positive and negative inputs into Chk1 regulation (Ng et al, 2004). Further evidence to support a 'de-repression mechanism' comes from the recent observation that when Chk1 is immunoprecipitated from unstimulated cells the kinase activity of the purified Chk1 can be increased upon washing with a stringent buffer (Walker et al, 2009). Activation of Chk1 therefore appears to occur through a mechanism that relieves repression of the kinase domain, be that intrinsic repression by the C-terminus or by other interacting proteins.

De-repression of Chk1 catalytic activity is achieved at least in part through the phosphorylation by ATR of multiple SQ sites in the SQ cluster domain between the kinase domain and the regulatory domain (Capasso et al, 2002; Zhao & Piwnica-Worms, 2001). The upstream signalling that leads to the phosphorylation of Chk1 by ATR has now been mostly delineated as discussed previously, and indeed it is in this step that Claspin functions. (See section 1.3 for the discussion on Claspin function) How exactly this phosphorylation leads to the activation of Chk1 however remains elusive. Phosphorylation of Ser345 appears to be especially critical. When this site is mutated to a non-phosphorylatable residue Chk1 kinase activity no longer increases upon stimulation. In addition this mutant protein is devoid of biological function. Phosphorylation of Ser345 however is not obligatory for Chk1 kinase activity. Walker et al. show that a Chk1 S345A mutant protein can exhibit substantial kinase activity under denaturing conditions. These conditions therefore mimic the phosphorylation induced activation of wild type Chk1 – either by inducing the same conformational change as phosphorylation or by removing an inhibitory protein that normally dissociates upon phosphorylation (Walker et al, 2009)

Amino acid point mutations in the Chk1 C-terminus can also generate a constitutively active kinase. Such mutations presumably disrupt the repressive effect of the C-terminus on the kinase domain. The activating mutations identified so far appear to cluster to two highly conserved domains highlighted in Figure 1.2. Mutation of the T residue in the TRFF motif of xChk1 to A, leads to a constitutively active kinase. Indeed deletion of this entire motif

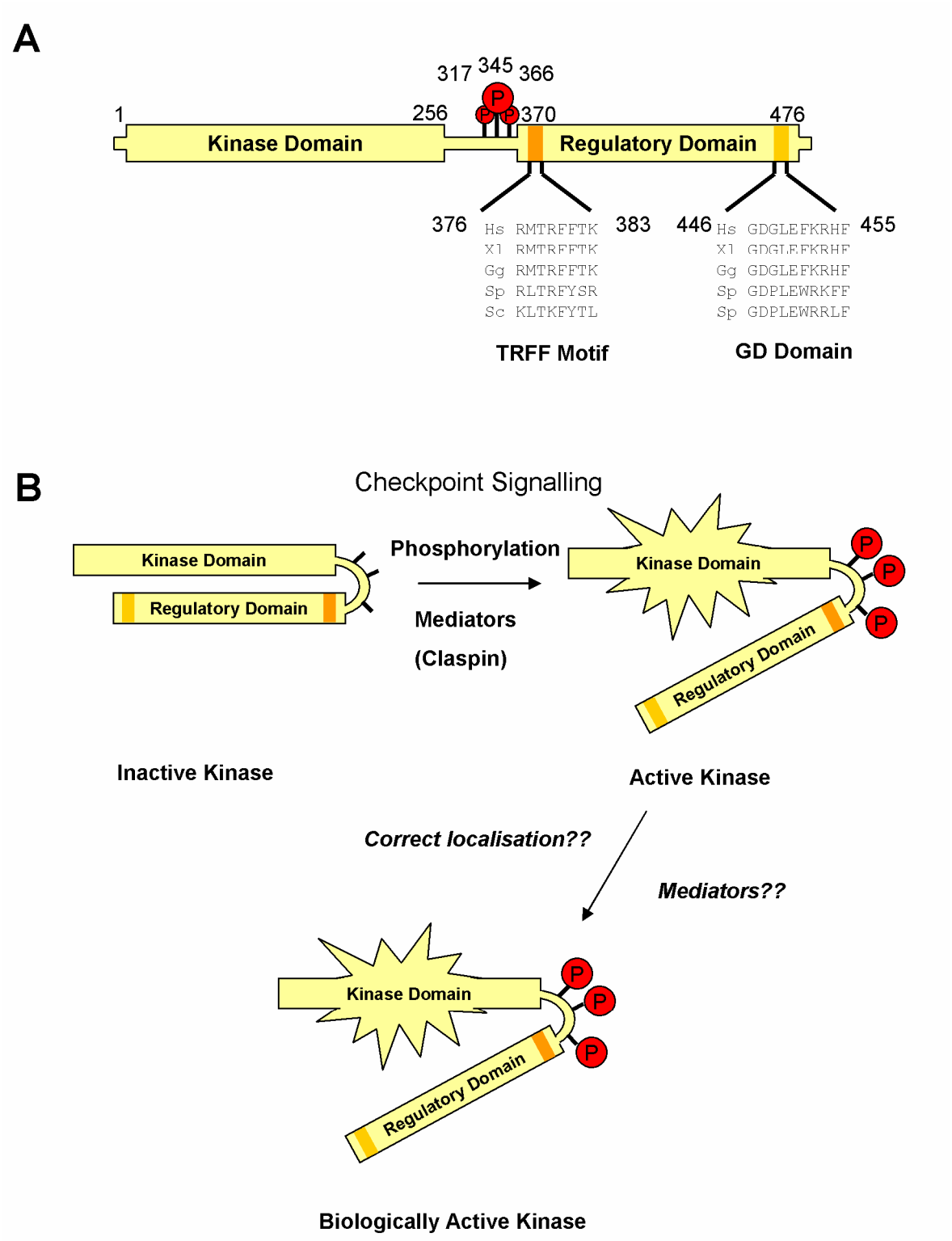


Figure 1.2 Model of Chk1 Activation
A) Schematic representation of Chk1 depicting the N-terminal Kinase domain and the C-terminal regulatory domain. Residue numbers refer to the amino acid residues in human Chk1. Important motifs in the regulatory domain are indicated. B) Chk1 is activated by upstream signalling which leads to its phosphorylation by ATR. This phosphorylation leads to an increase in its kinase activity. The localisation of Chk1 or other proteins are required for its biological activity.

(ΔKRMTRFF) leads to a constitutively active kinase (Wang & Dunphy, 2000). The same is also true in spChk1 where mutation of the first R in the *pombe* equivalent of the KRMTRFF leads to a superactive kinase (Kosoy & O'Connell, 2008). This kinase is functional in yeast and leads to an irreversible arrest in the G2 phase of the cell cycle. When the entire KRMTRFF motif was deleted this led to a non-functional protein. The intrinsic kinase activity of this protein was not determined therefore it is not possible to say whether this mutant has a higher kinase activity like xChk1. Furthermore, in vertebrate Chk1 mutation of the FF residues to AA also results in a kinase with much higher basal kinase activity but this mutant was not functional in checkpoint assays (Scorah et al, 2008). This indicates that simply increasing the kinase activity of Chk1 alone is not sufficient for biological function. Indeed it was shown that the TRAA mutant form does not localise correctly following DNA damage. Therefore in addition to regulating the kinase activity of the protein the KRMTRFF motif also regulates its sub-cellular localisation. Whether mutation of just the first R in this motif in vertebrate cells would lead to a correctly localised kinase with high kinase activity, as in *pombe*, is not known.

Mutations of residues in the GD domain of Chk1, also influence the function of Chk1. Mutations have been identified that both lead to an inactive kinase and a hyperactive kinase. Deletion of the GD residues in the GD domain in both spChk1 and scChk1 result in a non-functional kinase. In the case of scChk1 it is thought that this loss of function could be due to lack of binding to scRad9, a protein that has been shown to be required for its activation (Chen et al, 2009). However mutations in the second half of the GD domain lead to an active kinase but this did not always correlate with an increase in its biological activity. In scChk1 and hChk1 mutation of the Leucine residue in the GD domain to Arginine results in increased intrinsic kinase activity of Chk1. Interestingly in yeast this mutation no longer interacts with scRad9 but is constitutively phosphorylated on the yeast residues equivalent to S317 and S345. As with other mutations that lead to an increased kinase activity it appears that this mutation also results in a biologically inactive protein (Pereira et al, 2009). In spChk1 mutation of the central glutamic acid residue in the GD domain to alanine results in a non-functional protein but mutation to an aspartic acid residue leads to an increase

in the biological activity of the kinase, although interestingly this is not through an increase in kinase activity (Kosoy & O'Connell, 2008).

The overall model of Chk1 activation at the present time is one where the C-terminus of Chk1 binds to and inhibits the kinase domain. Mutations in the C-terminus described so far fall into three categories – some mutations lead to an increase in kinase activity but the protein is non-functional for its biological activity (ΔTRFF, TRAA, L->R in the GD domain etc); a point mutation has been identified that both leads to an increase in kinase activity and a corresponding increase in biological activity (spChk1 R382A) and mutations have been described that lead to a non-functional kinase (ΔGD). A distinction can be made therefore between mutations that simply lead to an increase in kinase activity or mutations that lead to an increase in the biological functional of Chk1. It is clear that simply over coming the repression exerted on the kinase domain either by naturally phosphorylation or artificially through mutation is not sufficient and adds an extra layer of regulation to control the activity of the kinase.

1.3. The function of Claspin in the DNA Damage Response

1.3.1. Claspin – Structure and Function

Claspin was originally identified as a novel protein that interacted with Chk1 upon activation of DNA damage signalling using xenopus egg extracts (Kumagai & Dunphy, 2000). This novel protein is large, very acidic and contains no previously defined motifs found in proteins involved in the DNA damage response such as FHA or BRCT domains. However it does contain S/TQ sites which are known to be preferentially targeted by the ATM/ATR kinases discussed previously.

Claspin is phosphorylated in an ATM/ATR dependent manner following checkpoint activation and this modification is necessary for the Claspin-Chk1 interaction (Kumagai & Dunphy, 2003). The phosphorylation leads to a reduction in the electrophoretic mobility of both full-length Claspin and the isolated C-terminus. The importance of this phosphorylation is illustrated by the fact that

either dephosphorylating Claspin (so it no longer binds to Chk1) or removing Claspin from Xenopus egg extracts leads to a failure of Chk1 phosphorylation during replication stress (Kumagai & Dunphy, 2000). The phosphorylation that regulates the Claspin-Chk1 interaction mainly occurs on the C-terminus of Claspin (Kumagai & Dunphy, 2003). Closer examination of the C-terminus of Claspin led to the identification of a domain that contains two tandem repeats which are essential for Claspin-Chk1 binding. This domain was termed the Chk1 activation domain (CKAD).

The consensus sequence of the tandem repeats is ExxxLC(S/T)GxF. The CKAD also contains SQ sites down-stream of the SG sites. As the binding of Chk1 to Claspin was mediated by phosphorylation the significance of each of these sites was tested. Binding studies showed that only mutation of the S in the SG motif to A in the isolated CKAD (but not mutation of the SQ sites) resulted in reduced binding when the residues were mutated singly or resulted in a complete lack of binding when the two SG sites were mutated to AG (Kumagai & Dunphy, 2003). In addition introduction of these mutations into full length Claspin resulted in an inhibition of the Chk1-Claspin interaction and a loss of Chk1 activation during replication stress (Kumagai & Dunphy,2003). These observations were subsequently shown to hold true for Human Claspin (Clarke & Clarke, 2005). In addition, the role of phosphorylation of a third repeat present in human and mouse Claspin was shown to be minimal, as mutation of the SG site in this motif led to no defect in Chk1 activation and only very slightly impaired binding of Chk1-Claspin (Clarke & Clarke, 2005). It is interesting to note that in both Xenopus and Human Claspin only the slowest migrating form of Claspin (hyperphosphorylated) interacts with Chk1 (Clarke & Clarke, 2005). Mutation of the 2AG sites in the Chk1 binding motif singly or in combination do not significantly alter the mobility shift induced by checkpoint signalling indicating that other residues are also phosphorylated (Kumagai & Dunphy, 2003).

Having identified the residues on Claspin necessary for interacting with Chk1, studies then went on to identify what region of Chk1 was required for interaction with Claspin. Binding assays using fragments of Chk1 identified that it was the kinase domain of Chk1 that binds to Claspin. A putative phosphate binding site on Chk1 was identified as the region that contacted the phosphorylated Claspin. Mutation of certain residues in this region of Chk1

inhibits the Chk1-Claspin interaction and no longer allows the upstream phosphorylation of Chk1 and activation (Jeong et al, 2003). In addition it appears that hypophosphorylated Chk1 appears to interact with phosphorylated Claspin more strongly that hyperphosphorylated Chk1.

Another study using purified recombinant proteins (ATR, TopBP1, Claspin and Chk1) to recapitulate the ATR mediated phosphorylation of Chk1 shows that Claspin greatly stimulates the kinase activity of ATR towards Chk1 but not other ATR substrates (Lindsey-Boltz et al, 2009). This activity was shown to increase further in the presence of damaged DNA and the reaction was dependent on protein-protein interactions. The specificity of Claspin for ATR mediated phosphorylation of Chk1 had been reported previously (Liu et al, 2006; Lupardus & Cimprich, 2006; Yan et al, 2006). This study also defines a minimal region on Claspin that can facilitate the phosphorylation of Chk1 by ATR. This domain contains the previously identified CKAD but in addition it also contains a region adjoining it which shows sequence homology with the yeast Claspin orthologue, Mrc1 (Lindsey-Boltz et al, 2009).

Given the importance of the phosphorylation induced Claspin-Chk1 interaction, the next obvious question is which kinase phosphorylates the SG residues. This issue however remains elusive and somewhat controversial. Claspin phosphorylation of the 2 SG residues in Xenopus extracts in dependent on ATR, but ATR is not thought to be the kinase that directly phosphorylates the residues (Kumagai & Dunphy, 2003). SG does not conform to the motif preferentially targeted by ATR and phosphorylation of these sites by ATR could not be detected (Kumagai & Dunphy, 2003). This has also been confirmed by inhibiting ATR with caffeine or siRNA in human cells and using phospho-specific antibodies to monitor Claspin phosphorylation at T916 (Bennett & Clarke, 2006; Chini & Chen, 2006). It has been suggested that Chk1 itself may actually phosphorylate Claspin at this residue (Chini & Chen, 2006) but this has been subsequently disputed using a Chk1-/- DT40 cell line (Bennett et al, 2008). It is clear however that Chk1 does affect the phosphorylation of Claspin. Early studies of the Chk1-Claspin interaction used a phospho-peptide to block the interaction of Claspin and Chk1. This blocking peptide prevented the interaction of Claspin and Chk1, reduced Chk1 phosphorylation but also led to a reduction in Claspin phosphorylation. This indicates that either Chk1 directly phosphorylates Claspin

or the Chk1-Claspin complex is required for further Claspin phosphorylation (Clarke & Clarke, 2005). A similar observation was made using Chk1-/- DT40 cells where a defect in Claspin phosphorylation (as judged by a mobility shift) was observed in the absence of Chk1 (Bennett et al, 2008).

A recent study has also raised a question mark as to the function of the phosphorylation of the SG sites on Claspin (Lindsey-Boltz et al, 2009). Using a defined system that only contained ATR, TopBP1, a minimal Claspin fragment and kd-Chk1 they show that only the WT but not 3AG mutant of Claspin stimulates phosphorylation of Chk1 by ATR. However they could detect no phosphorylation of the SG residues in this system suggesting that the phosphorylation of these residues is not essential in vitro and confirming that phosphorylation of these residues is unlikely to be mediated by ATR. However it has been shown previously that Chk1 and 3AG-Claspin can interact in vitro but not in vivo. This indicates that other proteins present within the cell may regulate this interaction, potentially increasing the importance of phosphorylation of the SG sites in vivo (Chini & Chen, 2006). Interestingly however even in the absence of phosphorylation of the SG in the reconstituted in vitro system, mutation to 3AG inhibited the phosphorylation of Chk1. This suggests that the mutation itself and not just the lack of phosphorylation may affect Claspin function.

From the above data it is possible to propose a model of how Claspin facilitates the activation of Chk1. Upon ATR activation, through the activity of an unknown kinase regulated by ATR, Claspin is phosphorylated on conserved SG residues in the CKAD. This phosphorylation results in the recruitment of inactive Chk1, which binds to Claspin via a putative phosphate binding site in the kinase domain of Chk1. The Claspin-Chk1 complex is a more efficient substrate for ATR than Chk1 alone. Claspin also appears to stimulate the autophosphorylation of Chk1 (Kumagai et al, 2004). Chk1 is released from Claspin upon activation leaving the phosphorylated Claspin molecule free to activate more Chk1 molecules and amplify the signal. (See Figure 1.3)

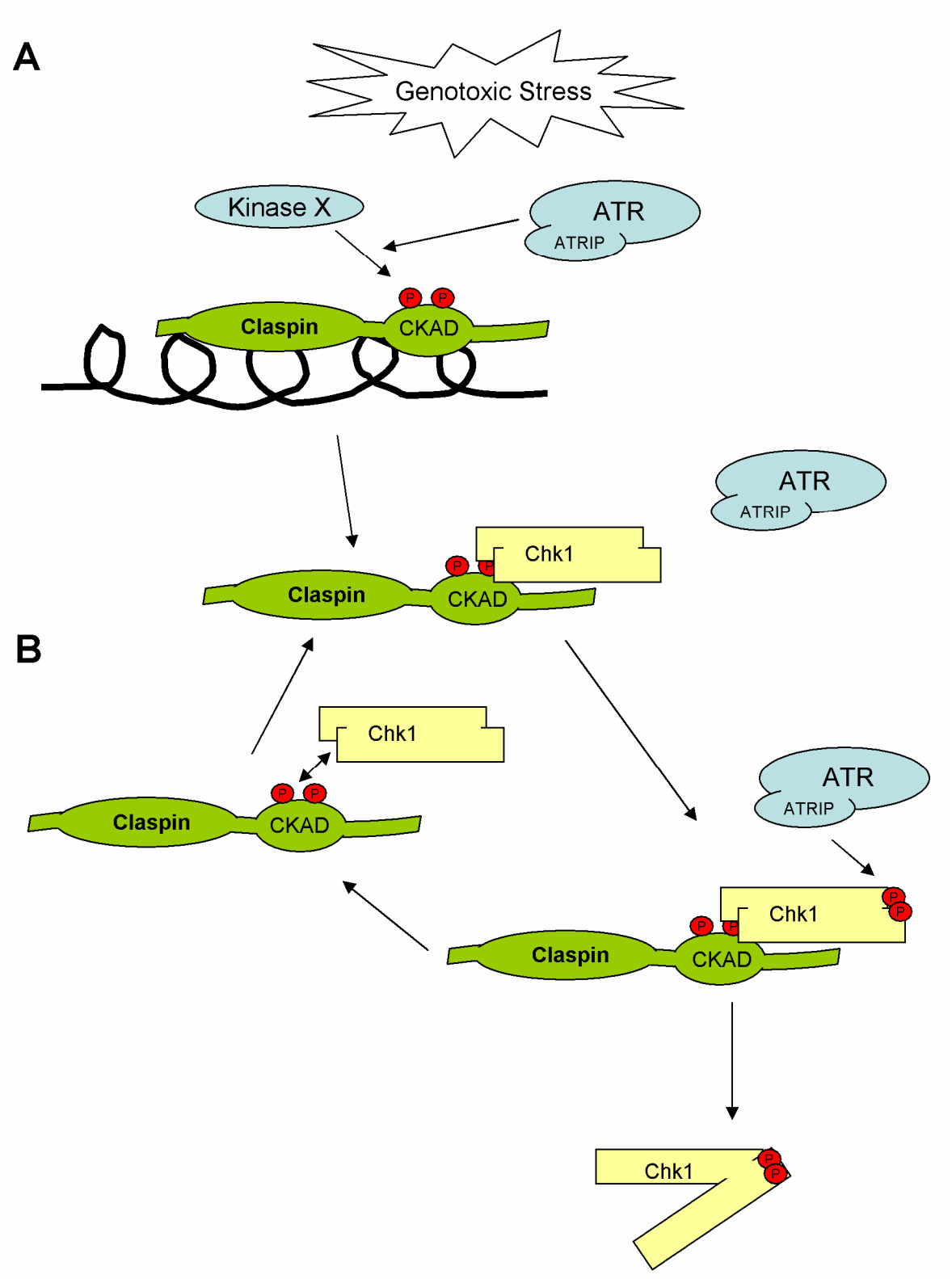


Figure 1.3 Claspin mediated phosphorylation of Chk1
A) Claspin is phosphorylated in an ATR dependent manner on conserved Chk1 binding motifs by an unknown kinase during replication stress. This generates a binding site for Chk1. B) The Claspin-Chk1 complex facilitates phosphorylation of Chk1 by ATR. This leads to Chk1 activation and the active Chk1 molecule dissociates from Claspin. This allows the 'active' Claspin to recruit more inactive Chk1 and facilitate further Chk1 phosphorylation and activation. The cycle of activation then continues.

1.3.2. Claspin and the DNA replication checkpoint

The first indication that Claspin plays a role in DNA replication came from the observation that Claspin binds to replicating chromatin in Xenopus egg extracts (Lee et al, 2003). Claspin binding to chromatin was shown to be dependent on the Cdc45 mediated unwinding of DNA suggesting that Claspin binding to chromatin is an early event in DNA replication. Claspin interacts with Cdc45 and also with the RPA, the RFC complex and MCM proteins (Lee et al, 2005). Binding of Claspin to chromatin is mediated through the N-terminus and a patch of basic amino acids has been shown to be required for this (Lee et al, 2005). In addition to binding to chromatin Claspin also binds directly to DNA (Sar et al, 2004). Claspin has a higher affinity for branched DNA structures that would be present at a replication fork. Therefore Claspin recruitment to chromatin might not only involve interaction with proteins on the chromatin but also with the DNA itself. In addition Claspin appears to have a ring shaped structure, a feature shared by other proteins involved in replication such as the MCM helicase and PCNA (Sar et al, 2004).

This binding of Claspin to chromatin is enhanced upon checkpoint activation suggesting that Claspin is either actively loaded onto chromatin during replication stress or modified Claspin has a higher affinity for chromatin than unmodified (Lee et al, 2003). Unlike the recruitment of Rad17 and ATR, Claspin recruitment does not require RPA and Claspin itself is not required for the recruitment of other checkpoint proteins during replication stress. Interestingly however Claspin does appear to interact with Rad17, another checkpoint protein and it has been suggested that Rad17 is required for Claspin accumulation on chromatin in response to replication stress (Lee et al, 2005; Wang et al, 2006).

The fact that Claspin plays an important role in Chk1 activation and the fact that it binds to replicating chromatin suggests that it the ideal candidate to activate checkpoint signalling during replication stress. Indeed Claspin is phosphorylated following replication stress and is required for the S-M checkpoint and replication slowing following DNA damage during S phase (Chini & Chen, 2003; Lin et al, 2004).

The S phase kinase Cdc7 has also been shown to regulate Claspin during the replication checkpoint (Kim et al, 2008). Cdc7 binds to and phosphorylates Claspin. Loss of Cdc7 leads to both a reduction in total levels of Claspin and a reduction in the phosphorylation and chromatin association of Claspin. This in turn leads to an impaired Chk1 activation during replication stress (Kim et al, 2008). How the phosphorylation of Claspin by Cdc7 mediates these effects is not known.

Overall there is ample evidence in the literature that Claspin plays an important role in the replication checkpoint through its ability to facilitate efficient activation of Chk1.

1.3.3. Claspin Plays a role in DNA replication

It was noted in early experiments using Xenopus egg extracts that chromatin replicated more slowly in the absence of Claspin (Lee et al, 2003). This suggests that in the absence of replication stress Claspin is required to promote normal replication. This notion was confirmed by DNA fibre analysis, examining replication progression directly in the absence of Claspin (Petermann et al, 2008). In the absence of Claspin, replication forks progressed more slowly, a phenomenon that had also been previously observed for Chk1 (Petermann et al, 2006). This may suggest that Claspin works by facilitating Chk1 mediated replication fork progression. However when both Claspin and Chk1 are simultaneously depleted from cells there is a greater decrease in replication fork progression than when the proteins are depleted singly. This suggests that Claspin and Chk1 have distinct roles in promoting normal replication. Similar findings were also reported elsewhere (Scorah & McGowan, 2009). This study revealed that in the absence of either Claspin or Chk1 replicating cells displayed a higher number of stalled forks and a higher number of fired origins. Indeed the inter-origin distance was decreased in the absence of Claspin as seen previously for Chk1. Under conditions of replication stress Chk1 inhibits origin firing by suppressing the activity Cdk2 however Claspin does not appear to be essential for Chk1 activation in this context. Claspin depletion did not lead to an increase in origin firing, indicating that it is not required to suppress origin firing under conditions of replication stress. Therefore Chk1 and not Claspin appears to be required to inhibit origin firing under replication stress. How

Claspin regulates origin firing and fork stalling during normal replication however is not known (Scorah & McGowan, 2009).

Consistent with a role in facilitating normal replication and in agreement with what is seen for ATR, Claspin knock-down also leads to increased fragile site instability. Fragile sites are regions in the genome that are particularly sensitive replication stress and, due to the difficulty in replicating through such lesions, often lead to chromosomal abnormalities (Casper et al, 2002; Focarelli et al, 2009). In addition Claspin is also required to facilitate PCNA ubiquitination during replication stress, which facilitates the activity of by-pass polymerases in order to replicate damaged DNA. It does so by regulating the recruitment of the Rad18 ubiquitin ligase to chromatin (Yang et al, 2008). This function of Claspin appears to be independent of ATR but may involve collaboration with another protein, Timeless (Yang et al, 2008).

Timeless forms a complex with its binding partner Tipin. Interestingly Tipin depletion leads to S phase slowing, an impaired DNA replication checkpoint and reduced Chk1 activation during replication stress (Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007). Indeed knock-down of Tipin or depletion from Xenopus egg extracts led to an inhibition of the accumulation of Claspin during replication stress and loading onto chromatin (Errico et al, 2007; Yoshizawa-Sugata & Masai, 2007). Taken together this suggests that a Claspin/Tipin/Timeless interaction may be important for the regulation of DNA replication.

1.3.4. Claspin – the molecular switch that turns on and off the G2-M checkpoint?

Claspin expression levels are regulated during the cell cycle (Bennett & Clarke, 2006). Claspin levels fluctuate with cell cycle progression and are highest in S/G2 phases of the cell cycle and lowest in G1 cells. Claspin has been shown to be an E2F target gene (Iwanaga et al, 2006) but the fluctuation of Claspin throughout the cell cycle is thought to be mediated primarily through proteasomal degradation. The regulation of Claspin at the protein level is complex. Proteins with both positive and negative effects on Claspin levels have

been discovered. In addition Claspin stability following genotoxic stress and during a normal cell cycle appear to be regulated in a different manner.

Following genotoxic or replication stress Claspin is stabilised. This appears to be mediated at least in part through the action of a ubiquitin protease USP28 (Zhang et al, 2006). USP28 is phosphorylated by ATM following DNA damage suggesting that this may lead to its activation, but USP28 also stabilises Claspin in the absence of DNA damage (Zhang et al, 2006). In addition another ubiquitin specific protease, USP7, has also been implicated in stabilising Claspin, again under both stressed and non-stressed conditions (Faustrup et al, 2009). Thus the stabilisation of Claspin following genotoxic stress involves multiple factors. Indeed it has been shown that Chk1 itself regulates Claspin levels and this may be an important part of the mechanism by which Claspin is stabilised (Chini et al, 2006).

Claspin levels are regulated by ubiquitination and it appears that both the APC^{Cdh1} and the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligases can control the degradation of Claspin. β-TrCP binds to its substrates via a conserved phospho-DSGXXS motif and, once bound, targets its substrates for degradation via the proteasome. Claspin contains this motif in its N-terminus and if the pS residues are mutated to A this prevents interaction with β-TrCP and leads to the stabilisation of the Claspin (Peschiaroli et al, 2006). Indeed it appears that this degradation of Claspin is what regulates checkpoint recovery following genotoxic stress (Mailand et al, 2006). Expression of a mutant, non-degradable form of Claspin induces a more sustained G2-M arrest following genotoxic stress than does WT Claspin. The stabilised Claspin correlates with sustained Chk1 phosphorylation and prolonged G2-M arrest (Mailand et al, 2006).

Plk1 has been identified as a kinase that can regulate phosphorylation of the phospho-degron required for $SCF^{\beta\text{-}TrCP}$ mediated destruction of Claspin (Mamely et al, 2006). Plk1 phosphorylates the N-terminus of Claspin and this phosphorylation is reduced upon mutation of the S residues in the degron to A (Mailand et al, 2006). Depletion of Plk1 appears to phenocopy the expression of the non-degradable form of Claspin, with Claspin levels remaining high in mitotic cells and recovery from G2 arrest impaired in the absence of Plk1 (Mamely et al, 2006). There is precedent in the literature for Plk1 regulation of checkpoint

recovery involving Claspin. Xenopus Plx1 binds to and phosphorylates Claspin, which leads to Claspin dissociation from chromatin and a defect in checkpoint adaptation (Yoo et al, 2004). ATR was identified in this study as the kinase that regulated Plx1 binding to Claspin via its Polo box domain (PBD). The residues on human Claspin that facilitate PBD binding of Plk1 have not been identified. It has been shown however that Plk1 and Claspin interact via the PBD of Plk1 and the C-terminus of Claspin (Mamely et al, 2006). How this then leads to Plk1 phosphorylating the N-terminus of Claspin is not clear.

In addition to being degraded upon entry into mitosis following escape from G2 arrest, Claspin levels are kept low in the G1 phase of the cell cycle through the activity of the APC^{Cdh1} ubiquitin ligase. Claspin interacts with Cdh1 via its N-terminus through a LLK motif which is not a canonical Cdh1 degron sequence (Gao et al, 2009). Expression of a version of Claspin lacking this LLK motif resulted in higher levels of Claspin expression during G1 phase and unscheduled Chk1 activation in G1 phase. Building on the previous observation that the $SCF^{\beta\text{-}TrCP}$ mediated degradation of Claspin leads to checkpoint recovery it has been proposed that ubiquitination of Claspin and Plk1 by APC^{Cdh1} is the molecular mechanism that controls the activation of the G2 arrest (Bassermann et al, 2008). To activate a G2-M arrest the APC^{Cdh1} is reactivated, which leads to the ubiquitination of both Plk1 and Claspin. Plk1 destruction by the APC^{Cdh1} leads to an inhibition of the $SCF^{\beta\text{-}TrCP}$ mediated destruction of Claspin but Claspin levels remain high even though Claspin is also a substrate of APC^{Cdh1}. It appears that USP28 counteracts the APC^{Cdh1} mediated degradation of Claspin following DNA damage to keep Claspin levels high and maintain the checkpoint. (See Figure 1.4) Why USP28 displays such specificity for Claspin over Plk1 in order to lead to its stabilisation is not clear. In addition this model does not explain how the APC^{Cdh1} is then switched off to facilitate recovery, allowing Plk1 levels to increase and Claspin levels to decrease through the activity of the $SCF^{\beta\text{-}TrCP}$.

Overall it is clear that both the degradation and stabilisation of Claspin play an important, yet complicated, role in the activation, maintenance and recovery from the G2-M arrest.

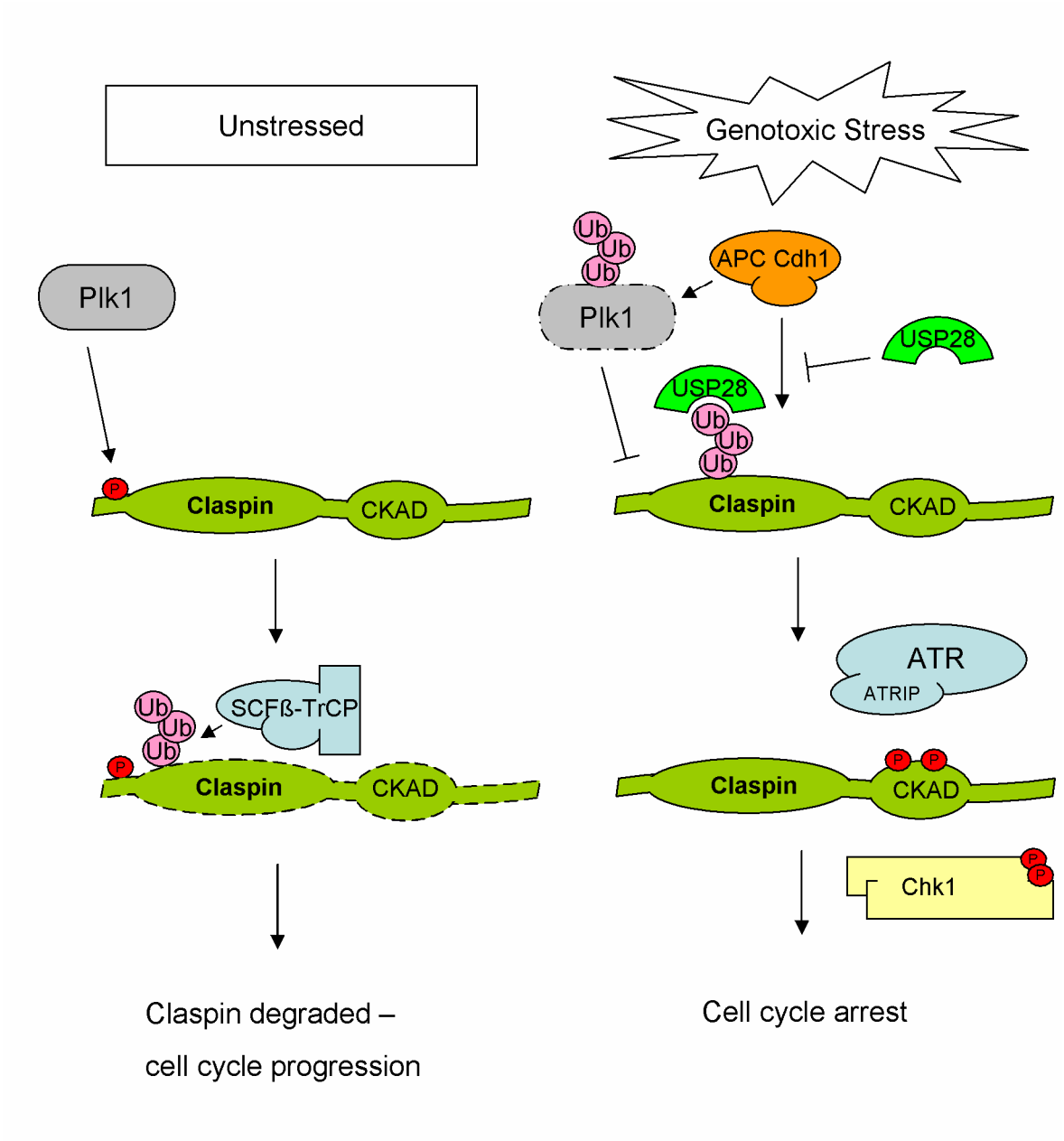


Figure 1.4 Claspin Stabilisation and Degradation following genotoxic stress
In unstressed conditions Plk1 phosphorylates Claspin facilitating ubiquitination and degradation by the $SCF^{\beta\text{-}TrCP}$ complex. Upon genotoxic stress APC^{Cdh1} is activated which leads to the destruction of Plk1 and inhibition of the $SCF^{\beta\text{-}TrCP}$ mediated degradation of Claspin. APC^{Cdh1} also targets Claspin for degradation but this is counteracted by the activity of USP28. Stabilised Claspin in conjunction with checkpoint signalling then activates the cell cycle checkpoint.

1.3.5. Claspin and Cancer

Many mediator proteins involved in DNA damage signalling are either mutated in cancers or inherited mutations in these genes predisposes to cancer. e.g. Brca1, Nbs1, ATM etc. Therefore a potential role for Claspin mutations in cancer has been investigated. A screen for germline Claspin mutations in 125 Finnish cancer breast families revealed 7 mutations in the CLSPN gene but none of which appeared to correlate with cancer susceptibility (Erkko et al, 2008). Only three exon mutations resulted in an amino acid change (G6D, ΔQ1195 and S1280L) and of these only one had not been previously reported (ΔQ1195). None of these mutations are located in any region of Claspin that has been shown to be critical for function (Erkko et al, 2008).

Another study sequenced the CLSPN gene in families with either a history of breast cancer or a multi-cancer phenotype and from cancer cell lines (Zhang et al, 2009). Eight mutations not present in the population controls were described. I236V and A1146S were found to be germline mutations from families with a history of cancer. Six mutations were found in cancer cell lines. Analysis of the functional consequence of these mutations revealed that only one of the mutations, I783S, resulted in impaired Claspin function.

Claspin has also been evaluated as a proliferation marker in cancer tissue (Tsimaratou et al, 2007). Claspin has been shown to be over expressed in cancer cell lines compared with normal cells and in human tumours compared with normal tissue both at the mRNA and protein level (Lin et al, 2004; Tsimaratou et al, 2007; Verlinden et al, 2007). Claspin expression, as judged by immunocytochemistry, correlates strongly with that of Ki67, a marker of proliferation currently in use. The labelling index of Claspin was found to be lower than Ki67 but this is probably due to the tight S phase specific expression of Claspin.

Claspin also appears to be a target of the E7 oncoprotein of the HPV-16 virus, infection with which can lead to squamous cell carcinomas. In addition to being defective for a whole host of cell cycle checkpoint mechanisms including p53 inhibition activations of hTERT etc cells infected with HPV-16 also fail to activate a G2-M DNA damage, even in the presence of DNA damage. In order to

overcome the G2-M arrest the E7 protein promotes the destruction of Claspin through deregulation of the normal process that regulates Claspin turnover (Spardy et al, 2009). In contrast with the inhibition of the DNA damage response by the oncoprotein E7 the SV40 large T antigen stimulates the DNA damage response. The LT antigen binds to Claspin and this potentially leads to the stabilisation of the protein (Hein et al, 2009). In this case Claspin may be playing a protective role, preventing DNA damage during replication but the functional significance of Claspin stabilisation by the SV40 LT antigen remains unclear.

Further study will be necessary to determine whether Claspin is inactivated by mutation during tumorigenesis. Evidence currently shows that Claspin is over expressed in cancer cells but whether this alone is enough to deregulate the cell cycle is not known, but unlikely. However over expressed Claspin is probably promoting progression through S phase and thus contributing to cancer progression. This may explain the low number of mutations associated with this gene.

1.4. Mrc1 – the functional Claspin orthologue in yeast?

DNA damage signalling components have been widely studied in two model yeast organisms; budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*) (Hartwell, 1967; Nurse et al, 1976). Soon after the identification of Claspin in Xenopus a novel protein was reported in both budding and fission yeast termed Mediator of the Replication Checkpoint (Mrc1) (Alcasabas et al, 2001; Tanaka & Russell, 2001). scRad53/scChk1 and spChk1 are activated following DNA damage in budding and fission yeast respectively. Two adaptor proteins facilitating the activation of these kinases, scRad9 and spCrb2, are necessary for activation in response to DNA damage but not in response to replication stress (Gilbert et al, 2001; Saka et al, 1997; Schwartz et al, 2002). Therefore there was a missing link as to whether these kinases (scRad53 and spCds1) required additional factors to facilitate their activation following replication stress. To address this, genetic screens were carried out. In budding yeast the aim was to identify mutants that failed to grow in the presence of

chronic replication stress (Alcasabas et al, 2001). In fission yeast the screen was devised to identify mutant strains that were sensitive to hydroxyurea (HU), which induced replication stress, but which could be rescued by over-expression of spCds1, indicating that the protein identified would play a role in the activation of this kinase (Tanaka & Russell, 2001). scMrc1/spMrc1 was identified.

spMrc1 is required specifically for spCds1 activation following replication stress but not for spChk1 activation following DNA damage. spCds1ΔspMrc1Δ double mutant cells show the same sensitivity to HU as either single mutant. However both spCds1ΔspChk1Δ and spMrc1ΔspChk1Δ double mutants show greater sensitivity to HU than the single mutants alone (Tanaka & Russell, 2001). This indicates that spMrc1 acts in the same pathway as spCds1 and that spChk1 function is not impaired in the absence of spMrc1. Consistent with this is the observation that spMrc1 cells are unable to activate the S-M checkpoint which is dependent on spCds1 activation but remain competent for the G_2-M checkpoint which depends of the activity of spChk1 (Tanaka & Russell, 2001).

spMrc1 is regulated by Rad3/Tel1 (ATR/ATM) following replication stress. An spMrc1 electrophoretic mobility shift consistent with phosphorylation by Rad3/Tel1 kinases (but not by spCds1) is observed following replication stress. Rad3 and Tel1 generally target S/TQ sites for phosphorylation and it followed that mutation of the S/TQ sites present in spMrc1 led to a substantial reduction in phosphorylation of spMrc1 but also greatly sensitised the cells to killing by HU (Zhao et al, 2003). There was also a substantial impairment of the spCds1 mediated S-M checkpoint. This checkpoint mediated phosphorylation of spMrc1 and not just its mere presence is therefore essential for its role in mediating the activation of spCds1.

Consistent with spMrc1 playing an adaptor role in the activation of spCds1, the two proteins have been shown to interact (Tanaka & Russell, 2001). Indeed it appears that it may be the interaction of spMrc1 and spCds1 that is regulated by checkpoint signalling. Two residues in spMrc1 were identified as being important for the cells resistance to HU – S604 and T645 (Zhao et al, 2003). S604 is phosphorylated by Rad3/Tel1 following HU treatment but its role in mediating spMrc1 function is not clear – but may be involved in regulating

spMrc1 chromatin association. Phosphorylation of this site is not essential for the S-M checkpoint and is not required for spCds1 interaction. Interestingly the other site (T645) lies within the region of spMrc1 that has been shown previously to be required for interaction with spCds1 via the FHA domain of spCds1 (Tanaka & Russell, 2001). Loss of the C-terminus of spMrc1 (Δ610-1019) prevents interaction with Cds1, whereas loss of residues 1-162 has no effect. Phosphorylation of the T645 site is required for the interaction of spMrc1 and spCds1 and for the activation of the spCds1 dependent S-M checkpoint (Zhao et al, 2003). These observations were further extended by Xu et al, who have also identified another site, T653, as being just as critical as T645 in the response to replication stress (Xu et al, 2006). Mutation of just these 2 sites, termed tandem TQ repeats, completely abolished spCds1 phosphorylation following HU treatment (Xu et al, 2006). Once phosphorylated by Rad3 the TQ repeats form consensus FHA phospho-threonine binding sites which then interact with the FHA domain of spCds1. This appears to be the first, so-called 'priming', step in spCds1 activation. It appears that the spCds1-spMrc1 interaction facilitates the phosphorylation of spCds1 by spRad3 on T11. Phosphorylation of this residue promotes intra-molecular binding between the pT11 residue of one spCds1 molecule to the FHA domain of the other. Auto-phosphorylation then occurs leading to full activation of the kinase (Tanaka & Russell, 2004; Xu & Kelly, 2009).

spMrc1 also interacts with chromatin (Zhao et al, 2003) and has been shown to directly interact with DNA (Zhao & Russell, 2004). A region between amino acids 160 – 317 in the N-terminus of the protein is required for the direct interaction with DNA. spMrc1 appears to prefer to interact with branched DNA structures over linear dsDNA. It was also possible to identify two key residues that mediate the interaction with DNA, K235 and K236. Mutation of these sites in the N-terminus of the protein rendered the protein fragment no longer able to bind DNA. Significantly it was also shown that this DNA binding activity is required for the checkpoint function of spMrc1.

It was noted that in the absence of spMrc1, modest spChk1 activation is observed (Tanaka & Russell, 2001). As spChk1 is only activated following DNA damage this suggests that spMrc1 prevents replication induced DNA damage during an otherwise unperturbed cell cycle. Indeed further studies show that spMrc1 has

functions in DNA replication that are independent of its role in the activation of spCds1. The sensitivity to HU in the spCds1Δ mutant can be suppressed by mutations in either spOrc1 or spCdc45, proteins required for replication initiation and elongation respectively. This suggests that simply reducing the rate of replication in spCds1Δ cells increases viability (Nitani et al, 2006). However the same study, now examining the spMrc1Δ mutant cells, showed that when replication was perturbed by mutating spOrc1 the cells were more sensitive to replication stress, not less. Reducing the number of viable origins would mean that the origins that did fire would have to be viable for longer in order to complete replication. spMrc1 therefore plays a role in maintaining viable forks in order to promote viability. However mutating spCdc45 or spMCM components in the spMrc1Δ cells, as with spCds1Δ cells, leads to an increase in viability following HU treatment suggesting that mutations which slow the elongation phase of DNA replication promote viability in the absence of spMrc1. This same mutation in spCdc45 promoted recovery from a replication arrest induced by HU in the spMrc1Δ background (Nitani et al, 2006).

Most of the observations that are valid for spMrc1 are also valid for scMrc1. scMrc1 is not required for the DNA damage checkpoints but is required for the S-M checkpoint, the intra-S checkpoint, and to delay late origin firing in the presence of replication block. All of these functions serve to maintain viability of cells subjected to prolonged replication stress (Alcasabas et al, 2001). Subsequent analysis has shown that scMrc1 is required to facilitate activation of scRad53 following replication stress. As seen with spMrc1Δ cells, scMrc1Δ cells also accumulate sufficient DNA damage during S phase to lead to the activation of scRad9. This indicates that scMrc1 prevents the accumulation of replication associated DNA damage. In contrast with spMrc1, which did not require spCds1 for its phosphorylation, scMrc1 phosphorylation is compromised in the absence of scRad53 indicating a role for scRad53 in the phosphorylation of scMrc1, possibly via some sort of an amplification loop (Alcasabas et al, 2001). Further in vitro characterisation of the role of scMrc1 in the activation of scRad53 shows that scMrc1 is required to stimulate the kinase activity of scMec1 (the upstream kinase that phosphorylates scRad53) toward scRad53. It does so, not by stimulating the catalytic activity of scMec1, but instead by promoting enzyme-

substrate association (Chen & Zhou, 2009). scMrc1 is also required to maintain scMec1 levels at stalled replication forks (Naylor et al, 2009).

Aside from its function under conditions of DNA synthesis inhibition scMrc1 also appears to play important roles in a normal S phase. scMrc1 cells progress more slowly through S phase even in the absence of stress (Szyjka et al, 2005). This appears to be due to a general overall slowing of replication fork progression due to the generation of structures in the DNA that must be resolved by helicases. The function of scMrc1 in normal replication however seems to be separate from its checkpoint role (Osborn & Elledge, 2003). Unlike in the complete absence of scMrc1, where damaged DNA accumulates, no such damage is observed in a checkpoint defective scMrc1 protein (scMrc1-AQ). Further studies show that scMrc1 specifically localises with the replication fork during S phase. scMrc1 associates with chromatin following origin firing and then dissociates from chromatin once S phase is complete (Osborn & Elledge, 2003). These data strongly indicate that scMrc1 plays a role in a normal S phase. Further analysis has revealed that scTof1 and scCsm3 are partially required for the recruitment of scMrc1 to the replication fork, even though it appears that they also play roles in S phase that are independent of scMrc1 recruitment (Bando et al, 2009; Hodgson et al, 2007). Finally, evidence has recently been provided that scMrc1 also interacts with scPol2, the catalytic subunit of DNA polymerase ε, required for leading strand DNA synthesis. Two independent contact sites have been established; the C-terminus of scMrc1 interacts with the C-terminus of scPol2 and the N-terminus of scMrc1 interacts with the N-terminus of scPol2. The interaction between the C-termini of scMrc1 and scPol2 appears to occur constitutively at replication forks but the N-terminal interaction is subject to checkpoint regulation. Indeed it has also been shown that scMrc1 is required to maintain scPol2 at a stalled replication fork (Lou et al, 2008). scMrc1 has also been shown to interact with the MCM helicase and it has been suggested that scMrc1 plays a role in coupling the replication fork with the helicase (Katou et al, 2003; Szyjka et al, 2005). Taken together this places scMrc1 at an ideal location to couple the unwinding of the DNA with the synthesis of the two daughter strands. It also makes scMrc1 an ideal candidate to activate checkpoint signalling should something go wrong with DNA

replication. It would be interesting to test whether this coupling function is conserved in vertebrate Claspin.

It can be seen from the description of the yeast Mrc1 homologues that many common features are conserved with vertebrate Claspin. They are both large acidic phospho-proteins. In common with yeast Mrc1, Claspin binds chromatin and DNA, it is required for the S-M checkpoint following replication stress and although Claspin activates Chk1 (not Chk2 the vertebrate spCds1/scRad53 orthologue) the mechanism of activation seems quite similar to what is currently known about Chk1 activation by Claspin. It is interesting to note that spMrc1 activates spCds1^{Chk2} whereas Claspin activates Chk1. This data however fits in well with the observation that vertebrate Chk1 has taken over most of the roles played by spCds1^{Chk2} in the replication checkpoints (Canman, 2001; Chen & Sanchez, 2004). It has also been observed that spMrc1 is cell cycle regulated peaking in S phase and this is thought to restrict spCds1 activity to S phase (Tanaka & Russell, 2001). A similar observation has been made with Claspin. spMrc1 has also been shown to be regulated by the Tel2 and Hsk-1 orthologues of human Clk-2 and Cdc7 respectively (Shikata et al, 2007; Shimmoto et al, 2009). Recent work has also shown that human Claspin is regulated in a similar manner (Collis et al, 2007; Kim et al, 2008). Although spMrc1 and human Claspin only share roughly 20% similarity at the amino acid level it does indeed appear that spMrc1 is the functional homologue of vertebrate Claspin.

1.5. The DT40 Model System

The DT40 cell line was first developed in 1985. It is a chicken suspension cell line, of lymphoid origin, immortalised by transformation with RAV-1, an avian leukosis virus (ALV). Hyline SC chickens were infected with the RAV-1 virus then the resulting tumours were re-implanted into syngeneic chickens and the DT40 cell line was derived from one of these transplantable tumours (Baba et al, 1985). The cell line shows elevated expression of c-myc due to the insertion of viral DNA upstream of the c-myc gene. Further studies have also shown that this cell line does not express functional p53. p53 is either not detectable (Takao et al, 1999) or only detectable following genotoxic stress (Tanikawa et al, 2000). Indeed it was later shown that p53 is indeed expressed in DT40 cells but levels

are kept very low due to the action of a negative regulator of p53, YY1 (Sui et al, 2004).

The utility of this cell line however really only became clear when it was demonstrated that transfected DNA integrated by homologous recombination at very high frequencies (Buerstedde & Takeda, 1991). This high ratio of targeted to random integration of transfected DNA makes DT40 ideal for reverse genetics in somatic cells. Gene targeting by homologous recombination had been shown previously but largely confined to work in mouse ES cells. (Doetschman et al, 1987) Therefore having a cell line that can be used to knock-out proteins with relative ease greatly facilitates the characterisation of proteins at both the molecular and cellular level. Indeed the benefits of the DT40 cell line have been exploited by researchers over the past 20 years.

The cell line is genetically stable and nearly euploid, with one extra copy of chromosome 2 and one extra mini-chromosome (Winding & Berchtold, 2001). However caution has recently been expressed as to how stable the DT40 genome actually is, with some slight variations of the 'normal' DT40 karyotype observed depending on the source of the cells (Chang & Delany, 2004). The diploid chicken karyotype consists of 8 pairs of macro-chromosomes, 2 sex chromosomes (ZW) and 30 pairs of mini-chromosomes (Brown et al, 2003). DT40 are about 10μm in diameter, with a large nucleus. They grow quickly under optimal growth conditions, with a doubling time of approximately 8 -10 hours when grown at 39.5°C. (Winding & Berchtold, 2001) On a practical note the quick doubling time and the fact that the cells grow in suspension greatly facilitate the process of gene targeting.

Due to the popularity of the chicken as a resource for biological research and the commercial value of the poultry industry great effort has been invested into generating genome resources for the chicken research community. This has obviously benefited research with the DT40 cell line. Genome linkage maps (Groenen et al, 2000), chicken BAC libraries (Crooijmans et al, 2000), EST databases (Abdrakhmanov et al, 2000; Boardman et al, 2002) and complete sequencing of the Chicken Genome (Hillier et al, 2004) provide valuable tools for the DT40 community.

It is not surprising therefore that the DT40 system has been used to address questions in areas of research pertaining not only to immunology such as B cell receptor signalling, immunoglobulin diversification etc but also to the areas of chromosome biology, cell cycle, DNA damage checkpoints and signalling and DNA repair (Dhar et al, 2001; Sonoda et al, 2001b; Winding & Berchtold, 2001; Yamazoe et al, 2004).

The short doubling time and the suspension cell nature of DT40 cells make them very amenable to cell cycle and DNA damage studies. They are particularly easy to handle for flow cytometry and enrichment for cells in different phases of the cell cycle is possible by elutriation (Franklin & Sale, 2006; Gillespie & Henriques, 2006; Gillespie & Walker, 2006). DT40 cells lack a functional G_1-S arrest and instead arrest in G_2 phase in response to DNA damage. The cells lack functional p53 as discussed earlier which permits longer cell survival in the presence of genotoxic insults and allows p53 independent features of pathways to be investigated. Numerous DT40 knock-outs have been generated for proteins involved in the maintenance of genomic stability and this has provided valuable information as to the function of these proteins. (Table 1-1) In addition to using reverse genetics to probe the basic functions of proteins it is also possible to make targeted mutations in proteins allowing identification of critical residues, which facilitates a more in-depth study of protein function (Arakawa et al, 2006; Ayoub et al, 2009).

Reagents have recently been made available that facilitate the generation of compound DT40 knock-outs through the recycling of resistance markers (Arakawa, 2001). Resistance markers flanked by mutant LoxP sites can be excised once targeting has been confirmed allowing either the reuse of the same targeting vector to target the second allele of the gene or the use of a targeting vector bearing the same resistance marker to target a different gene. In addition novel strategies are being employed in order to increase the benefits to using DT40 cells for example promoter-hijack strategies in order to generate conditional knockouts and the use of a temperature sensitive degron to allow tight temporal control over the expression of proteins (Samejima et al, 2008; Su et al, 2008).

DT40 Knock-out	Reference
Homologous Recombination/Inter-strand Crosslink Repair	
Rad54-/-	(Bezzubova et al, 1997)
Rad52-/-	(Yamaguchi-Iwai et al, 1998)
Rad51$^{On/Off}$	(Sonoda et al, 1998)
Rad51B/C/D-/-	(Takata et al, 2000)
XRCC2/3-/-	(Takata et al, 2001)
SNM1A/B(Artemis)/C-/-	(Ishiai et al, 2004)
Brca1-/-	(Martin et al, 2007)
CtIP-/-/-	(Yun & Hiom, 2009)
FANC C-/-	(Hirano et al, 2005)
FANC D1 (Brca2)-/-	(Ayoub et al, 2009)
FANC D2-/-	(Yamamoto et al, 2005)
FANC G-/-	(Yamamoto et al, 2003)
FANC J-/-	(Bridge et al, 2005)
FANC L-/-	(Seki et al, 2007)
Blm-/-	(Wang et al, 2000)
WRN-/-	(Imamura et al, 2002)
Fbh1-/-	(Kohzaki et al, 2007)
Non-homologous End Joining	
Ku70-/-	(Takata et al, 1998)
DNAPKcs-/-/-	(Fukushima et al, 2001)
DNA LigV-/-	(Adachi et al, 2001)
Mismatch Repair	
MSH6-/-	(Kobayashi et al, 2006)
MSH3-/-	(Nojima et al, 2005)
Translesional Synthesis	
Polκ-/-	(Okada et al, 2002)
Rev3 (Polζ)-/-	(Sonoda et al, 2003)
POLQ-/-	(Yoshimura et al, 2006)
Rad18-/-	(Yamashita et al, 2002)
Polη-/-	(Kawamoto et al, 2005)
POLN-/-	(Yoshimura et al, 2006)
Rev1-/-	(Simpson & Sale, 2003)
Polλ-/-	(Tano et al, 2007)
Nucleotide Excision Repair	
XPA-/-	(Okada et al, 2002)
XPG-/-	(Kikuchi et al, 2005)
XPF-/-	(Nojima et al, 2005)
Base Excision Repair	
Parp1-/-	(Hochegger et al, 2006)
Polß-/-	(Tano et al, 2007)
Fen-1-/-	(Kikuchi et al, 2005)
DNA damage Signalling	
Nbs1-/-	(Tauchi et al, 2002)
Mre11 $^{On/Off}$	(Yamaguchi-Iwai et al, 1999)
ATM-/-	(Takao et al, 1999)
ATR $^{On/Off}$	N Lowndes, Personal communication
Chk1-/-	(Zachos et al, 2003)
Chk2-/-	(Rainey et al, 2008)
Rad9-/-	(Kobayashi et al, 2004)
Rad17-/-	(Kobayashi et al, 2004)
H2AX-/-	(Sonoda et al, 2007)
53BP1-/-	(Iwabuchi et al, 2006; Nakamura et al, 2006)

Table 1-1 List of DT40 Knock-out cell lines of genes involved in the DNA Damage Response

Overall this model system has proved to be very important in elucidating the functions of a wide variety of pathways something which is likely to continue into the future.

1.6. Hypothesis and Aims

It is clear that there is a lot of biochemical evidence now available regarding Claspin. However a gap exists between in vitro data and what happens in the context of a cell. Currently there is no clean system, amenable to genetic manipulation, in order to study the function of Claspin on a cellular level. The development of a DT40 knockout (discussed in section 1.5) therefore would be an ideal tool in bridging this gap.

In addition to the benefits already described, the DT40 system potentially has particular advantages over other systems for studying Claspin function. This is the only system identified to date where a genetic deletion of Chk1, the kinase regulated by Claspin, has been possible (Zachos et al, 2003). Therefore this system has the greatest chance of also producing a viable Claspin knockout. However, the DT40 system has also been used to gain important information regarding the function of essential genes through the generation of conditional knock-out cell lines.

The system also benefits from the fact that many other mediator proteins implicated in the activation of Chk1 have already been knocked out (see Table 1-1) which would allow a thorough examination of role of the individual proteins (or combinations thereof) in the activation of Chk1 in an isogenic background. It is also intended to examine the role Chk1 itself plays in regulating Claspin since this issue has been raised in the literature. A basic characterisation of the chicken homologue of Claspin should also yield interesting results.

The generation of a Claspin-/- cell line will greatly facilitate the characterisation of the role Claspin plays in the activation of Chk1 and has the potential to allow the discovery of the precise roles it plays during replication.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. General Reagents

Melford Laboratories Ltd. (Ipswich, UK)

Tris Base Ultrapure, Tris Hydrochloride Ultrapure, Agarose, Magnesium chloride, Magnesium sulphate.

Fisher Scientific (Loughborough, UK)

Sodium chloride, Sodium hydroxide, Hydrochloric acid, Acetic acid, Sodium acetate, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Sodium pyrophosphate, Maltose, Glucose, Sucrose, Manganese (II) chloride, Potassium hydroxide, Potassium chloride, EDTA, Glycerol, Sodium Dodecyl Sulphate, Ethanol, Methanol, n-Butanol, Dimethyl sulphoxide, Chloroform, Sodium Citrate, Potassium dihydrogen phosphate, Ammonium Persulphate, Potassium Acetate.

Buffer	Composition
20 × SSC	3M Sodium Chloride, 0.3M Sodium citrate pH 7.0
50 × Denhardts Reagent	1% BSA, 1% Ficoll 400, 1% Polyvinylpyrrolidone
1 × PBS-A	160mM Sodium Chloride, 3mM Potassium chloride, 8mM Disodium hydrogen phosphate, 1mM Potassium dihydrogen phosphate
1 × PE	160mM Sodium Chloride, 3mM Potassium chloride, 8mM Disodium hydrogen phosphate, 1mM Potassium dihydrogen phosphate, 1mM EDTA
50 × TAE	2M Tris Acetate, 50mM EDTA
1 × Semi Dry Blotting Buffer	48mM Tris-Cl pH 9.2, 39mM Glycine, 1.3mM SDS - 20% Methanol added just prior to use
10 × Tris Buffered Saline Tween	200mM Tris-Cl pH 7.6, 1.37 M Sodium Chloride, 1% Tween 20
1 × SDS-PAGE Running Buffer	250mM Tris-HCl, 1.92M Glycine, 0.01% SDS
1 × TE	10mM Tris-HCl pH 8.0, 1mM EDTA
LB Broth (and Agar)	10g of NaCl, 10g of tryptone, 5g of yeast extract per 1000mls of water, then autoclaved. For 1000mls of LB-agar 20g of Agar was added prior to autoclaving. (Ampicillin to a final concentration of 100µg/ml or kanamycin to a final concentration of 50µg/ml was added as required)
NZY Broth (and Agar)	5g of NaCl, 2g of $MgSO_4 \cdot 7H2O$,5g of yeast extract, 10g of NZ amine (casein hydrolysate) per 1000mls of water then autoclaved. For 1000mls of NZY-agar 20g of agar was added prior to autoclaving. Top Agar was NZY Broth containing 0.7% agar then autoclaved.

Table 2-1 List of Commonly Used Buffers and Reagents

2.1.2. Molecular Biology – Analysis of DNA and RNA

2.1.2.1. Recombinant DNA Technology

Cambio (Cambridge, UK)

Klenow Polymerase.

New England Biolabs UK (Hitchin, Hertfordshire, UK)

Mung Bean Nuclease, KpnI Phosphorylated Linkers, T4 DNA Ligase (400U/µl), Restriction Enzymes (PciI).

Promega (Southampton, UK)

Shrimp alkaline phosphatase.

GE Healthcare Life Sciences (Little Chalfont, UK)

Nanovue Spectrophotometer.

Roche Diagnostics Ltd. (Burgess Hill, UK)

Restriction Enzymes (KpnI, XbaI, SalI, SfuI, SpeI, NheI, ScaI, BclI, NcoI, MluI, BglII, XhoI), Rapid DNA Ligation Kit.

Invitrogen (Paisley, UK)

Restriction Enzymes (HindIII, EcoRI, BamHI, NotI, SmaI, SacI, PstI), Chemically Competent DH5α E. coli, SOC Media, TOPO® TA Cloning Kit for Sequencing, ZeroBlunt® TOPO® Cloning kit for Sequencing.

Eppendorf (Histon, Cambridge, UK)

Biophotometer (Spectrophotometer), Refrigerated Microcentrifuge 5415 R, Microcentrifuge 5415 D.

2.1.2.2. DNA Purification and Clean Up

Qiagen Ltd. (Crawley UK)

Qiaquick Gel Extraction Kit, Qiaquick Nucleotide Removal Kit, Qiaquick PCR Purification Kit, QiaexII Gel Extraction Kit, Qiafilter Maxi Prep Kit, Qiaprep Mini Prep Kit.

Fermentas (York, UK)

20mg/ml Glycogen.

2.1.2.3. PCR

Invitrogen (Paisley, UK)

Platinum Taq Polymerase, Pfx Polymerase, Superscript II RT-PCR kit.

Agilent Technologies UK Ltd (Stockport, UK)

Quick Change II Site Directed Mutagenesis Kit (Stratagene).

Applied Biosystems (Warrington, Cheshire, UK)

GeneAmp PCR Core Kit, GeneAmp RNA PCR Core Kit.

VHBio (Gateshead, UK)

10mM dNTP Mix, Oligonucleotide Supplier.

New England Biolabs (Hitchin, Hertfordshire, UK)

Phusion DNA Polymerase.

Takara Bio Europe/Clontech (Saint-Germain-en-Laye, France)

SMART RACE cDNA Amplification Kit.

Biorad (Hemel Hempstead, UK)

PTC-200 Thermal Cycler.

RT-PCR	
1F	5′-AATGTTGGTCGACAGGACGGTGACTCTTCTGTGC-3′
2F	5′-AATGTTGGTCGACTGCCAGTGAAACAGAAGAACAGC-3′
3F	5′-AATGTTGGTCGACGAAGAGAAACAAGTTGAAGACGGT-3′
4F	5′-AATGTTGGTCGACAGATGGACTTGTTTCAGAGGG-3′
5F	5′-AATGTTGGTCGACAGAAGAGCTGAACGCTGTGC-3′
6F	5′-AATGTTGGTCGACCCTTACGGGGTTTCTTTGG-3′
1R	5′-CTGCTTCTAGATCATTGCCAGAGCAGATTCG-3′
2R	5′-CTGCTTCTAGACTCTGTTGAAGGTGGTTTGGG-3′
3R	5′-CTGCTTCTAGACAAATTTTCCAGAGCAAAG-3′
4R	5′-CTGCTTCTAGACACTGTTCTCGTTCAAATCTCTC-3′
5R	5′-CTGCTTCTAGAATGGGAAAAGCAACGAAGC-3′
6R	5′-CTGCTTCTAGAATTGTGAAGGTTGGGAGTGG-3′
7R	5′-CTGCTTCTAGAAAGTTGCAGTTGGAGTCGGG-3′
10R	5′-AGACAGCAGGAGCAGGGAGAACAACG-3′
B	5′-CTCGAGTGCGGCCGCTCATCCTCAGAAAACCGTTC-3′
RACE	
5′-RACE 1	5′-TCCACCTGCTGGCCCTCTGAGCTTGG-3′
5′-RACE 3	5′-CGCTGTCCAGCAGACCCTGTCGAATCCG-3′
3′-RACE 1	5′-AGAGAAGGACAGCGACATAGAAGACCC-3′
3′-RACE 5	5′-GCCCAGGGTTGACAGCACAGAGC-3′
3′-RACE 7	5′-ATCTCTTGCCAAACGGCCCAGGGTTGAC-3′
Site Directed Mutagenesis	
cClaspin M1Δ For	5′-GCGTCCGGCCCGGGCCGCGGCGGCTCCCG-3′
cClaspin M1Δ Rev	5′-CGGGAGCCGCCGCGGCCCGGGCCGGACGC-3′
cClaspin S1236P For	5′-CTATTGCCCAGGAATCCATTTGAAACATTCAGACCTGCC-3′
cClaspin S1236P Rev	5′-GGCAGGTCTGAATGTTTCAAATGGATTCCTGGGCAATAG-3′
PCR – primers for targeting vector arms	
G13F	5′- GGTCGACCAGACCCGTCATACCTATACGTGAGC -3′
G10R	5′- CATCGATTGGGCAGTGACCTTCTCTTTTTCC -3′

G9F	5′- CAGATGTGGGAGAGGAAAAGGAGG -3′
G9R	5′- CTATGTCTGCTGATCTGCTTTCCG -3′
3F	5′-AATGTTGGTCGACGAAGAGAAACAAGTTGAAGACGGT-3′
4R	5′-CTGCTTCTAGACACTGTTCTCGTTCAAATCTCTC-3′

Table 2-2 Primer Sequences used in PCR, RT-PCR, RACE and Site Directed Mutagenesis
Restriction enzyme sites added to primers are coloured. SalI in blue; XbaI in green; NotI in red, ClaI in orange.

2.1.2.4. Agarose Gel Electrophoresis/Southern Blot

Sigma (Poole, Dorset)

Ethidium Bromide, Orange G, Xylene Cyanole, Bromophenol Blue, Whatman 3MM Chromatography Paper, Methylene Blue.

Cambridge Bioscience Ltd (Cambridge, UK)

GelRed DNA Stain.

Fermentas (York, UK)

O'GeneRuler™ 100 bp DNA Ladder, O'GeneRuler™ 1 kb DNA Ladder, O'GeneRuler™ 1 kb Plus DNA Ladder

Biorad (Hemel Hempstead, UK)

Biospin 30 columns, Mini Sub-cell GT Cell and Sub-cell GT Cell Submerged Horizontal Gel Systems.

Melford Laboratories Ltd. (Ipswich, UK)

Agarose (Molecular Biology grade).

GE Healthcare Life Sciences (Little Chalfont, UK)

Hybond-XL (30cm × 3m roll), EPS601 Power Supply, Rediprime II DNA labelling Kit. Redivue α-32P-dCTP (3000Ci/mmol, 10mCi/ml), Autoradiography Cassettes with intensifying screens.

Perkin Elmer (Beaconsfield, UK)

EasyTide α-^{32}P-dCTP (3000Ci/mmol, 10mCi/ml).

Thermo Fisher Scientific (Cramlington, UK)

Hybaid Shake'n'stack hybridisation oven and bottles. Hybridisation mesh.

UVitec (Cambridge, UK)

UVitec Crosslinker (CL-E580).

Agilent Technologies UK Ltd (Stockport, UK)

Stratalinker 1800 (Stratagene).

2.1.2.5. DNA/RNA Purification

Gen-Probe Life Sciences Ltd (Manchester, UK)

Nucleon BACC2 DNA Extraction Kit.

Qiagen Ltd. (Crawley UK)

RNeasy Mini Kit.

Amsbio (Abindgon, UK)

RNABee.

2.1.2.6. cDNA Library Screen

GE Healthcare Life Sciences (Little Chalfont, UK)

Hybond-N (22.5cm×22.5cm sheets), Rediprime II DNA labelling Kit. Redivue α-32P-dCTP (3000Ci/mmol, 10mCi/ml), Autoradiography Cassettes with intensifying screens.

Agilent Technologies UK Ltd (Stockport, UK)

Stratalinker 1800 (Stratagene), Uni-ZAP XR Pre-made Chicken Fibroblast cDNA library.

Sigma (Poole, Dorset)

Polyvinylpyrrolidone, Salmon Sperm DNA, PolyA RNA, Corning Square Bioassay Dishes, Whatman 3MM Chromatography paper.

Biorad (Hemel Hempstead, UK)

Biospin 30 columns.

Melford Laboratories Ltd. (Ipswich, UK)

X-gal, IPTG.

2.1.2.7. Plasmids and Probes

2.1.2.7.1. pCR4-cClaspin

Two cCLSPN mRNA fragments were amplified by RT-PCR using Pfx polymerase. The following PCR primer pairs were used to amplify a 5′ fragment (7F-2R) and a 3′ fragment (2F-10R) that overlapped in a region containing a unique SfuI restriction site: 7F-2R: 7F – 5′-GCTCGAAGGTTTCTTACGCGGCAGC-3′ and 2R - 5′-CTGCTTCTAGACTCTGTTGAAGGTGGTTTGGG-3′ and 2F-10R: 2F - 5′-AATGTTGG TCGACTGCCAGTGAAACAGAAGAACAGC-3′ and 10R 5′-AGACAGCAGGAGCAG GGAGAACAACG-3′. The individual PCR fragments were cloned into pCR4-TOPO. Clones were screened ensure that the clones selected contained the cDNA fragments in the same orientation. The SpeI/SfuI 5′ fragment from pCR4-7F-2R was ligated into SpeI/SfuI digested pCR4-2F-10R (the 3′ fragment). The resulting

plasmid contained the full length Claspin cDNA inserted in the same orientation as the T7 promoter. The cDNA sequence was verified by sequencing.

2.1.2.7.2. pET28(a)+ - Claspin fragment

The cClaspin fragment was amplified by PCR using the following primers: Forward (EcoRI) 5'·ccg gaa ttc gaa gag aaa caa gtt gaa gac ggt 3', Reverse (NotI) 5'·ctc gag tgc ggc cgc cac aaa ttt tcc aga gca aag 3'. The resulting PCR product was digested with EcoRI/NotI and ligated with pET28(a)+ precut with EcoRI and NotI. This vector allows the expression of the cClaspin fragment containing an N-terminal His_6 – T7 tag separated by a thrombin cleavage site and a C-terminal His_6 tag under the control of the T7 RNA polymerase.

2.1.2.7.3. pEGFP-N1-cClaspin

The pCR4-cClaspin vector was digested with SmaI and ScaI. This releases the cClaspin cDNA but removes the last 4 amino acids and the stop codon. This fragment was then ligated into SmaI digested pEGFP-N1 (Clontech) in order to fuse the cClaspin cDNA in-frame with the GFP cDNA. Plasmids were screened to ensure the cDNA had inserted in the correct orientation. The entire cDNA sequence was verified by sequencing.

2.1.2.7.4. pCR4-SF.cClaspin

pCR4-SF.cClaspin was generated using InFusion Cloning (Clontech). The primers were designed such that they overlapped the region surrounding SmaI digested pCR4-cClaspin by 15 bases. The SF tag was amplified using Pfx polymerase using the following primers: 5′-CCGCCGCCATGGCCCCTCCGCTAGCTCCTTTCTCG-3′ and 5′- GACTGCGTCCGGCCCCCAAGCTTGGTACCGAGC -3′. The template was pcDNA3.1-N SF TAP (Dr. J. Gloeckner, Helmholtz Zentrum München). The resulting PCR product was recombined with SmaI digested pCR4-cClaspin using InFusion Cloning from Clontech according to manufacturer's instructions to generate pCR4-SF.cClaspin. The entire cDNA sequence was verified by sequencing.

2.1.2.7.5. pTRE-Tight:SF.cClaspin

pTRE-Tight (Clontech) was digested with NheI and ligated to the SpeI SF.cClaspin cassette from pCR4-SF.cClaspin. The orientation of the inserted cDNA was confirmed and the entire cDNA sequence was verified by sequencing.

2.1.2.7.6. pTRE-Tight:cClaspin.EGFP

pTRE-Tight (Clontech) was digested with XbaI and SalI and ligated to the XbaI/SalI cClaspin.EGFP cassette from pEGFP-N1-cClaspin. The entire cDNA sequence was verified by sequencing.

2.1.2.7.7. pLox-Neo-GFP and pLox-Bsr-GFP

The Multi Cloning site was removed from pEGFP-C2 (Clontech) by digestion with BamHI/BglII and re-ligating the resulting vector. The resulting plasmid was digested with MluI/PciI to liberate the GFP expression cassette. This fragment was blunt ended with Klenow and then ligated with KpnI linkers. The resulting fragment was then digested with KpnI and ligated into KpnI digested pLox-Neo and pLox-Bsr (gift from JM Buerstedde) to yield pLox-Neo-GFP and pLox-Bsr-GFP.

2.1.2.7.8. pTET-Off Advanced Puro

pTET-Off advanced was digested with XhoI to remove the Neomycin resistance cassette. The resulting plasmid was either self ligated to generate pTet-Off Advanced – Neo (no selectable marker) or ligated with an XhoI Puro resistance cassette from pLox-Puro.

2.1.2.7.9. pHIS

pHIS was generated by ligating a HindIII/BamHI fragment from the Histidinol resistance cassette (JM Buerstedde) into the HindIII/BamHI sites of pPUR (Clontech). This removes the puromycin resistance cassette from pPUR and replaces it with the Histidinol resistance gene, allowing its expression from the SV40 promoter.

2.1.2.7.10. pSuperior/Frt-Hygro

pSuperior.neo (Oligoengine) was digested with EcoRI/XmaI and blunt ended using Klenow. This plasmid backbone was then ligated a blunt PvuII cassette from pcDNA5/FRT/TO to generate the desired plasmid. This vector was then digested with BglII and XhoI and ligated with the appropriate annealed shRNA oligos.

	Primers	Description
A	G13F: 5′-GGTCGACCAGACCCGTCATACCTATACGTGAGC-3′ G7R: 5′-GATCGATGAATAGAAGTGATGCGTGCTATGG-3′	Gel Purified PCR product (1648bp)
B	G10F: 5′-CTTTGACTCAGGTTGTAATCTTAGG-3′ 5R 5′-CTGCTTCTAGAATGGGAAAAGCAACGAAGC-3′	PCR product digested with PstI then gel purified (988bp)
C	G14F: 5′-GCTACAGCTATCTTGACAAGTGTG-3′ G13R 5′-AAGCAGAGCCCTGCGGCTC-3′	Gel Purified PCR product (584bp)
D	G15F: 5′-GAAGAGGGCAGTGATGGCTCG-3′ G14R 5′-GGAGCAGCGGAGGAGGGTTG-3′	Gel Purified PCR Product (460bp)
E	3F: 5′-AATGTTGGTCGACGAAGAGAAACAAGTTGAAGACGGT-3′ 5R 5′-CTGCTTCTAGACACTGTTCTCGTTCAAATCTCTC-3′	PCR product digested with SacI then gel purified (2007bp)

Table 2-3 Southern Blotting Probes

2.1.3. Molecular Biology - Analysis of Protein

Sigma (Poole, Dorset)

Tween 20 , Benzamidine, Aprotinin, Leupeptin, Okadaic acid, EGTA, Sodium orthovanate, Sodium fluoride, ß-glycerophosphate, Dithiothreitol, Triton-X-100, Brij-35, PMSF, Sodium Pyrophosphate, Protease Inhibitor Cocktail I, Phosphatase Inhibitor cocktail I, Phosphatase Inhibitor cocktail II, Protein A-Sepharose Beads, Anti-flag M2 Affinity gel, Flag Peptide, Ponceau S solution, TEMED, BSA, ATP.

Invitrogen (Paisley, UK)

1M HEPES solution, Novex Precast gels, Simply Blue Safe Stain.

Stratech Scientific LTD (Suffolk, UK)

StrepTactin Sepharose (#2-1201-010), D-Desthiobiotin (10X Buffer E).

Merck Biosciences (Nottingham, UK)

NP-40.

Thermo Fisher Scientific (Cramlington, UK)

Bradford Assay Reagent, BCA assay reagent.

Fermentas (York, UK)

High Range, Spectra™ Multicolor Broad Range Protein Ladder, PAGE Ruler Unstained Protein Ladder.

GE Healthcare Life Sciences (Little Chalfont, UK)

Full Range Rainbow Molecular Weight Markers, Amersham ECL™ Western Blotting Detection Reagents, Redivue L-S35 Methionine 1000Ci/mmol 10mCi/ml, Amplify Fluorographic Reagent.

Perkin Elmer (Beaconsfield, UK)

EasyTide γ-^{32}P-ATP (3000Ci/mmol, 10mCi/ml).

Millipore (Watford, UK)

Chk1 (Recombinant human active, N-terminally GST tagged, expressed in Sf21 cells).

Biorad (Hemel Hempstead, UK)

Gel Dryer (Model 583) attached to a Gel Master Gel Dryer Vacuum System (Welch), Empty Micro Biospin chromatography columns.

React Scientific (Troon, UK)

Supported Nitrocellulose Membrane (30cm × 3 m) 0.45µm pore size.

Severn Biotech (Kidderminster, UK)

30% acrylamide solution (37.5:1), 40% acrylamide solution (125:1).

New England Biolabs UK (Hitchin, Hertfordshire, UK)

Goat Anti-rabbit IgG (H&L) HRP secondary antibody, Horse Anti-mouse IgG (H&L) HRP secondary antibody, Lambda Protein Phosphatase, 3 × red Loading Buffer Pack.

Promega (Southampton, UK)

Donkey Anti-Goat HRP secondary antibody, Quick Coupled T7 TNT Kit.

Genetic Research Instrumentation (Braintree, Essex, UK)

AE-6450 Dual Mini Vertical P.A.G.E. Apparatus.

Thistle Scientific Ltd. (Uddingston, Glasgow, UK)

Western Blot Transfer Apparatus - Biometra Fastblot B34 w/o cooling.

Eppendorf (Histon, Cambridge, UK)

Biophotometer (Spectrophotometer), Refrigerated Microcentrifuge 5415 R, Microcentrifuge 5415 D.

2.1.4. Cell Biology

2.1.4.1. Tissue Culture

BD Biosciences

T25, T75 and T175 Tissue Culture Flasks (Straight Neck, Standard TC Treated), 96 well, 24 well, 12 well and 6 well Tissue Culture Plates (Flat Bottomed,

Standard TC Treated), 35mm, 60mm and 100mm Tissue Culture Dishes (Standard TC Treated.

Thermo Fisher Scientific (Cramlington, UK)

1.5ml Nunc Cryovials.

Thermo Fisher Dharmacon (via Abgene Ltd., Epsom, Surry, UK)

The negative control siRNA used was siGENOME NON TARGETING Control #2 (#D-001210-02-20). Both shClaspin.1 and shCdk2.1 were provided as pre-annealed 2'-deprotected desalted oligos. The siEDD pool was a Smart Pool designed by Dharmacon against the chicken EDD mRNA sequence (XM_424053). The lyophilised oligos were resuspended in the supplied resuspension buffer then aliquotted and stored at -80°C.

siRNA	Sequence
shClaspin.1 (cClaspin)	Sense Sequence A.A.G.A.A.G.A.A.A.G.G.A.G.A.G.G.G.A.G.U.U Antisense Sequence C.U.C.C.C.U.C.U.C.C.U.U.U.C.U.U.C.U.U.U.U
shCdk2.1 (cCdk2)	Sense Sequence C.U.G.C.A.C.U.A.C.G.A.U.C.C.C.A.A.C.A.U.U Antisense Sequence U.G.U.U.G.G.G.A.U.C.G.U.A.G.U.G.C.A.G.U.U
siEDD Pool A	Target Sequences: G.A.C.A.A.T.A.C.C.T.G.A.T.G.A.A.T.C.T C.G.A.G.C.A.G.G.A.T.C.A.T.C.A.A.G.T.A C.G.A.A.A.G.T.T.C.C.G.G.A.C.T.G.T.T.T G.A.T.G.G.A.G.C.C.T.C.A.T.T.T.G.A.T.A

Table 2-4 siRNA Duplex Sequences

Invitrogen (Paisley, UK)

DMEM (high glucose, with pyruvate, no glutamine), RPMI 1640, 200mM Glutamine, Tryptose Phosphate Broth, 2.5% Trypisin, MEM Vitamins, Geneticin (G418), OptiMEM, Lipofectamine 2000, Tetracycline, Zeocin.

Sigma (Poole, Dorset)

DMEM minus Cysteine and Methionine, Penicillin G, Streptomycin, Amphotericin, Puromycin, Histidinol, ß-mercaptoethanol, EBSS, Doxycycline, Chicken Serum, Aphidicolin, Hydroxyurea, Caffeine, Doxorubicin, Etoposide, Cycloheximide, Anisomycin, Emetine, Thapsigargin, Tunicamycin, Dithiothreitol, 30% Hydrogen Peroxide, MG132, Leupeptin, Bromo-deoxyuridine, Nocodazole.

Oligoengine (Seattle, US)

Suitable target sequences for shRNA were designed against the isolated cCLSPN mRNA isoform 1 or chicken CDK2 mRNA (EF182713) using the Oligoengine 2.0 software (sequence underlined in Table 2-5). The target sequences were then made into the appropriate oligo sequence to allow the expression of the oligo as a hairpin. The oligos were synthesised to produce BglII and XhoI overhangs once annealed.

shRNA Oligo	Sequence
shCdk2 S	5′-GATCCCCCTGCACTACGATCCCAACATTCAAGAGATGTTGGGATCGTAGTGCAGTTTTTC-3′
shCdk2 AS	5′-TCGAGAAAAACTGCACTACGATCCCAACATCTCTTGAATGTTGGGATCGTAGTGCAGGGG-3′
shClaspin S	5′-GATCCCCAAGAAGAAAGGAGAGGGAGTTCAAGAGACTCCCTCTCCTTTCTTCTTTTTTTC-3′
shClaspin AS	5′-TCGAGAAAAAAAGAAGAAAGGAGAGGGAGTCTCTTGAACTCCCTCTCCTTTCTTCTTGGG-3′

Table 2-5 shRNA oligo sequences

Autogen Bioclear (Calne, UK)

Blasticidin (Invivogen), Foetal Bovine Serum, Tetracycline Free Foetal Bovine Serum, Dialysed Foetal Bovine Serum.

Roche Diagnostics Ltd. (Burgess Hill, UK)

Hygromycin B.

Axon Medchem Ltd. (Groningen, Netherlands)

BIRB-796.

Merck Biosciences (Nottingham, UK)

Rapamycin, SB202190, SB600125, JNK Inhibitor VIII, Wortmannin, Bafilomycin A1.

Dr. David M. Sabatini, Whitehead Institute for Biomedical Research

Torin1.

Lonza (Slough, UK)

Amaxa® Nucelofection Kit T and L, Nucleofector II.

Biorad (Hemel Hempstead, UK)

Gene Pulser II, 0.4cm electroporation cuvettes.

Promega (Southampton, UK)

Dual Luciferase Assay Kit, Glomax Luminometer.

2.1.4.2. Flow Cytometry

Stratech Scientific LTD (Suffolk, UK)

Anti-mouse FITC (#515-095-072), Anti-rabbit FITC (#111-096-047).

Dako Ltd (Ely, Cambridgeshire, UK)

Mouse Anti-BrdU Antibody - clone Bu20a (#M0744).

Sigma (Poole, Dorset)

Propidium Iodide.

Qiagen Ltd. (Crawley UK)

100mg/ml RNase A.

Millipore (Watford, UK)

Rabbit Anti-pS10 Histone H3 Antibody (#06-507).

SantaCruz Biotechnology (via Insight Biotechnology, Wembley, UK)

Rabbit Anti-pS10 Histone H3 Antibody (#sc-8656-R).

BD Biosciences

FACS Calibur Flow Cytometer, BD FACSFlow, BD FACS Rinse, BD FACS Clean, 5ml Polystyrene Round Bottom Tubes, 15ml Polystyrene Conical Tubes.

2.1.4.3. Metaphase Spreads/FISH

Sigma (Poole, Dorset)

Demecolchine, Dextran sulphate, Igepal CA 630, Formamide (#F9037).

Dr. Darren Griffin, Farmachrom, University of Kent.

Biotin labelled Ch23 specific BAC (LEI0090), (Digoxygenin labelled Ch23 specific BAC (LEI0102) not used in this study).

Qbiogene (Cambridge, UK)

FixoGum Rubber Cement.

Qiagen Ltd. (Crawley UK)

100mg/ml RNase A.

GE Healthcare Life Sciences (Little Chalfont, UK)

Cy3 labelled Streptavidin.

Vector Labs (Peterborough, UK)

Vectasheild mounting medium containing 1.5µg/ml DAPI.

2.2. Methods

2.2.1. MOLECULAR BIOLOGY – ANALYSIS OF DNA and RNA

2.2.1.1. Recombinant DNA Technology

2.2.1.1.1. Restriction Digests

Typically 5-10µg of DNA was incubated with 20U of restriction enzyme in the appropriate buffer with a final volume of 30µl, at the recommended temperature for 2 hours. If the digest was allowed to proceed overnight then the amount of restriction enzyme was reduced to avoid star activity. If a double digest was to be preformed then the most appropriate buffer was chosen for digestion. If no suitable buffer could be found then the digest would be carried out sequentially adjusting the salt condition for the second enzyme and then digesting with the second enzyme. To prevent re-ligation of the vector the DNA was dephosphorylated. To dephosphorylate the vector DNA 2U of Shrimp Alkaline Phosphatase was included when setting up the digest. Before further processing for blunting or digestion with other enzymes the restriction enzymes were heat inactivated (if possible) at 70°C for 10 minutes.

2.2.1.1.2. End Blunting

Where necessary, in order to create compatible ends for cloning, sticky ends were converted into blunt ends. Depending on the type of overhang different enzymes were chosen. Klenow Polymerase was used to fill in 5′ overhangs. Mung bean nuclease was used to remove 3′ and 5′ overhangs.

2.2.1.1.2.1. Klenow Fill in

1-5µg of purified DNA (or heat-inactivated restriction digests supplemented with Mg and 40µM dNTP's) was diluted in reaction buffer supplemented with 40µM dNTP's. 1U of enzyme per µg of DNA was added. The reaction was incubated at 25°C for 15 minutes. To terminate the reaction EDTA was added to a final concentration of 10mM and the reaction was heated to 70°C for 10 minutes.

2.2.1.1.2.2. Mung Bean Nuclease Digestion

1-5µg of purified DNA (or heat inactivated restriction digest – mung bean nuclease is active in restriction enzyme buffer) was dissolved in 1 × Mung Bean

Nulcease digestion buffer. 2U of nuclease per µg of DNA was added and the reaction incubated at 30°C for 30-60 minutes. The DNA was purified by agarose gel electrophoresis or spin column purification.

2.2.1.1.3. Linker Ligations

Pre-annealed phosphorylated linkers were ligated to blunt ended DNA in order to create sticky ends. A 100 fold molar excess of linkers to DNA ends was added to the blunt ended DNA. The ligation was carried out in 1 × Ligation buffer containing 400U of T4 DNA ligase overnight at room temperature. The reaction was then heat inactivated and without purification the ligated linkers were digested to completion with the appropriate restriction enzyme in order to generate sticky ends.

2.2.1.1.4. Annealing Oligos

2µg of sense and anti-sense oligos were placed in a 1.5ml screw cap micro tube. This was brought up to a final volume of 50µl with annealing buffer (100mM NaCl, 50mM HEPES). The oligos were then boiled for 5 minutes and then immediately placed in a beaker containing ≈ 300mls of boiling water. This was then allowed to cool to room temperature slowly. The annealed oligos were stored at -20°C until use.

2.2.1.1.5. Vector-Insert Ligations

Prior to ligation the DNA was purified, either by gel purification or by clean up columns (Qiaquick spin columns), and quantified using the Nanovue Spectrophotometer. (GE Healthcare). Ligations were carried out using the Rapid DNA Ligation Kit following manufacturer's recommendations. A typical ligation contained approximately 50ng of vector and 2 – 5 fold molar excess of insert diluted in 1 × ligation buffer containing ATP. 5U of T4 DNA ligase was then added. Ligations were incubated at room temperature for up to an hour and then either frozen or transformed into chemically competent bacteria.

2.2.1.1.6. Cloning of PCR products

PCR products (see below) were cloned using the TOPO Cloning kits from Invitrogen. Standard Taq polymerase (Platinum Taq/AmpliTaq) adds a non-template A overhang to PCR products. Therefore the TOPO-TA cloning kit can be used to clone these products. High fidelity polymerases (Phusion

Polymerase/Pfx Polymerase) with proofreading ability and exonuclease activity remove this overhang and generate blunt ended PCR products. Therefore the TOPO-Blunt cloning kits are used for these products. Alternatively blunt PCR products are purified and A overhangs can be added.

The TOPO ligation reaction consisted of 1µl of TOPO vector, 4µl of purified PCR product and 1µl of the salt solution. For short PCR products this was incubated at room temperature for 10-20 minutes but for long PCR products it was incubated for 60-90 minutes. The reaction was used immediately for transformation into DH5α *E. coli*.

2.2.1.1.7. Adding A overhangs to PCR products

The PCR product was purified using the PCR purification kit. 1 × PCR Buffer supplemented with 200µM dATP was added. 1U of Taq polymerase was added and the reaction is incubated at 72°C for 30 minutes. To prevent loss of the added A's the reaction is used immediately for TA cloning.

2.2.1.1.8. Transformations

Typically $1/10^{th}$ – $1/5^{th}$ (2-5µl) of a ligation reaction or 10-100ng of super-coiled plasmid was added to 50µl of chemically competent DH5α *E coli* cells in a 14ml round bottom tube. These were incubated on ice for 30 minutes and then heat shocked at 42°C for 45 seconds. The bacteria were then re-incubated on ice for 2 minutes and 250µl of SOC media was added. The bacteria were allowed to recover at 37°C with shaking before being spread onto the appropriate selective plates. To ensure single colonies were obtained both 50µl and 250µl of the transformation mix was plated out.

2.2.1.1.9. DNA Clean up

Prior to ligation the DNA was 'cleaned up' using one of the following Qiagen kits following manufacturer's instructions. In brief the appropriate solubilisation buffer containing a high salt concentration was added to the digest or gel. Once dissolved (the gel was heated to 50°C in order to solubilise it) the mixture was applied to a silica based spin column which binds DNA in the presence of high salt concentration. The column was washed with Ethanol and the DNA eluted in a low salt buffer containing 10mM TrisHCl pH 8.0.

QiaexII Gel extraction kit – for purification of large fragments of DNA from agarose gels.

Qiaquick Gel Extraction Kit – for routine purification of DNA from agarose gels.

Qiaquick PCR purification kit – for routine clean up of restriction digests, PCR products etc.

Qiaquick Nucleotide Removal Kit – for the clean up of restriction enzyme digests.

2.2.1.1.10. Ethanol Precipitation

A crude DNA purification can be carried out by precipitating DNA from solution using Ethanol. To the volume of the DNA in solution (restriction digest etc) was estimated. To this was added 3M Sodium Acetate pH5.2 (volume equal to $1/10^{th}$ the volume of DNA) followed by ice cold 100% Ethanol (volume equal to twice the volume of DNA + sodium acetate). The solution was mixed and incubated on ice for 30 minutes. The tube was then spun at 16,100 x g for 30 minutes at 4°C. The pelleted DNA was washed once with 70% Ethanol to remove the excess salt, allowed to air dry for a couple of minutes and then resuspended in an appropriate butter e.g. 10mM Tris-HCl pH 8.0 or TE buffer. To precipitate DNA from a dilute solution 40-50µg of glycogen was added to the solution prior to the addition of ethanol to aid in the precipitation of the DNA.

2.2.1.1.11. Plasmid Preps

Plasmid preps were carried out using the Qiaprep Maxi/Mini prep kits from Qiagen. Miniprep DNA was used for routine cloning and DNA sequencing. Maxi prep DNA was used for transfecting cells. From an overnight culture (3mls for a miniprep, and 100mls for a maxi prep) the bacteria were pelleted. The pellet was then resuspended in a buffer containing RNaseA and lysed by the addition of a SDS/NaOH solution. The SDS and proteins were precipitated by the addition of potassium acetate. The precipitate was removed (by centrifugation for the minipreps or by filtration for the maxipreps) and the cleared lysate containing the plasmid was bound to a column (silica based spin-column for minipreps and an ion-exchange column for maxipreps). The columns were washed and the DNA was eluted. For maxipreps the DNA was precipitated by the addition of

isopropanol followed by a high speed centrifugation step. The resulting pellet was re-dissolved in an appropriate volume of 10mM TrisHCl pH 8.0.

2.2.1.2. Nucleic Acid Quantitation

The concentration and quality of nucleic acids (DNA/RNA/oligos) was estimated by UV spectroscopy, by either using the Nanovue spectrophotometer or the Biophotometer with UV transparent cuvettes, Uvettes. The A_{260} reading was used in order to determine the concentration of nucleic acid using the following conversion factors. An A_{260} reading of 1 was equivalent to 50µg/ml for DNA, 40µg/ml for RNA and 30µg/ml for DNA oligos. An A_{260}/A_{280} ratio of greater than 1.8 was classified as a 'clean' DNA solution. For greater accuracy A_{260} readings were only taken between 0.1 and 1, the DNA was diluted as appropriate.

2.2.1.3. DNA sequencing

DNA sequencing was carried out by the Molecular Technology Service at the Beatson Institute. DNA sequencing is carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, routinely using approximately 500ng of plasmid DNA as template and 20ng of sequencing primer. The resulting sequencing reactions are analysed using an Applied Biosystems 3130xl (16 capillary) sequencer. The data was analysed using Contig Express. High quality sequence data obtained in triplicate was used to determine new sequence.

2.2.1.4. Polymerase Chain Reaction

2.2.1.4.1. PCR

Standard PCR was carried out using either Platinum Taq or AmpliTaq PCR kits. The PCR reactions were assembled in thin walled domed capped PCR tubes and typically consisted of 1-10ng of plasmid DNA or 50-100ng of genomic DNA, 1 × PCR Buffer, 200µM each dNTP, 0.2µM forward and reverse primers and 1-2.5U of polymerase in a final volume of 50µl. A typical PCR cycle consisted of initial denaturation at 94°C for 1 minute, followed by 25-35 cycled of 94°C for 30 sec, annealing at 45-65°C for 30 sec followed by 72°C for 1min per kb. A final extension of 10 minutes at 72°C was carried out and the PCR product was then cooled to 4°C until use.

High fidelity PCR was carried in order to amplify DNA for sequence analysis, for cloning and for amplifying genomic DNA. The procedure is the same as above except that a high fidelity polymerase is used according to manufacturer's instructions. Unless stated elsewhere the primer sequences used are given in Table 2-1.

2.2.1.4.2. RT-PCR

Routine RT-PCR was carried out using the GeneAmp RT-PCR Core kit. For cDNA cloning the Superscript II RT-PCR kit coupled with a Pfx Polymerase amplification step was used following the manufacturers instructions. Typically 2µg of total RNA was reverse transcribed into cDNA by MuLV reverse transcriptase using both random hexamers and Oligo dT's as primers. $1/10^{th}$ of the cDNA generated was used as the template in the corresponding PCR's. Primer Sequences used are given in Table 2-2.

2.2.1.4.3. RACE

Rapid Amplification of cDNA Ends was carried out using the SMART RACE kit from Clontech following manufacturer's instructions. 3′ RACE cDNA is generated using a Universal Primer Sequence attached to $dT_{(30)}VN$ to prime synthesis. 3′ RACE PCR's are carried out using forward primers designed towards the 3′ end of the gene of interest and the reverse primer from the universal primer sequence attached to the $dT_{(30)}VN$ sequence. 5′ RACE cDNA is generated by priming cDNA synthesis using a $dT_{(25)}VN$ primer and including a universal primer sequence ending in a $dG_{(3)}$ sequence. Once the BD PowerScript™ Reverse Transcriptase reaches the end of the transcript it adds a non-template specific $dC_{(3)}$. This then anneals to the $dG_{(3)}$ containing primer and this primer serves as a template to add the universal primer sequence to the ends of all transcripts transcribed. The 5′ RACE PCR's are carried out using a reverse primer designed towards the 5′ end of the gene of interest and the forward primer from the universal primer sequence added to the 5′ ends of all transcripts. The RACE primer sequences used are given in Table 2-2.

2.2.1.4.4. Site directed mutagenesis

Site Directed Mutagenesis was carried out using the QuickChange II Kit from Stratagene according to manufacturer's instructions. Briefly the mutagenesis reaction was assembled by adding 10ng of plasmid, 125ng of the forward and

reverse mutagenesis primers, 1× PCR buffer, 1 × dNTP's and 2.5U of PfuUltra HF DNA polymerase in a final volume of 50µl. The PCR reaction was cycled as per manufacturer's instructions. Following the cycle 10U of DpnI restriction enzyme was added and incubated at 37°C for 1 hour to digest the parental DNA. The remaining DNA was then transformed into XL1-Blue Supercompetent *E. coli* cells. Plasmid was isolated from the colonies that grew and the DNA was sequenced in order to identify a plasmid containing the desired mutation. The primer sequences used for mutagenesis are given in Table 2-2.

2.2.1.5. Agarose Gel electrophoresis

Agarose gel electrophoresis was used for the routine analysis of DNA from restriction digests and PCR's etc or as the first step in Southern blotting. 0.8% - 2% agarose gels in 1×TAE buffer was boiled in the microwave, allowed to cool and poured into the casting tray. Prior to pouring Ethidium Bromide (final conc. 1µg/ml) or GelRed (10,000×) was added to visualise the DNA. (GelRed is a sensitive, non-toxic, non-mutagenic dye with a similar excitation and emission spectrum of EtBr) The DNA was mixed with 6×loading dye (10 mM Tris-HCl pH 7.6, 0.15% orange G, 0.03% Xylene Cyanol FF, 60% glycerol, 60 mM EDTA) and loaded into the gel along with DNA ladder for size estimation (O'GeneRuler 100bp, O'GeneRuler 1kb and O'GeneRuler 1kb Plus, Fermentas). The gel was typically run at between 8-10V/cm and the DNA visualised using a transilluminator or the Syngene Genius Bio imaging system with GeneSnap Software.

2.2.1.6. Southern Blotting

20µg of purified DNA was first digested with the appropriate restriction enzyme (200-300U) in a final volume of 300µl overnight at 37°C. The digested DNA was concentrated by EtOH precipitation and re-dissolved in 30µl of TE buffer. The DNA was loaded onto a 0.8% agarose gel (15cm × 15cm × 0.7cm) and run at 1.2-1.5V/cm overnight. The gel was examined using the transilluminator in order to confirm that it had run for long enough. In order to facilitate the transfer of high molecular weight DNA from the gel a small amount of depurination was induced by incubating the gel with 0.25M HCl for 30 minutes. This introduces nicks into the DNA breaks the DNA into smaller pieces when denatured by alkali. The gel was then incubated with denaturation buffer (0.5M NaOH, 1.5M NaCl) for

1 hour with gentle agitation. Finally the gel was neutralised in neutralisation buffer (1M TrisHCl pH 8.0, 1.5M NaCl) for 1 hour with gentle agitation at room temperature. In order to transfer the DNA from the gel to the nylon membrane 2 sheets of 3MM paper were wrapped around a glass plate 16cm × 16 cm. The glass support was placed on rubber bungs in a Pyrex dish. The dish was filled with 20×SSC and the 3MM paper was allowed to dip into the buffer. The top left hand corner of the gel was cut off for orientation and the gel was inverted and placed on the wet 3MM paper. Air bubbles were removed. 4 wide strips of strong plastic were then placed under the edges of the gel to prevent buffer from bypassing the gel and entering the paper towels directly. The nylon membrane was placed on top and the air bubbles were removed. 2 sheets of 3MM paper the same size as the gel were placed on top and were allowed to wet through, followed by stacks of paper towels placed on top. Another glass plate was placed on top of this and was weighted using some lead pots. This set up allows for the buffer in the Pyrex dish to travel through the gel and membrane into the paper towels, carrying the DNA with it. The DNA will be bound by the membrane. The transfer was allowed to take place overnight. Once transferred the DNA was UV cross linked to the membrane at 120mJ/cm^2 using the Stratalinker 1800 (Stratagene) or the UVitec crosslinker (UVitec). The membrane was then stored dry until use. In order to ensure successful transfer of the DNA from the gel onto the membrane, following transfer the membrane was incubated with a Methylene blue stain (0.04% Methylene blue in 0.5M NaOAc pH 5.2) for 20-30 minutes. The membrane was then washed gently with water. DNA stains blue on the white background. To remove the methylene blue stain the membrane was then washed in 1% SDS/1 × SSC for 20 minutes at room temperature.

2.2.1.7. Probe Labelling and hybridisation

DNA probes (usually 500bp-1500bp in length) were labelled using random priming with the Rediprime II Kit. 25-50ng of probe DNA (generated by PCR amplification of the target DNA and gel purified) was dissolved in 45µl of TE buffer, boiled for 5 minutes then cooled rapidly on ice to generate single stranded DNA. This was added to the labelling tube which contained lyophilised reaction buffer, dNTP's and Klenow Polymerase. To this 5µl of α-32P-dCTP (3000Ci/mmol, 10mCi/ml) was added, then mixed and incubated at 37°C for 60

minutes. This generally yielded a probe of specific activity of greater than 1 × 10^9 cpm/µg DNA. The probe was boiled and cooled rapidly before adding to the hybridisation buffer.

Hybridisation was carried out in hybridisation bottled on a rotisserie in hybridisation oven set to 65°C. The membrane was prehybridised in Church Buffer (0.25M Na_2HPO_4 Buffer pH 7.2, 7% SDS, 1mM EDTA) for 1-2 hours at 65°C. Fresh pre-warmed Church buffer was then added followed by the denatured labelled probe. The membrane was hybridised overnight at 65°C. After hybridisation the membrane was washed twice with wash buffer I (20mM Na_2HPO_4 Buffer pH 7.2, 5% SDS, 1mM EDTA) and twice with wash buffer II (20mM Na2HPO4 Buffer pH 7.2, 1% SDS, 1mM EDTA) then exposed to X-ray film in an autoradiography cassette at -70°C for 1-2 days. The film was then developed in a Kodak X-Omat 3000RA automatic film processor.

In order to strip the membrane prior to re-hybridisation the membrane was first incubated with 50mls of 0.4M NaOH for 30 minutes at 42°C then with 50mls 0.2M Tris-Cl pH7.5, 0.1% SDS, 0.1 × SSC and incubate for 30 minutes at 42°C. This was followed either directly with pre-hybridisation or a 10 minute wash in Wash Buffer II to remove the stripping buffer. Throughout the hybridisation process and exposure to film it is important not to allow the membrane to dry out as to do so make it more difficult to remove the probe.

2.2.1.8. Genomic DNA purification

Genomic DNA was purified using the Nucleon BACC2 DNA purification Kit. Briefly approximately 1-2 ×10^7 DT40 cells were harvested and washed once in ice cold PBS and transferred to a 1.5ml microtube. The cells were then resuspended in 1ml of Nuclear lysis buffer (Reagent A) (10mM TrisHCl pH 8.0, 320mM Sucrose, 5mM $MgCl_2$ and 1% Triton-X-100) and incubated on ice for 5 minutes. The nuclei were pelleted at 1,300×g for 5 minutes and the supernatant was discarded. The pellet was then resuspended 2ml of Reagent B, an SDS containing buffer and transferred to a polypropylene 15ml screw capped tube. The tube was rotated on an end over end rotary wheel for 2-4 hours at room temperature to allow the pellet to completely solubilise. 750µg of RNase A was then added to the samples in order to remove RNA and incubated at 37°C for 30 minutes. The sample was

deproteinised by adding 0.5mls of 5M Sodium perchlorate and 2mls of Chloroform and mixing well following each addition. Without remixing the phases, 300µl of Nucleon resin was added to the upper phase. The nucleon resin reacts covalently with protein via the protein amino groups thus attaching the remaining protein to the resin facilitating the separation of the proteins from the DNA. To separate the phases the tube was spun at 1300×g for 3 minutes. The upper phase (≈2.5mls) was then removed and added to 5mls of ice cold Ethanol to precipitate the DNA. The DNA was pelleted by centrifugation at 1,300×g for 15 minutes. The resulting pellet was washed once with 70% Ethanol and resuspended in 500µl of 8mM NaOH. Once dissolved the pH was adjusted by adding HEPES to a final concentration of 10mM. The DNA was quantified using the NanoVue spectrophotometer and stored at 4°C.

2.2.1.9. Total RNA purification

RNA was purified from cells using RNABee, a reagent containing phenol and guanidine isothiocyanate. Briefly, approximately 5×10^6 cells were washed and resuspended in 1ml of RNA Bee solution. 0.2mls of chloroform was added to this and shaken vigorously. It was then incubated on ice for 5 minutes before being centrifuges at 16,100 x g for 10 minutes. The upper phase containing the RNA (≈500µl) was then added to 500µl of isopropanol to precipitate the RNA. After incubation at room temperature for 10 minutes the RNA was pelleted by centrifuging at 16,100 x g for 15 minutes at 4°C. The resulting pellet was then washed twice with 1ml of 70% Ethanol and allowed to air dry. The pellet was resuspended in RNase free water and quantified using the NanoVue spectrophotometer and stored at -80°C. If necessary the RNA was further purified using the RNeasy Mini Kit from Qiagen.

2.2.1.10. cDNA library screening

2.2.1.10.1. Titering the Library

A chicken Uni-ZAP XR cDNA Library was screened for the presence of a cClaspin cDNA according to manufacturer's instructions. In order to determine the titre of the library the host bacteria were first prepared. A colony of XL1-Blue MRF' from a freshly streaked plate was inoculated into 50mls of LB containing 10mM $MgSO_4$ and 0.2% maltose. This was grown for 37°C for 4-6 hours and then the bacteria were pelleted and resuspended in 25mls of 10mM $MgSO_4$. The bacteria

were then diluted in 10mM $MgSO_4$ to an O.D. of 0.5. To aliquots of the diluted bacteria was then added a 10 fold serial dilution of the library from 1:10 – 1:10,000. The phage were allowed to attach to the bacteria at 37°C for 15 minutes. Then 6mls of molten NZY top agar (kept at 48°C), 30µl of 0.5M IPTG and 100µl of 250mg/ml X-gal was added to the bacteria. This was mixed and immediately poured onto a dry pre-warmed 150mm NZY agar plate. The top agar was spread evenly over the top of the plate. Once the top agar had set the plates were inverted and incubated at 37°C for 6-8 hours to allow plaques to form. From a plate with a reasonable number of plaque forming unit's on it the titre of the library and the background (the number of blue pfu's) can be determined.

2.2.1.10.2. Plating the Bacteriophage

Having just determined the titre of the library an appropriate number of plaques can be plated out for screening. The library screen was preformed on ten 24.5 cm × 24.5 cm square bioassay dishes with 1.5×10^5 pfu/plate – therefore 1.5×10^6 pfu's were screened. The XL1 Blue MRF' bacteria were prepared as above and were resuspended in 10mM $MgSO_4$ at an OD of 0.5. The appropriate volume of phage was added to 18mls of bacteria and the phage were allowed to attach to the bacteria at 37°C for 15 minutes. Per plate 1.8mls of this phage-bacteria suspension was added to 20mls of molten NZY top agar (kept at 48°C). This was mixed and immediately poured onto a dry pre-warmed square NZY agar plate. The top agar was spread evenly over the top of the plate. Once the top agar had set the plates were inverted and incubated at 37°C for 6-8 hours to allow plaques to form. Once the plaques were visible the plates were removed from the incubator and stored at 4°C until use.

2.2.1.10.3. Performing the Plaque Lifts

To perform the lifts square sheets of Hybond-N were placed onto the plate. The plaque DNA was allowed to bind to the membrane for 2 minutes. A needle was used to insert orientation marks. The membrane was then removed carefully and placed in a tray of denaturation buffer (0.5M NaOH, 1.5M NaCl) for 2 minutes. Next the membrane was transferred to neutralisation buffer (0.5M Tris-HCl pH 8.0, 1.5M NaCl) for 2 minutes and finally rinsing buffer (0.2M Tris-HCl, 2×SSC). The membrane was then dried in 3MM paper and the DNA was crosslinked to the membrane using the Stratalinker 1800 at 120mJcm^{-2}. The

procedure was repeated for the replica membrane (the needle marks were made in the same place on the replica membrane for orientation) and for the rest of the plates.

2.2.1.10.4. Screening and Hybridisation

A fragment of cCLSPN mRNA was cloned by Dr. Elizabeth Black by carrying out an RT-PCR using the following primers: forward 5′-ccggaattcgaagagaa acaagttgaagacggt-3′ and reverse 5′-ctcgagtgcggccgccacaaattttccagagcaaag-3′. The resulting PCR product was cloned into the EcoRI and NotI sites of pET28a(+) using the restriction sites engineered into the PCR primers. To generate the probe to screen the library this plasmid was then digested with EcoRI/NotI to liberate the 814bp fragment used as the probe. The chicken GAPDH probe was obtained by digesting pSPT19-GAPDH with EcoRI and the liberated fragment was gel purified. The probes were labelled with α-32P-dCTP as described previously.

Hybridisation was carried out in a square Tupperware box in a shaking water bath set at 68°C - processing 10 membranes at the time. The membranes were pre-hybridised in hybridisation solution (6×SSC, 5×Denhardts Reagent, 0.5% SDS, 1µg/ml PolyA RNA and 100µg/ml Salmon Sperm DNA) for 3 hours. 50ng of labelled denatured probe was then added and the probe was allowed to hybridise overnight. Following two washes with each of the wash buffers (I: 2× SSC, 0.1% SDS, II: 1×SSC, 0.1% SDS, III: 0.1×SSC, 0.1% SDS) the membranes were wrapped in Saran wrap and exposed to film at -70°C in autoradiography cassettes. The films were developed in a Kodak X-Omat 3000RA automatic film processor.

2.2.2. MOLECULAR BIOLOGY – ANALYSIS OF PROTEIN

2.2.2.1. Whole Cell Lysates

Cells were treated or not as appropriate, pelleted at 250×g, then washed once with ice cold PBS. Unless cells were lysed immediately they were snap frozen on dry ice and stored at -70°C until use. Cell pellets were lysed in whole cell extract buffer (400mM Potassium Chloride, 20mM HEPES, 5mM EDTA, 10mM EGTA, 1mM DTT, 0.4% Triton-X-100, 10% Glycerol, 5µg/ml Leupeptin, 285µM

PMSF, 1mM Benzamidine, 5µg/ml Aprotinin, 5mM Sodium Fluoride, 50ng/ml Okadaic Acid, 1mM Sodium Orthovanadate, 10mM ß-glycerophosphate, 5mM Sodium pyrophosphate) then incubated on ice for 20-30 minutes. The samples were then sonicated briefly to break up the chromatin (Sonics VCX130 Sonicator 2 sec on 2 sec off for 10 seconds on 30% amplitude) and spun at 16,100 x g in a refrigerated microfuge for 15 minutes to pellet the cellular debris. The cleared lysate was then transferred to a fresh tube and an aliquot removed for quantitation. The remainder was snap frozen on dry ice and stored at -70°C until use.

2.2.2.2. Protein Quantitation

2.2.2.2.1. Bradford Assay

The Bradford Assay relies on the blue colour generated when Comassie Brilliant Blue reagent binds to protein side chains. A standard curve of BSA was generated in a 1:10 dilution of the corresponding lysis buffer at the following concentrations: 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and 63µg/ml and 0mg/ml. The protein samples of unknown concentration were diluted 1:10 in dH_2O. 10µl of each standard (in duplicate) and 10µl of the diluted samples were placed in a cuvette. 1ml of working Bradford Assay reagent (50% Bradford Assay reagent: 50% dH2O) was then added to each cuvette. The absorbance at 595nm of the standards and samples were then read using a Biophotometer. The concentration of protein in the unknown samples was determined by comparison with the standard curve.

2.2.2.2.2. BCA Assay

Where substances in the lysis buffer interfered with Bradford Assay reagent (e.g. SDS) a BCA assay was carried out. This assay works by the reduction of the Cu^{2+} ions by the peptide bonds to Cu^{+} and the subsequent chelation of these ions by bichinconic acid to yield a purple colour. A standard curve was generated in the same way as for the Bradford Assay. 20µl of each standard (in duplicate) and 20µl of a 1:10 dilution of the protein samples was placed in a 5ml round bottom tube. 980µl of dH2O was then added to each tube. 1ml of working BCA assay reagent (25:24:1 Reagent A: Reagent B: Reagent C) was then added to each tube. The tubes were capped and then mixed and then incubated at 65°C for 1 hour to allow colour development. The BCA assay reagent turns from green to

purple. The samples were read in the Biophotometer at 562nm. The concentration of protein in the unknown samples was determined by comparison with the standard curve.

2.2.2.3. SDS-PAGE

SDS-PAGE was carried out in order to separate proteins so they could be analysed further by Western Blotting or to directly analyse protein by staining the gel. The mini vertical gel system from Atto was used. To cast the resolving gel a solution containing 6-15% acrylamide (acrylamide:bisacrylamide 37.5:1), 375mM Tris-HCl pH 8.8 and 0.1% SDS was made. To polymerise the gel ammonium persulphate and TEMED was added to a final concentration of 0.1% and 0.08% respectively. The gel mix was placed in the gel casting apparatus and over-laid with water-saturated butanol. Once set the stacking buffer (5% acrylamide (acrylamide:bisacrylamide 37.5:1), 125mM Tris-HCl pH6.8, 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED) was layered on top and the combs were inserted to allow the loading of samples. Once set the gel apparatus was correctly assembled and 1 × Running Buffer was added to the upper and lower chambers of the tank.

Samples to be analysed were added to an equal volume of 2 × SDS-PAGE loading buffer (120mM Tris-HCl pH6.8, 20% Glycerol, 5% SDS, Bromophenol blue supplemented with 100mM DTT before use) and were boiled for 5 minutes to denature the proteins. The samples were centrifuged briefly to remove debris and the samples were then loaded into the wells. Molecular weight markers were also run to estimate the size of the proteins to be analysed. The gels were then run at 180V at constant voltage until the dye front had just entered the running buffer.

To examine Chk1 phosphorylation by an electrophoretic mobility shift a 10% acrylamide gel but used and the acrylamide to bisacrylamide ratio was changed to 125:1.

To examine Claspin phosphorylation by an electrophoretic mobility shift a 6% acrylamide gel (37.5:1) was used but the gel was run for 2 hours 15 minutes at 180V until just the high molecular weight protein were left on the gel.

2.2.2.4. Western Blotting

Western Blotting was carried out using the semi dry blotting technique. Once the SDS-PAGE was run it was placed on top of 6 sheets of 3MM paper pre-soaked in dry blot buffer and cut to the size of the gel. On top of this was placed the nitrocellulose membrane and 6 more sheets of soaked 3MM paper. Air bubbles were removed by gently rolling with a marker pen. The 'sandwich' was placed on the transfer apparatus such that the gel was closest to the negative electrode. The proteins were transferred as standard at 20V, 200mA, 8W for 1 hour 20 minutes.

In order to transfer Claspin and other high molecular weight proteins onto the membrane they were transferred at 12V, 200mA, 8W for 2 hours and 30 minutes.

Ponceau S stain was used to ensure even transfer of the proteins onto the membrane. Then the membrane was blocked in blocking buffer (5% Marvel (non-fat dried milk powder) solution in 1 × TBS-T) for 1 hour at room temperature with gentle agitation. The membrane was then incubated with the appropriate primary antibody. (See Table 2-6 for the dilution of antibody used and what blocking buffer was used) The membrane was then washed three times for 10 minutes each with 1 × TBS-T with gentle agitation. The membrane was then incubated with the secondary antibody coupled to horseradish peroxidase for 1 hour at room temperature. The appropriate secondary antibodies were diluted 1:5000 in blocking buffer. After this incubation the membrane was washed as before and the bound secondary antibody was detected using Enhanced Chemiluminescence and X-ray film. The film was developed in a Kodak X-Omat 3000RA automatic film processor.

Antigen (Ab Type)	Supplier	Dilution and incubation conditions
cClaspin (pR)	In house (HF64B)	1:2500-1:5000 in 5% Marvel in TBS-T overnight at 4°C
Chk1 (mM)	SantaCruz (G-4)	1:1000 in 5% Marvel in TBS-T overnight at 4°C
Actin (mM)	Sigma (AC-40)	1:1000 in 5% Marvel in TBS-T overnight at 4°C
ATR (pG)	SantaCruz (N-19)	1:1000 in 5% Marvel in TBS-T overnight at 4°C
pS345 Chk1 (mR)	Cell Signalling (133D)	1:1000 in 5% BSA in TBS-T overnight at 4°C

Antigen (Ab Type)	Supplier	Dilution and incubation conditions
Cdk2 (pG)	SantaCruz (M2-G)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
EDD (pG)	SantaCruz (N-19)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
Tubulin (mM)	Sigma (DM1A)	1:5000 in 5% Marvel in TBS-T overnight at 4˚C
Strep II (mM)	Calbiochem (#71590-3)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
GFP (mM)	SantaCruz (B2)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
c-myc (mM)	SantaCruz (C-8)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
pS6 (pR)	Cell Signalling (#2211)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
pS6K (mM)	Cell Signalling (1A5)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
S6 (mM)	Cell Signalling (54D2)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
S6K (pR)	Cell Signalling (#9202)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
pJNK (mR)	Cell Signalling (81E11)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
pATF2 (pR)	Cell Signalling (9221)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
p p38 (pR)	Cell Signalling (9211)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
p cJun (S63) (pR)	Millipore (#06-828)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
JNK (mR)	Cell Signalling (56G8)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
ATF2 (mR)	Cell Signalling (20F1)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
p38 (pR)	Cell Signalling (9212)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
cJun (pR)	SantaCruz (D)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C
peIF2α (mR)	Cell Signalling (119A11)	1:1000 in 5% BSA in TBS-T overnight at 4˚C
eIF2α (pR)	SantaCruz (FL-315)	1:1000 in 5% Marvel in TBS-T overnight at 4˚C

Table 2-6 List of Primary antibodies
mM – mouse monoclonal; mR – rabbit monoclonal; pR – rabbit polyclonal; pG – goat polyclonal

2.2.2.5. Densitometry

Densitometry of Western blots was carried out using the 'Analyse -> Gels' function in ImageJ (NIH).

2.2.2.6. In vitro transcription translation

Coupled in vitro transcription-translation reactions were carried out using the T7 Quick Coupled TNT kit from Promega. A typical reaction consisted of 40µl of TNT mix (containing T7 RNA polymerase, nucleotides, amino acids (except Methionine) salts and RNAsin Ribonuclease inhibitor as well as the reticulocyte protein translation machinery), 1µg of plasmid DNA (with the cDNA downstream of a T7 promoter e.g. pCR4-TOPO, pBluescript or pcDNA3) 20µM Methionine , made up to 50µl with nuclease free water. The reactions were incubated at 30°C for 60-90 minutes. In order to in vitro translate cClaspin 2µl of T7 PCR Enhancer Solution (25mM Magnesium Acetate, 2.5mM Potassium Chloride) was added to the mix and the reactions were incubated at 30°C for at least 3 hours.

In order to label the translated proteins with radioactive Methionine 2µl of L-[35S]-Methionine (≈1000Ci/mmol, 10mCi/ml) was added instead of the cold Methionine. Reaction products were separated by SDS-PAGE. The S35 Signal was amplified by fluorography using Amplify™. The gel was first rinsed in dH2O and then fixed in freshly prepared fixing solution (50% Methanol/10% Glacial Acetic Acid). This was incubated at room temperature with gentle shaking for 30 minutes. This fixing solution was replaced with Amplify solution for another 30 minutes. The gel was finally incubated in 10% glycerol for 5 minutes prior to drying to prevent cracking. The gel was then dried and exposed to X-ray film in an autoradiography cassette fitted with intensifying screens.

2.2.2.7. Immunoprecipitation

cClaspin was immunoprecipitated form DT40 cells using the HF64B antibody. Approximately 2 × 10^7 DT40 cells were treated or not as desired and then harvested, washed once in PBS and lysed in 200µl of NET-N Lysis buffer (20mM Tris-HCl pH 7.5, 10% Glycerol, 137mM NaCl, 2mM EDTA, 1% NP-40, 1×Protease Inhibitor Cocktail, 1×Phosphatase Inhibitor Cocktail I, 1×Phosphatase Inhibitor Cocktail II) for 20 minutes, sonicated briefly and then spun at 16,100 x g at 4°C for 15 minutes in order to clarify the lysate. The protein concentration was determined by Bradford Assay. 1mg of lysate was diluted in a final volume of lysis buffer and 5µl of pre-immune serum from the rabbit used to generate the HF64B antibody and 40µl of a 50% slurry of washed, equilibrated Protein A-Sepharose beads was added. This pre-clearing was carried out in order to

remove the proteins that bind non-specifically to either the beads or IgG. The tubes were incubated at 4°C for 1 hour on an end-over-end rotator. The beads were then pelleted and the pre-cleared lysate transferred to a new tube. To this was added 5µl of HF64B antibody incubated at 4°C on a rotary wheel for 1 hour. Then 40µl of washed and equilibrated Protein A – Sepharose beads was added and incubated overnight at 4°C. The beads were then washed three times with lysis buffer and once with PBS with lysis buffer and used as required. For direct analysis of the purified protein the beads were boiled in SDS-PAGE sample buffer in order to remove the bound proteins.

2.2.2.8. Phosphatase Treatment

Claspin was immunoprecipitated from treated lysates as described (Section 2.2.2.7) and the beads were washed × 3 with lysis buffer not containing phosphatase inhibitors and × 2 with PBS. The final wash was in 1 × Lambda Protein Phosphatase buffer. The beads were then incubated with 20µl of 1 × Lambda Protein Phosphatase Buffer containing 1mM $MnCl_2$ and 80U/µl of Lambda protein Phosphatase. The reactions were incubated at 30°C for 30 minutes. 10µl of 3 × SDS-PAGE loading buffer was added to terminate the reaction. The protein was then analysed by SDS-PAGE and Western blotting to determine its phosphorylation status.

2.2.2.9. TAP Purification and Proteomics

2×10^8 DT40 cells stably expressing Strep II – FLAG tagged cClaspin were treated as required. The cells were then pelleted and washed twice with PBS. The cell pellet was then lysed in 1.5mls of lysis buffer (150mM NaCl, 30mM Tris-HCl pH 8.0, 1 × Protease Inhibitor, 1 × Phosphatase Inhibitor I, 1 × Phosphatase Inhibitor II. 01% SDS, 1% Triton-X-100) and incubated on ice for 30 minutes. The lysate was then sonicated and then spun at 16,100 x g at 4°C for 15 minutes to clarify the lysate. The cleared lysate was then filtered through a 0.22µm filter.

Streptactin Purification: To the cleared lysate was added 200µl of a 50% slurry of pre-washed and equilibrated Streptactin Beads and rotated at 4°C over night. The beads were pelleted at 1700×g for 1 minute. The pellet was transferred to an empty micro biospin column and washed three times with 500µl of wash buffer (150mM NaCl, 30mM Tris-HCl pH8.0, 0.1% Triton-X-100, 1 × Protease

inhibitor/Phosphatase inhibitor I and II). To elute the bound proteins 500µl of 1 × Desthiobiotin Elution buffer (100mM Tris pH 8.0, 150mM NaCl, 1mM EDTA, 2.5mM Desthiobiotin, 1 × Protease inhibitor/Phosphatase inhibitor I and II) was added to the column. The column was then sealed and was mixed on the rotary mixer for 20 minutes at room temperature. The column was placed over a 2ml collection tube and spun to remove the eluted proteins from the column.

FLAG Agarose Purification: The eluate was placed in a fresh micro biospin column and 150µl of anti-FLAG agarose beads (50% slurry of pre-washed and equilibrated with 1 × desthiobiotin elution buffer) was added. It was then rotated at 4°C for 2 hours to allow binding of SF-Claspin to the FLAG beads. The eluate was then discarded and the beads collected and washed three times with wash buffer and once with 1×TBS. On the last wash the remaining wash buffer was removed by doing a final spin with no buffer in the column or collection tube. The column was resealed and 200µl of FLAG peptide (200µg/ml in TBS) was added to elute the proteins off the beads. This was mixed on the rotary mixer for 20 minutes and then spun to elute the proteins. The sample was then concentrated by vacuum drying, resuspended in loading buffer and the proteins were separated on a 10% Novex Gel. The gel was then stained with Simply Blue Safe Stain to visualise the purified proteins.

Bands of interest were analysed by mass spectrometry by the Beatson Institute Proteomics Facility. Briefly the gel pieces were excised and digested with sequencing grade trypsin. The resulting peptides were subject to LC-MS/MS using an Ultimate 3000 capillary LC system (Dionex) interfaced to a QSTAR XL hybrid quadrupole time of flight tandem mass spectrometer (Applied Biosystems). The data generated was compared with the data in the IPI_gallus_v340 database. Multiple reaction monitoring (MRM) analysis was also employed using the 4000 QTRAP instrument to validate and relatively quantify phosphorylation sites.

2.2.2.10. Kinase Assay

SF-Claspin was translated using the IVT system from Promega as described in section 2.2.2.6. The tagged claspin was then bound to Streptactin Sepharose beads as described in section 2.2.2.9 but only using 20µl of beads. (Purified beads from a blank lysate were used as a negative control.) This purified Claspin

was washed with 1 × Kinase assay buffer and then used as the substrate for the kinase assay. The Chk1 kinase was commercially available recombinant active Chk1 and was diluted to an appropriate concentration in dilution buffer (20mM HEPES, 1mM EDTA, 0.01% Brij 35, 5% Glycerol, 0.1% 2-mercaptoethanol, 1mg/ml BSA). Reactions were carried out by assembling the appropriate amounts of substrate and kinase and bringing the final volume up to 10µl. 10µl of 2 × Kinase assay buffer (100mM HEPES, 20mM $MgCl_2$, 1.6mM DTT, 1.6mM EDTA, 20mM ß-glycerophosphate) was then added to each sample. To start the kinase assay 2µl of 1mM ATP was added and the reaction was incubated at 30°C for 1 hour. To stop the reaction 10µl of 3×SDS Loading buffer was added and the samples were boiled for 5 minutes. The proteins were then analysed by SDS-PAGE and western blotting as described previously.

To monitor the incorporation of phosphate the 1mM ATP solution was supplemented with a 1:30 dilution of γ-^{32}P-ATP (3000Ci/mmol 10mCi/ml) and processed as above. The samples were separated on a 6% SDS-PAGE and transferred to nitrocellulose membrane. This membrane was then exposed to X-ray film in an autoradiography cassette fitted with intensifying screens.

2.2.3. BIOINFORMATICS

2.2.3.1. DNA Sequence analysis

Routine DNA sequence analysis (restriction site mapping, generation of cloning strategies etc) and figure generation was carried using Vector NTI. DNA chromatogram analysis and sequencing analysis was carried out in Contig Express. Comparison between 2 nucleotide sequences was carried out using Align X. Identification of unknown sequences was carried out using BLAST, NCBI. Alignment of mRNA to genomic DNA was carried out using Spidey, NCBI.

2.2.3.2. Multiple Sequence Alignment

Multiple Sequence analysis and similarity plots were carried out using Align X program which is based on the ClustalW method. The residues in the output file were shaded according to similarity. The following Claspin orthologue sequences were used in the analysis: Chicken – conceptual translation of isolated chicken Claspin mRNA; Human - NP_071394.2; Mouse - NP_780763.2, Xenopus -

NP_001082041; Zebrafinch - XP_002195688.1; Cow - XP_585130.4; Chimp - XP_513311.2; Opossum - XP_001380658;

2.2.3.3. Tertiary Structure Prediction

The tertiary structure of Claspin was predicted using the Phyre Protein Fold recognition Server. (http://www.sbg.bio.ic.ac.uk/phyre)

2.2.3.4. Isoelectric Point (pI) Plot generation

The entire protein sequence was divided up into 50 amino acid peptides over-lapping by one amino acid using the Peptide Screening tool on the GeneScript Website (http://www.genscript.com/peptide_screening_tools.html). The pI of each of these individual peptides was calculated using the following tool - http://www.ebioinfogen.com/biotools/protein-isoelectric-point-calculator.htm. The pI of the peptide was plotted against the residue number (corresponding to the residue number of the central amino acid of each peptide) using Microsoft Excel.

2.2.4. CELL BIOLOGY

2.2.4.1. Tissue Culture

2.2.4.1.1. Culturing Suspension Cells (DT40/Cl18)

DT40 cells (ATCC CRl-2111) were cultured in high glucose DMEM containing pyruvate supplemented with 10% FBS, 1% heat inactivated chicken serum, 2mM Glutamine, 10μM β-mercaptoethanol, 50U/ml penicillin G and 50μg/ml Stereptomycin. The cells were grown in a humidified incubator at 39°C. Cells were passaged by simply diluting 1:10-1:20 into fresh media every 2-3 days to maintain the cells in exponential growth phase. Cl18 DT40 cells (a surface IgM negative variant DT40 cell line (Buerstedde et al, 1990) obtained from N Lowndes, NUI Galway) were also grown at 39°C in RPMI1680 supplemented with 10% FBS, 1% heat inactivated chicken serum, 2mM Glutamine and 50U/ml Penicillin G and 50μg/ml Streptomycin. These cells were diluted 1:100 every 2 days to keep them in exponential growth.

2.2.4.1.2. Culturing Adherent Cells (DF-1, CEF, HEK293-Tet Off)

DF-1 cells (CRL-12203) are immortalised non-transformed chicken embryo fibroblasts. They were grown at 39°C in high glucose DMEM containing pyruvate supplemented with 10% FBS and 4mM Glutamine. The cells were passaged at a ratio of 1:10-1:20 every 3-4 days.

The secondary Chick embryo fibroblasts were derived from frozen stocks of early passage primary fibroblasts that were previously isolated from 10 day old chicken embryos. The cells were grown at 39°C in high glucose DMEM containing pyruvate supplemented with 10% FBS, 10% tryptose phosphate broth, 1% heat inactivated chicken serum, 2mM Glutamine, 50U/ml Penicillin G and 50µg/ml Streptomycin. The cells were passaged at a ratio of 1:10 – 1:20 every 2-3 days.

The HEK293 Tet-Off cells (Clontech) were grown in at 37°C in high glucose DMEM containing pyruvate supplemented with 10% FBS and 2mM Glutamine. The cells were passaged at a ratio of 1:10 1:20 every 3-4 days.

2.2.4.1.3. Passaging adherent cells

Once the cells had reached confluence an aliquot of the cells was transferred to a new flask to allow continued growth of the culture. The media was first aspirated from the cells and they were then washed three times with 10mls of pre-warmed PBS. The cells were then washed with 10mls of pre-warmed PE. 1ml of pre-warmed 0.25% trypsin in PE was added to the flask (the volume of trypsin depends on the size of the flask/dish, 1ml is sufficient for a T75 flask) and the trypsin solution was distributed evenly over the surface of the flask/dish. The flask was then returned to the incubator for 2-3 minutes until the cells had detached form the plate. Once detached, 10mls of fresh pre-warmed media was added to the cells to inactivate the trypsin. An aliquot of this cell suspension was then added to a flask containing fresh media. The cells were then returned to the incubator to allow re-attachment of the cells. (Note: HEK293 Tet-Off cells are not very adherent and require 2-3 days before the cells attach to the plate following sub-culture.)

2.2.4.1.4. Cryogenic Preservation of Cell lines

For long term storage of cells, log phase healthy cells were trypisinsed if necessary and then resuspended in 90% FBS/10% DMSO and divided into 500µl aliquots in 1.5ml cryovials. Initial freezing was carried out in a Mr Frosty container (containing isopropanol) at -70°C to give a cooling rate of 1°C/minute. Once a temperature of -70°C was reached the cells were transferred to storage in liquid nitrogen vapour phase tanks at -180°C. To revive the cells the vials were retrieved and were quickly warmed up to 37°C by placing in a container of warm water. Once thawed the cells were added to pre-warmed media. The following day the cells were passaged or the media was changed depending on the confluency of the cells.

2.2.4.1.5. Counting Cells

Cells were counted using the automated Casy® Cell Counter and Analyser System (Innovatis). The appropriate dilution of cells (either directly for suspension cells or following trypsinisation for adherent cells) was automatically counted by the machine set to exclude debris from the calculation. This also allows for easy determination of the viability of the culture. For growth curve analysis measurements were taken in triplicate.

2.2.4.1.6. Transient Transfection of cells using Lipofectamine 2000

Lipofectamine 2000 was used in order to introduce siRNA or plasmid DNA into cells following manufactures instructions. Briefly the cells were either set up the day before transfection to be 90% confluent (for plasmid transfection) or 30-40% confluent on the day of transfection. The cells were set up in antibiotic free medium. Separately an appropriate amount of plasmid/siRNA and Lipofectamine 2000 was diluted in OptiMEM. For plasmid transfections the ratio of Lipofectamine 2000 to plasmid was typically 2.5µl:1µg and for siRNA it was 10µl:200pmol. Following a 5 minute incubation the diluted Lipofectamine 2000 and plasmid/siRNA was mixed and complexes were allowed to form for 20 minutes. The lipid – plasmid/siRNA complexes were then applied to the cells and the dish rocked gently to ensure even coverage of the cells with the complexes. Cells were returned to the incubator and harvested at the appropriate time to check for expression or knock-down.

2.2.4.1.7. Transient Transfection of Cl18 cells with plasmid DNA using Nucleofection

Cl18 DT40 cells were transiently transfected with plasmid DNA using the Amaxa nucleofection kit T as per manufacturer's instructions. Cells were passaged the day before to ensure that they were in log phase on the day of transfection. Per nucleofection 1×10^6 cells were mixed with 100µl of pre-warmed supplemented Solution T and 10µg of plasmid DNA. This was then mixed gently and transferred to an electroporation cuvette and subject to programme B-0023. The cells were then transferred to a 3-5mls of pre-warmed media and returned to the incubator.

2.2.4.1.8. Transient Transfection of DT40 cells with siRNA using Nucleofection

DT40 cells were transfected with siRNA using the Amaxa nucleofection kit L as per manufacturer's instructions. Cells were passaged the day before to ensure that they were in log phase on the day of transfection. Per nucleofection 1×10^6 cells were mixed with 100µl of pre-warmed supplemented Solution L and 100pmol of siRNA. This was then mixed gently and transferred to an electroporation cuvette and subject to programme B-0023. The cells were then transferred to a 3-5mls of pre-warmed media and returned to the incubator. The siRNA sequences are given in Table 2-4.

2.2.4.1.9. Targeted integration of targeting vector – DT40-MCM Cells

Cells to be transfected were set up the day before at 1×10^5 cells/ml. On the day of the transfection 5×10^6 cells were pelleted and resuspended in 500µl of supplemented Solution T (Amaxa) at room temperature. 5µg of linearised targeting vector DNA (purified by EtOH precipitation) was added and the Cells/DNA/Solution T mixture was divided amongst 5 nucleofection cuvettes. The cells were then subject to program A-0023. Immediately all the cells were pooled into 20mls of pre-warmed media. The cells were allowed to recover overnight at 39°C. The following day the total volume of media was brought up to 140mls and selective drug (G418 at 2mg/ml or Blasticidin-S at 30µg/ml) was added along with Amphotericin B at 2.5µg/ml. The cells were divided amongst 7 × 96 well plates using a multi-channel pipette. Colonies were visible after 2-3 weeks. Cells were screened for GFP expression using an Olympus CKX41 microscope.

2.2.4.1.10. Targeted integration of targeting vector – Cl18 Cells

Cells to be transfected were set up the day before at 1×10^5 cells/ml. On the day of the transfection 1×10^7 cells were pelleted and resuspended in 500µl of ice cold PBS. 30µg of linearised targeting vector DNA (purified by EtOH precipitation) was added and the Cells/DNA/PBS mixture was transferred to a pre-chilled 0.4cm cuvette. The cells were incubated on ice for 10 minutes then electroporated using a Biorad Gene Pulser II at 550V, 25µF at infinite resistance. The time constant is usually in the order or 0.8-1ms. The cells were returned to ice for a further 10 minutes and then added to 20mls of pre-warmed media. The cells were allowed to recover overnight at 39°C. The following day the total volume of media was brought up to 140mls and selective drug (G418 at 2mg/ml or Blasticidin-S at 30µg/ml) was added along with Amphotericin B at 2.5µg/ml. The cells were divided amongst 7 × 96 well plates using a multi-channel pipette. Colonies were visible after 7-10 days. Cells were screened for GFP expression using an Olympus CKX41 microscope.

2.2.4.1.11. Generation of stable DT40 cell lines using the Flp-In T-Rex system

This system from Invitrogen was developed in DT40 cells by Mark Walker. The cells were engineered to contain at least 2 stably integrated FRT sites in the genome. This allows Flp recombinase mediated site directed recombination of plasmid DNA containing FRT sites. In addition the cells also express the Tet Repressor which represses transcription of transgenes which contain a Tet operator sequence in their promoter. The transgene of interest is cloned into a plasmid containing TetO sequences or not depending on whether constitutive or regulated expression is desired (pcDNA5/FTR or pcDNA5/FRT/TO). This plasmid contains an FRT site down stream of which is a cDNA encoding for resistance to Hygromycin but missing the ATG start codon and a promoter. Upon targeted recombination of the FRT sites the hygromycin resistance gene is placed downstream of a promoter and regains the ATG start codon resulting in cells that are resistant to hygromycin allowing for selection of the clones. Random integration of the plasmid will not convey hygromycin resistance.

The Flp-In T-Rex cells were set up the day before at 1×10^5 cells/ml. On the day of the transfection 5×10^6 cells were pelleted and resuspended in 500µl of supplemented Solution T (Amaxa) at room temperature. 0.5µg of circularised

pcDNA5/FRT or pcDNA5/FRT/TO and 4.5µg of a Flp recombinase expression plasmid (pOG44) was added and the Cells/DNA/Solution T mixture was divided amongst 5 nucleofection cuvettes. The cells were then subject to program A-0023. Immediately all the cells were pooled into 20mls of pre-warmed media. The cells were allowed to recover overnight at 35°C. The lower temperature allows the Flp Recombinase (derived from yeast) to work. The following day the total volume of media was brought up to 140mls and Hygromycin was added to a final concentration of 1.5mg/ml. The cells were divided amongst 7 × 96 well plates using a multi-channel pipette. The plates were placed at 35°Cfor a further 2 days and then transferred to 39°C. Colonies were visible after 2-3 weeks

2.2.4.1.12. Expansion of drug resistant DT40 clones

Following the selection process clones were visible by eye in the 96 well plates. They could also be seen by a yellowing of the media in wells where clones were growing. Wells where clones were grown were inspected under the microscope to ensure no other clones were growing in the same well. Cells from wells with single clones in them were resuspended and then added to 800µl of fresh media in a 24 well plate. When the cells had reached confluence 500µl of the cells was added to 2.5mls of fresh media in a 6 well plate. Following this the cells were transferred to T25 flasks and larger. An aliquot of the cells from the clones was frozen down as soon as possibly to prevent in vitro evolution. Clones were usually maintained under antibiotic selection until they had reached the T25 stage.

2.2.4.1.13. Luciferase Assay

Cells to be assayed were transfected with 7.5µg of pTRE-Tight Luciferase (Clontech) and 0.25µg of pTK Renilla (Promega) by nucleofection as described. After 24 hours the cells were assayed for the presence of luciferase according to manufacturer's instructions. Briefly the cells were washed once in PBS and then lysed in 1 × Passive Lysis Buffer with gentle agitation on an orbital shaker for 15 minutes. In 10µl of each sample was placed into a 96 well plate and the luciferase activity (both Firefly and Renilla) was read using a Glomax Luminometer. 50µl of LAR II reagent and Stop and Glo Reagent was used with an integration time of 10 seconds. The data was normalised to take into account

the transfection efficiency (Firefly/Renilla) and the amount of protein between samples.

2.2.4.1.14. Irradiating Cells

As a method of inducing DNA damage cells were treated with either γ-IR or UV-C light. Cells were irradiated with γ-IR using an Alcyon II Cobalt-60 Teletherapy Unit. Dose rates varied from 1-2.5Gymin^{-1}. Cells were irradiated directly in the media in the culture flask. Control cells were brought to the Co-60 source but were not exposed to the ionising radiation. Cells were treated with UV-C light using a Stratalinker 1800 (Agilent Technologies, Stockport, UK) containing 254nm bulbs set to deliver 5000μJcm^{-2} (50Jm^{-2}). Prior to UV-C exposure the DT40 cells were first washed with PBS and placed in tissue culture plate suspended in a small volume of PBS. The cells were returned to full media following irradiation.

2.2.4.2. Flow Cytometry

2.2.4.2.1. Fixing cells

Cells were treated as required and pelleted at 250×g for 5 minutes in 15ml polystyrene tubes. The resulting pellet was then resuspended in 200μl of ice cold PBS. While vortexing, 2mls of ice cold 70% Ethanol was added drop wise to fix the cells. This minimises the formation of clumps and ensures uniform fixing of the cells. Fixed cells were stored at 4°C overnight or at -20°C for a couple of hours to several weeks before further analysis.

2.2.4.2.2. DNA Content

Cells were fixed as described and stored at -20°C for at least 30 minutes. The cells were pelleted and resuspended in 1ml of PBS containing 10μg/ml Propidium Iodide and 250μg/ml RNase A. The cells were stored in the dark for 30 minutes before analysis on the flow cytometer.

2.2.4.2.3. S phase

To monitor DNA replication, cells were tested for their ability to incorporate the thymidine analogue BrdU into their DNA. Cells were treated as required and 15 minutes before harvest 25μM BrdU was added to the culture. Cells were then fixed as above and stored until use. The cells were centrifuged at 250×g and the ethanol was removed. The cells were resuspended in 1ml of PBS and then 1ml of

4M HCl was added. The samples were incubated at room temperature for 15 minutes. The HCl treatment denatures the DNA and exposes the BrdU epitopes that are recognised by the anti-BrdU antibody. The cells were then washed once with PBS and once with PBT (0.5% BSA, 0.1% Tween 20 in PBS). The resulting pellet was resuspended in 200µl of PBT containing a 1:40 dilution of the anti-BrdU antibody and incubated at room temperature for 30 minutes. The cells were then washed with 1ml of PBT and resuspended in 200µl of PBT containing a 1:40 dilution of the FITC conjugated anti-mouse antibody. The cells were incubated for 30 minutes in the dark and then washed with 1ml of PBT. The cell pellet was then resuspended in 1ml of PBS containing 10µg/ml Propidium Iodide and 250µg/ml RNase A. The cells were stored in the dark for 30 minutes before analysis on the flow cytometer. Data was collected on a FACSCalibur flow cytometer and the data analysed using WinMDI software.

2.2.4.2.4. Mitosis

The number of mitotic cells was estimated by counting the number of cells that were positive for the mitosis specific marker, pS10 Histone H3, by flow cytometry. Cells were treated as required, then fixed as above and stored until use. The cells were centrifuged at 250×g and the ethanol was removed. The cells were then resuspended in 1ml of PBS containing 0.25% Triton-X-100 and incubated on ice for 15 minutes. The cells were then pelleted and resuspended in 100µl of 1% BSA in PBS containing a 1:50 dilution of the anti-pS10 H3 antibody followed by a 90 minute incubation at room temperature. The cells were washed once with 1% BSA in PBS and then resuspended in 100µl of 1% BSA in PBS containing a 1:30 dilution of the FITC conjugated anti-rabbit antibody. The cells were incubated for 30 minutes in the dark and then washed with 1ml of PBS. The cell pellet was then resuspended in 1ml of PBS containing 10µg/ml Propidium Iodide and 250µg/ml RNase A. The cells were stored in the dark for 30 minutes before analysis on the flow cytometer. Data was collected on a FACSCalibur flow cytometer and the data analysed using WinMDI software.

2.2.4.3. Metaphase Spreads

The day before analysis the cells were set up at 2×10^5 cells/ml. 2 hours before harvesting the cells were treated with Demecolcine at 0.1µg/ml to arrest cells in metaphase. The cells were then harvested by centrifugation at 250 × g for 5

minutes. To swell to cells they were resuspended in 20mls of 75mM KCl for 20 minutes and were then pelleted at 250 × g for 5 minutes. To fix the cells the resulting pellet was incubated with 20mls of freshly prepared fixative (ice cold methanol : acetic acid (3:1)). The cells were washed a further 4 times in fixative each time reducing the volume of fixative to 10mls, 5mls, 2mls and finally 1ml. The cells could be stored in fixative at -20°C for weeks. Glass slides were cleaned by incubating in methanol containing a few drops of 1M HCl for 5 minutes – then wiped dry with lint free tissue. The cells were 'splatted' onto glass slides by dropping them from a height for approximately 30cm onto the slide propped at an angle of approximately 30°. Once the fixative appeared to be drying out fresh fixative was dropped onto the slide to aid with the spreading of the chromosomes. The slides were then allowed to dry and age overnight. A few drops of Vectashield mounting medium containing DAPI was added and covered with a cover slip and sealed with nail varnish. The slides were visualised on a Zeiss Axioskop fluoresence microscope with an ×63 oil objective and analysed using Axiovision Software.

2.2.4.4. FISH

Metaphase spreads were prepared as described above. To prepare the slides for hybridisation the cytoplasm was first removed by incubation in 70% acetic acid for 10-20 s. The slides were dehydrated in an ethanol series – 70%, 80% and 100% for 5 minutes each and then allowed to air dry. They were then incubated at 55°C for 3 hours and passed through the same ethanol series, incubating the slides for 2 minutes this time. The slides were air dried and 95µl of RNase mix (100µg/ml RNase A in 2×SSC) was added to each slide. A 22×50mm coverslip was placed on top and the slide incubated at 37°C for 1 hour in a humidified chamber. The slide was then washed in 2×SSC for 5 minutes each. The slides were dehydrated in the ethanol series for 2 minutes each in 70%, 80% and finally 100% Ethanol and allowed to air dry. The slides were denatured for 90 secs in 70% Formamide in 0.6×SSC preheated to 75°C. The slides were then dehydrated for 2 minutes each in 70% ice cold Ethanol, 80% Ethanol at room temperature and 100% ethanol at room temperature and allowed to air dry. To prepare the probe for hybridisation 1µl of biotin labelled probe (Farmachrom, University of Kent) 1µl of herring sperm DNA and 4µl of hybridisation buffer (50% Formamide, 10% Dextran Sulphate, 2×SSC) was placed in a PCR tube. This was then

incubated at 75°C for 5 minutes and then allowed to re-anneal at 37°C for 45 minutes in a PCR machine. The probe was then applied to the denatured slide and covered with an 18mm×18mm coverslip, ensuring no air bubbles were trapped. The cover slip was then sealed with rubber cement and incubated in a humid chamber for at least 16 hours to facilitate hybridisation. The cover slip was removed and the slide was washed twice in 50% Formamide, 1×SSC at 37°C for 10 minutes. The slide was then placed in 2×SSC with 0.1% Igepal CA 630 for 1 minute at room temperature. The slide was then incubated in storage buffer (4×SSC, 0.05% Igepal CA 630) for 15 minutes and then into block buffer (4×SSC, 0.05% Igepal CA 630, 2% BSA) for 25 minutes at room temperature. 95µl of detection mix (0.5µl Streptavidin-Cy3 + 100µl 4xSSC, 0.05% Igepal CA 630, 1.5% BSA) was placed on each slide and covered with a 22mm×50mm coverslip. It was then incubated at 37°C in dark humid chamber for 35 mins. The coverslip was then removed and the slides washed 3 times for 3 minutes each in the dark with storage buffer with gentle agitation on an orbital shaker. The slide was then allowed to air dry. The slides were then counterstained with Vectashield containing DAPI to visualise the chromosomes, covered with a coverslip and sealed with nail varnish. The slides were visualised on a Zeiss Axioskop fluoresence microscope with an oil objective (×63) and analysed using Axiovision Software.

Chapter 3. Characterisation of Avian Claspin

3.1. Introduction

The principal aim of this project is to generate a cell line which is genetically deficient for Claspin expression. To this end the recombinogenic cell line, DT40, will be used. As the DT40 system is chicken in origin, the chicken Claspin (cClaspin) homologue must first be cloned and characterised. The description of this process is contained in this chapter.

3.2. Isolation of avian Claspin cDNA

When this project commenced in 2005 both Xenopus and Human CLSPN genes had been sequenced[1] (Kumagai & Dunphy, 2000). The first draft of the Chicken Genome Sequence had also just been released (Hillier et al, 2004). A BLAST search of the chicken genome sequences with human claspin mRNA sequence returned an mRNA sequence (XM_425782.1) that was similar to the human sequence, with the sequences sharing 42% overall identity. However this chicken CLSPN mRNA was approximately 1.33 times bigger than the human sequence with only the first $^2/_3$ of the mRNA homologous to human claspin and the remaining $^1/_3$ of the gene not homologous to any known human genes. (Figure 3.1-A) The conceptual translation of both the chicken and human genes showed that the resulting proteins shared 30% identity and 36% similarity at the amino acid level over the entire length of both proteins. When the non-homologous region of cClaspin was removed from this calculation, the % identical residues and % similar residues increased to 45% and 55% respectively. This strongly indicated that this sequence was the true sequence of the cClaspin homologue, albeit larger than expected. In order to confirm this predication it was necessary to isolate this mRNA sequence. The cDNA would also prove useful in the characterisation of cClaspin.

3.2.1. Uni-ZAP® XR cDNA Library Screen

The first approach taken in order to clone the full length cClaspin cDNA was to screen a commercially available Uni-ZAP® XR Chicken cDNA library. (Figure 3.1-B) The library was constructed from oligo dT primed cDNA isolated from secondary embryonic fibroblasts, cloned uni-directionally into the Uni-ZAP® XR

[1] Genbank accession number: Xenopus - AF297867; Human - AF297866

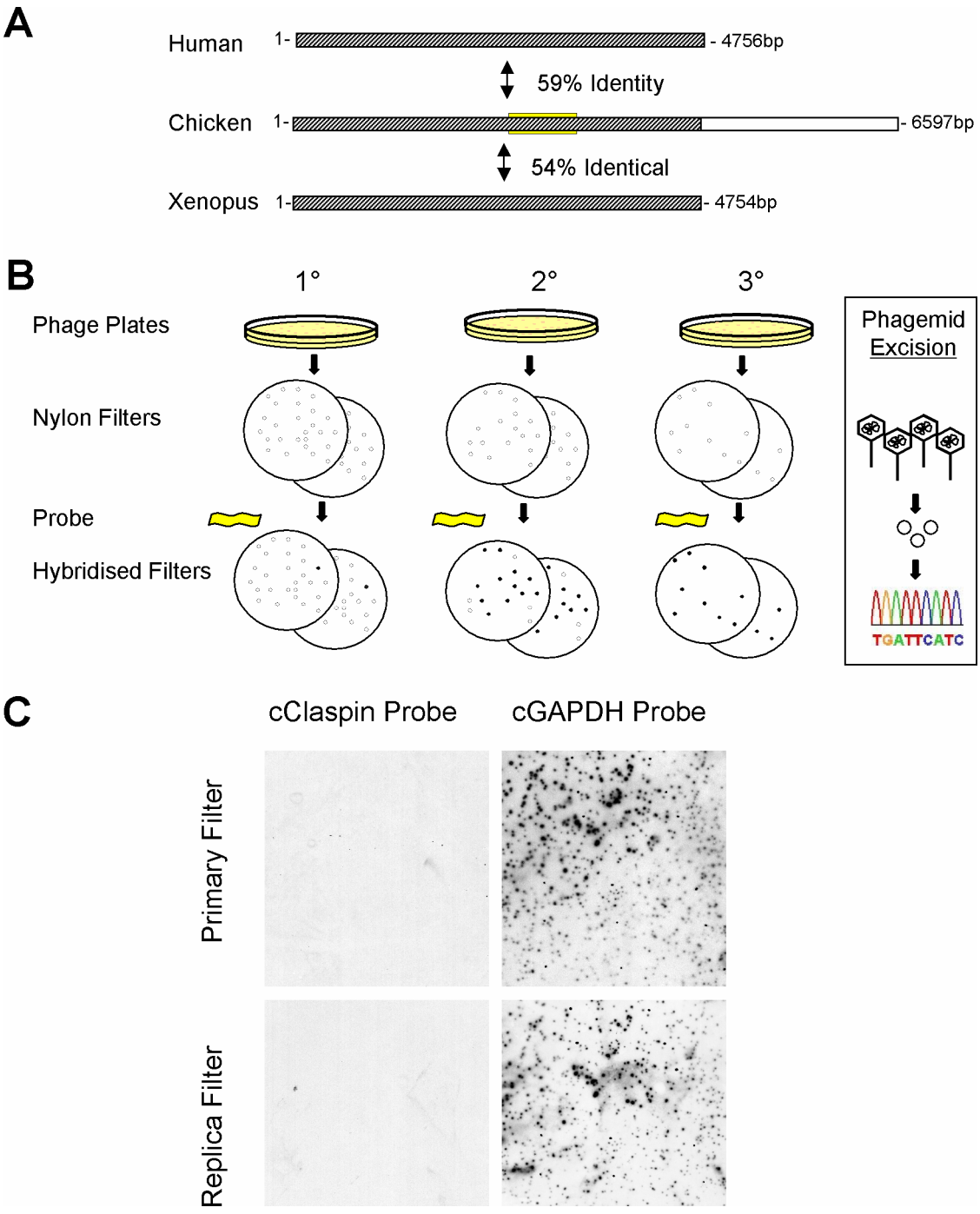


Figure 3.1 Lambda ZAP cDNA library screen
A) Schematic representation of the Human, Chicken and Xenopus mRNA sequences. Numbers indicate the % identity shared. The unshaded region shows the region of the cCLSPN mRNA not homologous to human or xenopus CLSPN. The location of the probe used to screen the library is indicated in yellow. B) Outline of Lambda ZAP cDNA Library screening. Primary screening allows the identification of few plaques containing the cDNA of interest (black dots) from a large number of plaques. Following phage purification the phagemid is excised and the cDNA sequenced. C) Representative films obtained when screening plaque filters with the Claspin or GAPDH probe.

vector. 1.5×10^6 pfu's were screened on duplicate filters using a ^{32}P labelled fragment of CLSPN mRNA, amplified in order to generate an anti-cClaspin antibody (Section 3.5). No positive hits, as judged by identical 'dots' being present on both filters, were obtained using this probe. (Figure 3.1-C) A possible explanation for this is that the average insert size in this library is 1.0kb. The cDNA's present in the library will therefore be enriched for short mRNA's and mRNA's only containing the 3′ ends. The probe used may be too far 5′ in the cDNA to detect the CLSPN mRNA's present in this library. Alternatively given that there is a high probability that the message detectable by the probe is present in very small numbers, more plaques may need to be screened in order to identify a positive one. To confirm the integrity of the DNA on the filters I reprobed 2 filters with a chicken GAPDH probe. Many positive plaques were identified ruling out the possibility that there was a problem with the filters or the hybridisation method. (Figure 3.1-C) Due to these difficulties encountered with library screening, an alternative strategy was adopted.

3.2.2. RACE and RT-PCR

In order to obtain the sequence of the 5′ and 3′ ends of the CLSPN mRNA, Rapid Amplification of cDNA Ends (RACE) was used. This would allow me to obtain enough sequence information in order to design primers for RT-PCR so that the entire cDNA could be cloned. As the CLSPN mRNA is quite long I chose to use the SMART™ RACE Kit from Clontech (Switching Mechanism at 5′ end of RNA Transcript). This kit uses a template switching mechanism in order to attach a 5′ specific primer to the end of mRNA transcripts. This relies on the non-specific terminal transferase activity of reverse transcriptase which adds $d(C)_3$ to the 5′ ends of RNA transcripts. This terminal transferase activity is highest once at the end of the message so it preferentially allows the amplification of full length cDNA's. However, partially degraded or fragmented RNA can also be amplified using this method.

Firstly I carried out a preliminary RT-PCR characterisation of the predicted cCLSPN mRNA. Primers were designed along the full length of the predicted mRNA in both the homologous and non-homologous regions. Primer pairs that would span the gap between the homologous and non-homologous region of the predicted mRNA were also used (4F, 5R in Figure 3.2-A). The results of this RT-

PCR are shown in Figure 3.2-B. Only the portions of the gene that were homologous to the human gene could be amplified. I was unable to amplify across the gap and unable to amplify any of the predicted gene that shared no homology with human CLSPN. This led me to believe that the prediction program was incorrect and I designed 3′ RACE primers close to where the homology ended rather than at the end of the predicted gene sequence as this appears not to be expressed in cells. The RT-PCR fragments were cloned and sequenced and the RACE primers were designed based on this sequence.

For the 5′ RACE PCR's I designed 3 'reverse' primers towards the very start of the gene. I chose regions of the gene that when translated showed some homology with human and xenopus Claspin. 2 of these primers gave me distinct PCR products which were cloned and sequenced. (Figure 3.2-C) The sequencing data from these cloned products allowed the identification the ATG start codon, which was surrounded by a Kozak sequence (Kozak, 1987). The 5′ UTR also contained an upstream in frame stop codon, indicating that I had identified the genuine start of the gene. As this method for RACE does not select for the presence of complete mRNA transcripts that contain the 5′ 7meG cap it is possible that I have not cloned the entire 5′ UTR sequence. However enough information was obtained to facilitate the generation of an accurate intron-exon map and to clone the cDNA.

The 3′ RACE proved more difficult. Four 'forward' primers were selected from the end of the sequence data obtained by RT-PCR. However, even though these primers amplified what appeared to be specific PCR products, when sequenced they shared no homology with the claspin genomic locus. (Figure 3.2-C) On closer examination of the Claspin Genomic locus, a region of DNA was identified that, when translated, gave rise to a peptide sequence that shared very high homology with the extreme C-terminus of both Human and Xenopus Claspin. This was immediately followed by a TGA stop codon. (Figure 3.2-D) An in frame stop codon was located approximately 100bp upstream of this potential Claspin stop codon so it was postulated that the last exon would be present between these two stop codons. This potential exon contained approximately 10 suitable AG splice acceptor sites so primers for RACE were designed at the extreme 3′ end of the potential exon so as to minimise the possibility of designing primers

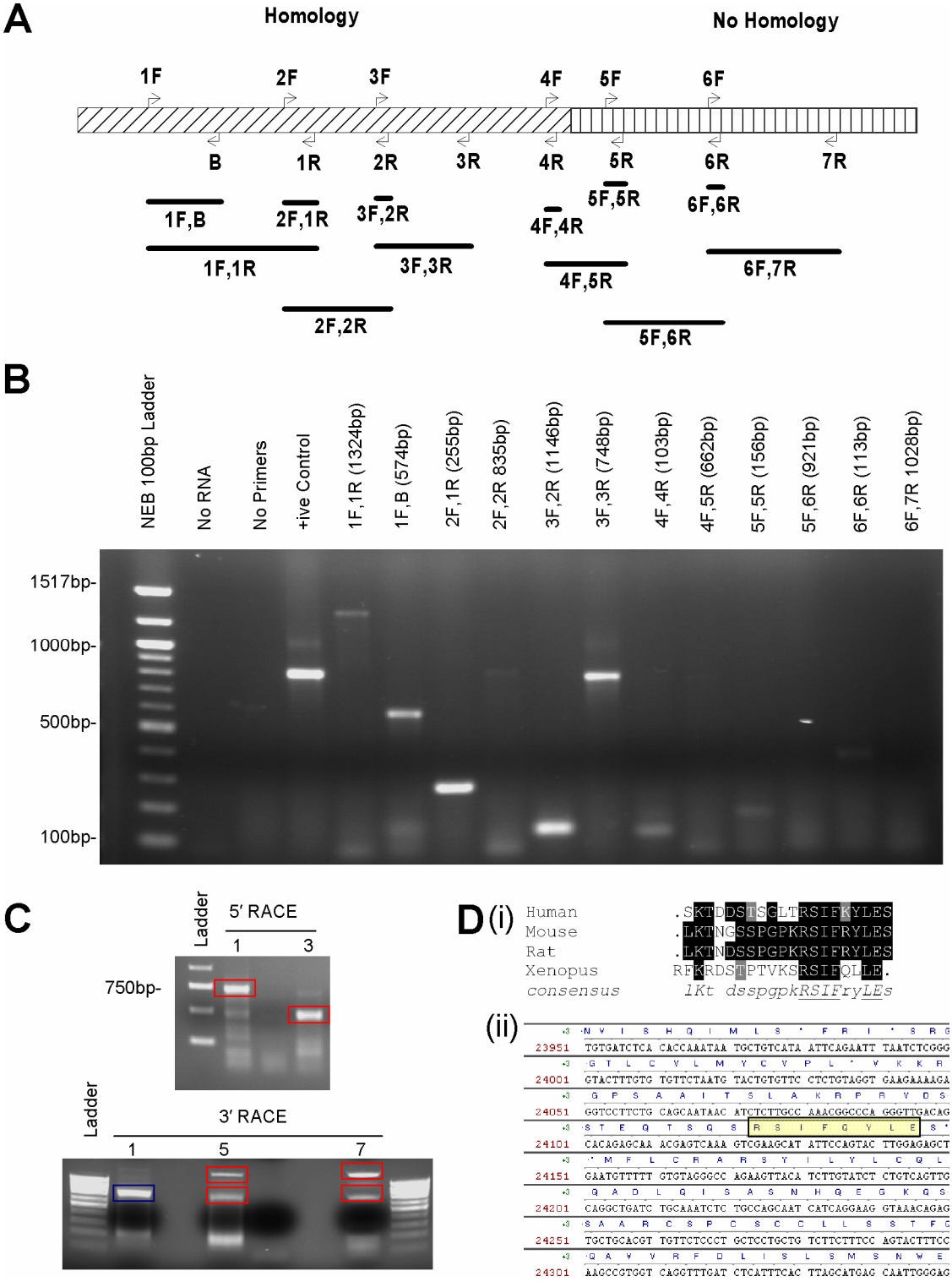


Figure 3.2 Isolation of Claspin mRNA Sequence by RT-PCR and RACE
A) Schematic representation of the location of primers designed along the predicted CLSPN mRNA sequence. B) RT-PCR results using primers illustrated in A. C) RACE products obtained. Each product obtained using a separate primer. Products boxed in red show CLSPN UTR sequence, blue shows unrelated sequence. D) (i) Alignment of C-terminal residues from indicated Claspin homologues. (ii) Highlighted area shows the identification of a homologous region to the C-terminal peptide in the cClaspin genomic locus.

across an intron-exon boundary. Using these newly designed primers I successfully amplified the last exon of the CLSPN gene. (Figure 3.2-C) The upper band contained 1165bp's of 3′ UTR sequence. The lower band also contained 3′ UTR sequence but was smaller by 626bp due to a splicing event within the UTR sequence. Both UTR's contains a polyadenylation signal 20bp upstream from the start of the poly(A) tail. Thus 2 distinct transcripts were isolated indicating a potential role for the 3′ UTR in regulating cClaspin expression.

Having obtained sequence from both ends of the mRNA, this then allowed the design of primers in order to amplify the entire cDNA. Attempts at cloning the entire cDNA in one fragment failed. Therefore 2 fragments were amplified, a 5′ fragment and a 3′ fragment that overlapped. In both cases single specific bands were obtained, indicating no alternative splicing taking place in the coding region of the gene. The overlapping region contained a unique restriction site (SfuI) which facilitated the ligation of the two fragments together in order to generate the full length cDNA. Once assembled, the cDNA was subject to 3 rounds of sequencing using primers spanning the entire cDNA. Using only the high quality sequencing data obtained, the cDNA sequence was assembled. This data was combined with the data obtained data for the UTR's to generate the sequence for entire mRNA. (Figure 3.3-A)

The mRNA sequence was then compared with the genomic DNA sequence available in the database. Some differences were observed and are summarised in Table 1.1. Most polymorphisms were silent and resulted in no change to the amino acid sequence. 3 amino acid changes were observed. At position 24 the residue encoded for by the genomic DNA sequence is a serine residue whereas in the mRNA sequence it was a glycine residue. At position 742 an asparagine residue was converted to an aspartic acid residue. As these changes should not significantly alter the structure of the protein they were left unchanged. In addition these residues were not conserved in Claspin from other species.

At position 1235 a proline residue was changed to a serine residue. As proline residues can contribute significantly to the secondary structure of a protein, and this residue is present in a highly conserved motif albeit of unknown function, this mutation was investigated further. At the nucleotide level this mutation

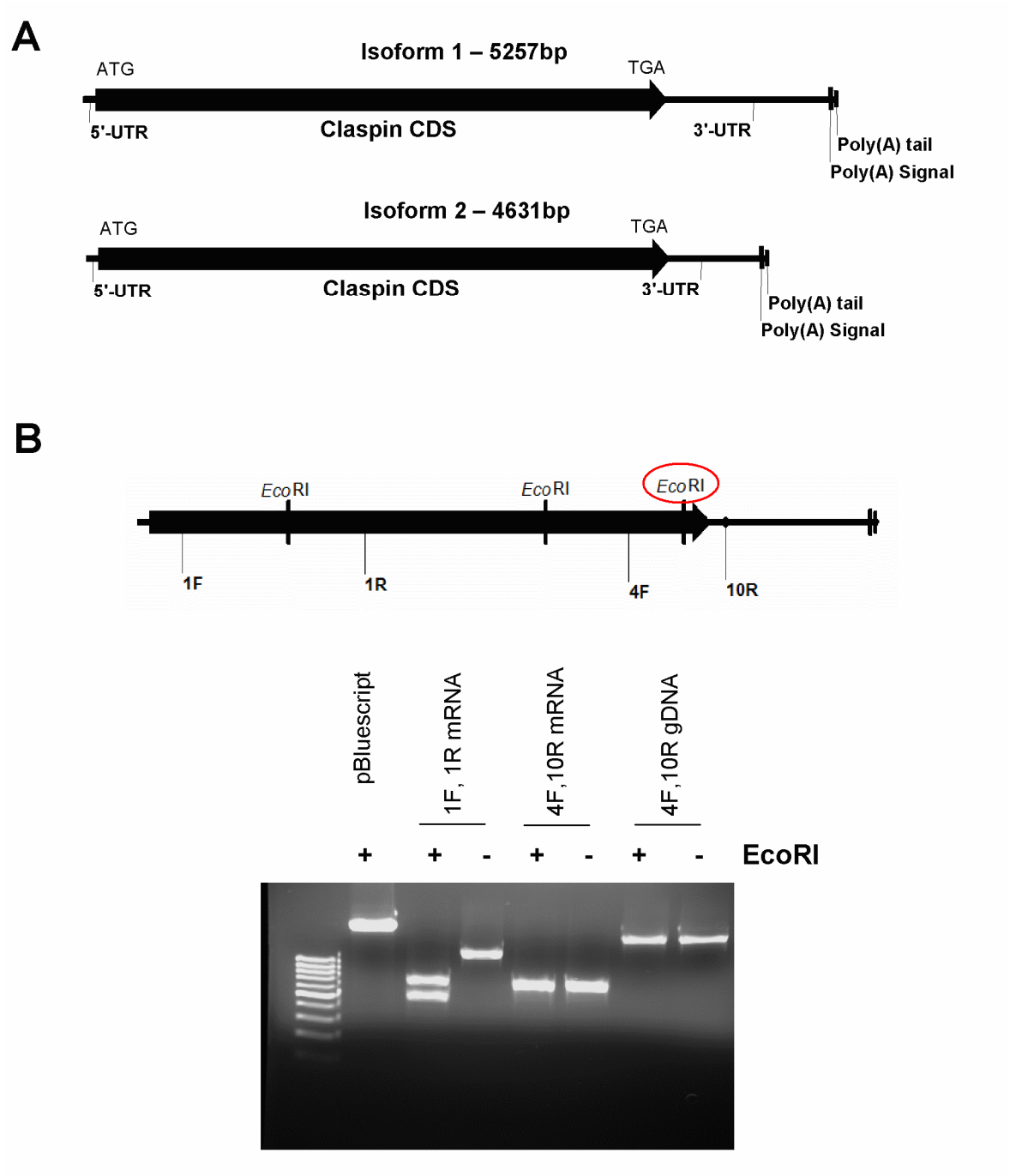


Figure 3.3 cCLSPN mRNA structure
A) Schematic representation of both cCLSPN mRNA isoforms isolated. Isoform 2 differs only in a 626bp deletion in the 3' UTR B) Investigation of P1235S mutation. The Claspin mRNA contains 2 EcoRI sites and the potential EcoRI site that would be generated if the P1235S mutation was correct circled in red. The indicated PCR products were amplified using Pfx polymerase with the primers indicated using either cDNA (RNA) or genomic DNA as the template. The products were then digested with EcoRI and analysed by agarose gel electrophoresis. pBluescript was linearised with EcoRI to confirm the activity of EcoRI. The predicted sizes of the DNA fragments are as follows: pBluescript – 2961bp; 1F,1R - 1324bp; 1F,1R + EcoRI – 762bp, 562bp; 4F,10R mRNA – 715bp; 4F,10R mRNA + EcoRI – 395bp, 320bp; 4F,10R Gen. DNA – 2026bp; 4F,10R Gen. DNA + EcoRI – 1491bp, 335bp.

Exon No.	mRNA Position	Base Change	Amino acid change
1 (5′ UTR)	1	C → G	-
1 (5′ UTR)	29	A → G	-
1 (5′ UTR)	34	delAG	-
2	126	T → C	No change
2	147	C → T	No Change
2	148	A → G	S24G
2	210	C → T	No change
7	1026	G → A	No change
8	1455	T → C	No change
12	2302	G → A	D742N
14	2540	A → G	K821R
19	3273	T → C	No change
21	3576	A → G	No change
23	3781	C → T	P1235S
25 (3′ UTR)	4841	T → C	-
25 (3′ UTR)	5189	A → G	-
25 (3′ UTR)	5193	G → T	-
25 (3′ UTR)	5256	G → A	-

Table 3-1 Differences between Claspin mRNA sequence isolated and Claspin genomic Locus sequence
mRNA position refers to base positions in isolated CLSPN mRNA isoform 1. The Claspin mRNA sequence isolated was compared with the corresponding genomic DNA sequence from the NCBI. Differences are listed.

resulted from a C->T transition. This introduced an extra EcoRI site into the DNA sequence allowing me to easily test which DNA sequence was correct. A PCR was carried out on genomic DNA and on cDNA using primers that spanned this mutation site using a high fidelity Taq polymerase. The resulting PCR products were then digested with EcoRI. If the proline residue was correct then the PCR product should be resistant to digestion by EcoRI. This turned out to be the case, indicating that the serine residue was introduced during either the reverse transcriptase step or the PCR amplification step when cloning the gene. For comparison the EcoRI digests pBluescript plasmid and a PCR product known to contain an EcoRI site, 1F, 1R. The P1236S mutation was reverted to a proline using site directed mutagenesis.

3.3. cClaspin Domain Structure

Translating the cDNA sequence isolated, gives the amino acid sequence of cClaspin. A multiple sequence alignment, comparing cClaspin, hClaspin and xClaspin shows that the proteins are highly similar sharing 78% overall homology and 35% identity. (Figure 3.4-A) (See Appendix D for a complete multiple sequence alignment of Claspin orthologues from various species) Based on this homology is it possible to map the domains already identified in xClaspin and hClaspin with the homologous sequences in cClaspin (Lee et al, 2005; Sar et al, 2004). Figure 3.5-A shows this in schematic form. The identity plot in Figure 3.5-B (based on the multiple sequence alignment in Figure 3.4-A) shows the regions of the protein that are highly conserved. The N-terminus of the protein contains the region of the protein shown to interact with DNA and chromatin. This region of the protein is not very highly conserved at the amino acid level.

The C-terminal portion of the protein has been shown to mediate Chk1 activation through phosphorylation of conserved motifs in the Chk1 activation domain. The consensus sequence for this motif is ExxLC(pS/pT)GxF. Three repeats of this motif are present in human and chicken with only two present in xenopus. Once phosphorylated this serves as a binding site for Chk1 which facilitates its activation by ATR through an unknown mechanism. The kinase that phosphorylates the S/TG residues remains unknown but is dependent on ATR. Interestingly a BRCT binding motif (pSxxF) is contained within the Chk1 binding motif (Manke et al, 2003). It has not been shown that this motif

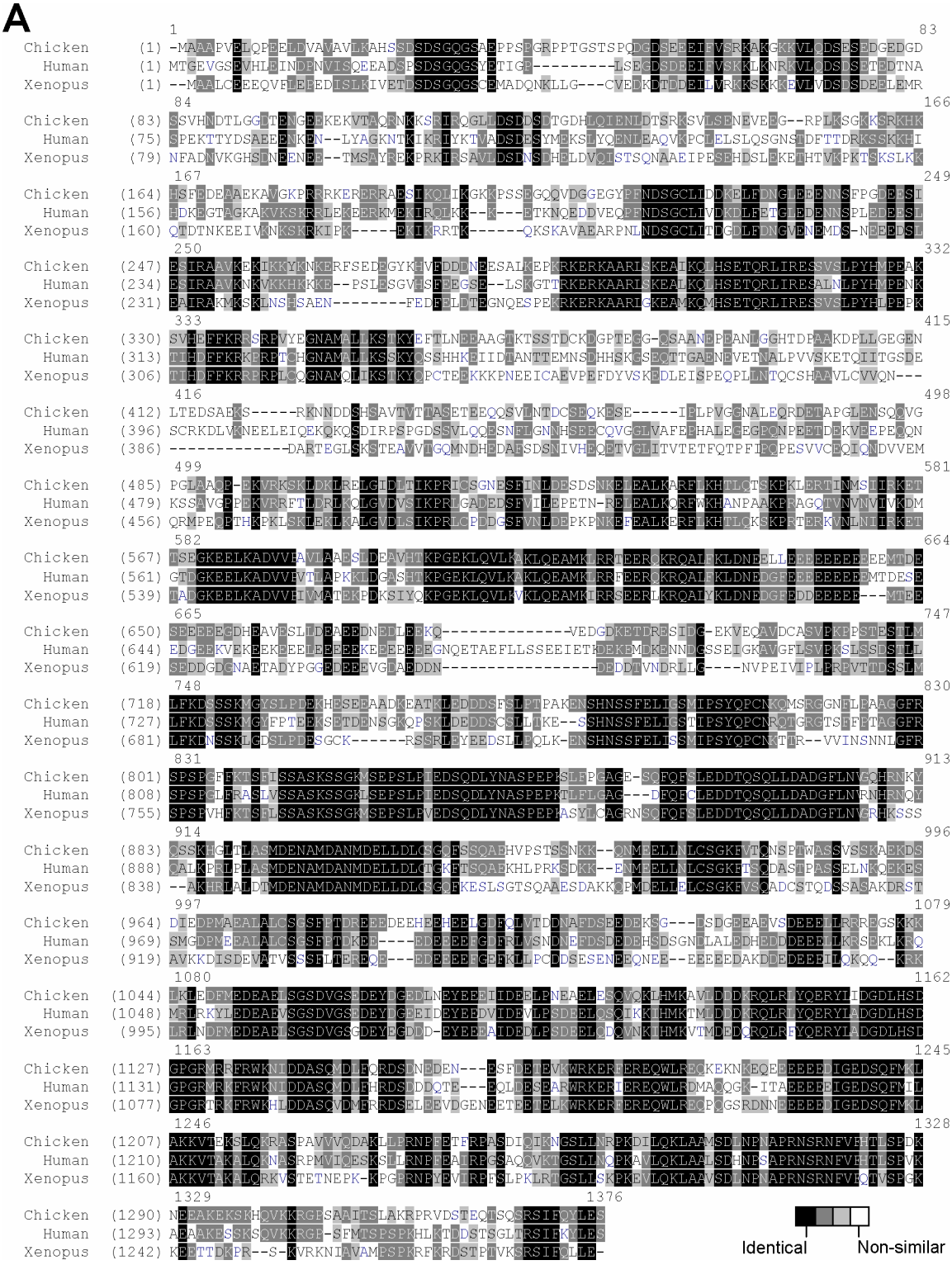


Figure 3.4 Claspin Amino Acid sequence Alignment
A) Clustal Alignment of the Human, Xenopus and Chicken Claspin amino acid sequences. The figure was generated using AlignX.

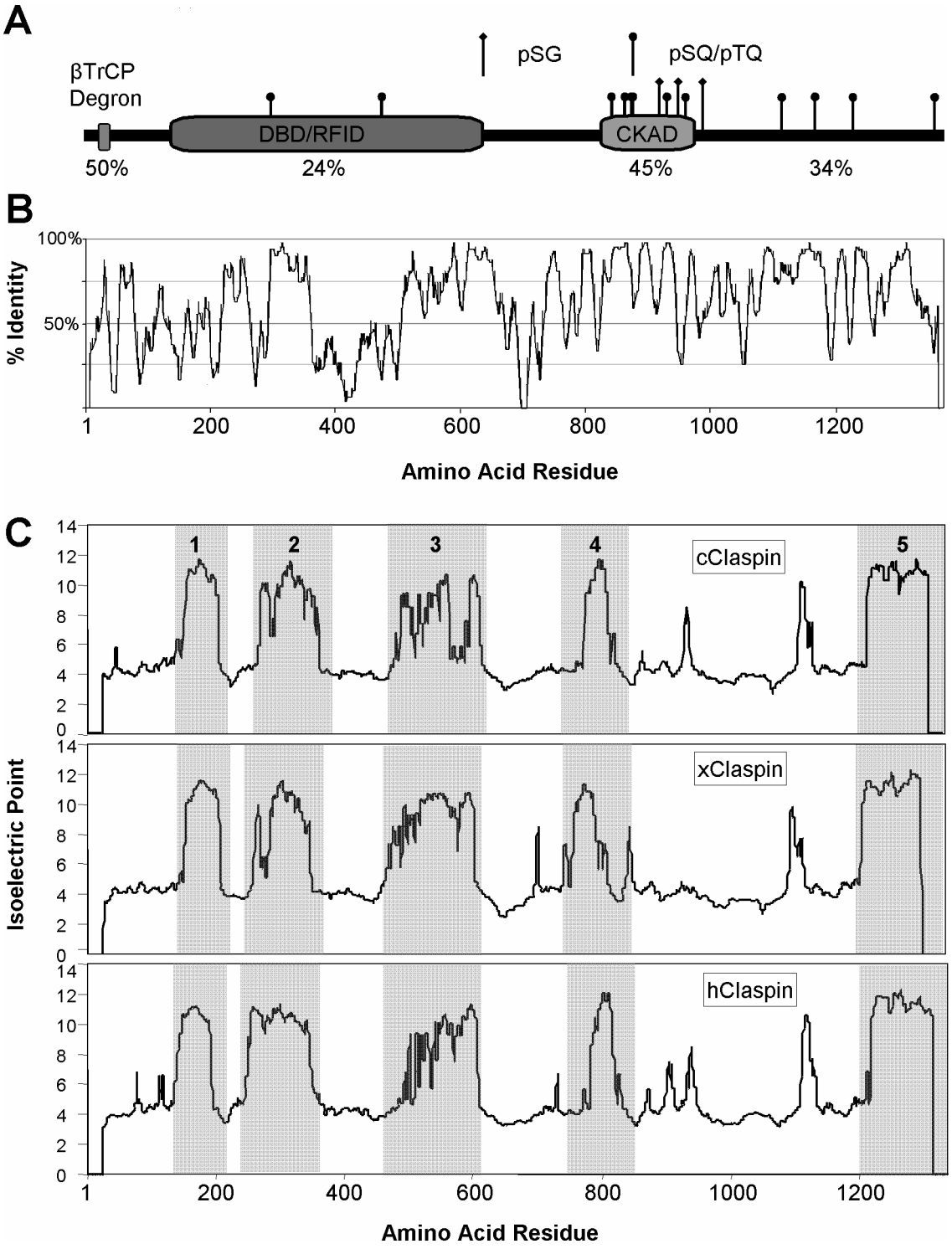


Figure 3.5 Claspin Domain structure
A) Schematic representation of the cClaspin Domain structure. Domains are based on homology with those identified in Xenopus and Human Claspin. Numbers indicate the % identity shared within the domains indicated. Values are derived from the multiple sequence alignment in Appendix D. B) Similarity plot based on chick/human/frog claspin alignment in Figure 3.4. (Window size = 10) Amino acid residues broadly mirror the domain structure present in A. C) Iso-electric Point Plot of chick/frog/human Claspin. (Window size = 50)

actually mediates any interaction between Claspin and any other BRCT containing family members, but may hint at another layer of complexity in the way Claspin mediates Chk1 phosphorylation. High sequence conservation is observed in the Chk1 Activation domain. The rest of the C-terminal portion of the protein also shares high sequence homology.

It has been noted previously that even though overall Claspin protein is quite acidic the protein contains patches of basic residues which appear to be important for the correct functioning of the protein (Lee et al, 2005). An isoelectric point plot of the protein reveals the 4 basic patches identified in this study (patches 2-5 in Figure 3.5-C) and also identifies another basic patch at the N-terminus of the protein. These basic patches are highly conserved suggesting that they play an important role in the overall structure and function of the protein.

Tertiary structure predication was attempted using the Phyre Protein Fold recognition server (Kelley & Sternberg, 2009). However no significant homology with any known structures was returned. Regions of low complexity and coiled coil regions were however identified.

3.4. Analysis of in vitro translated cClaspin

I used coupled in vitro-transcription-translation in order to confirm my cloned cDNA did indeed code for protein. The cDNA had been cloned into the pCR4-TOPO vector for cloning which contained the T7 promoter. This promoter facilitates transcription of genes by the T7 RNA polymerase. I used the T7 Quick Coupled TNT Kit from Promega, which allows the coupled transcription and translation of proteins in one tube. ^{35}S-methionine was included in the reaction mix in order to radioactively label any proteins produced. Initial attempts at translating the protein failed. (Figure 3.6-A(i)) This system is generally less efficient at translating large proteins. However by optimising the reaction by increasing the reaction time and adjusting the salt condition allowed the detection of a band by SDS PAGE which ran at approximately 250kDa. These conditions also allowed the translation of EDD, an E3 ubiquitin ligase, which has a calculated molecular weight of 309kDa. It has been reported previously that although the predicted molecular weight of Claspin is 150-160kDa, on SDS-PAGE

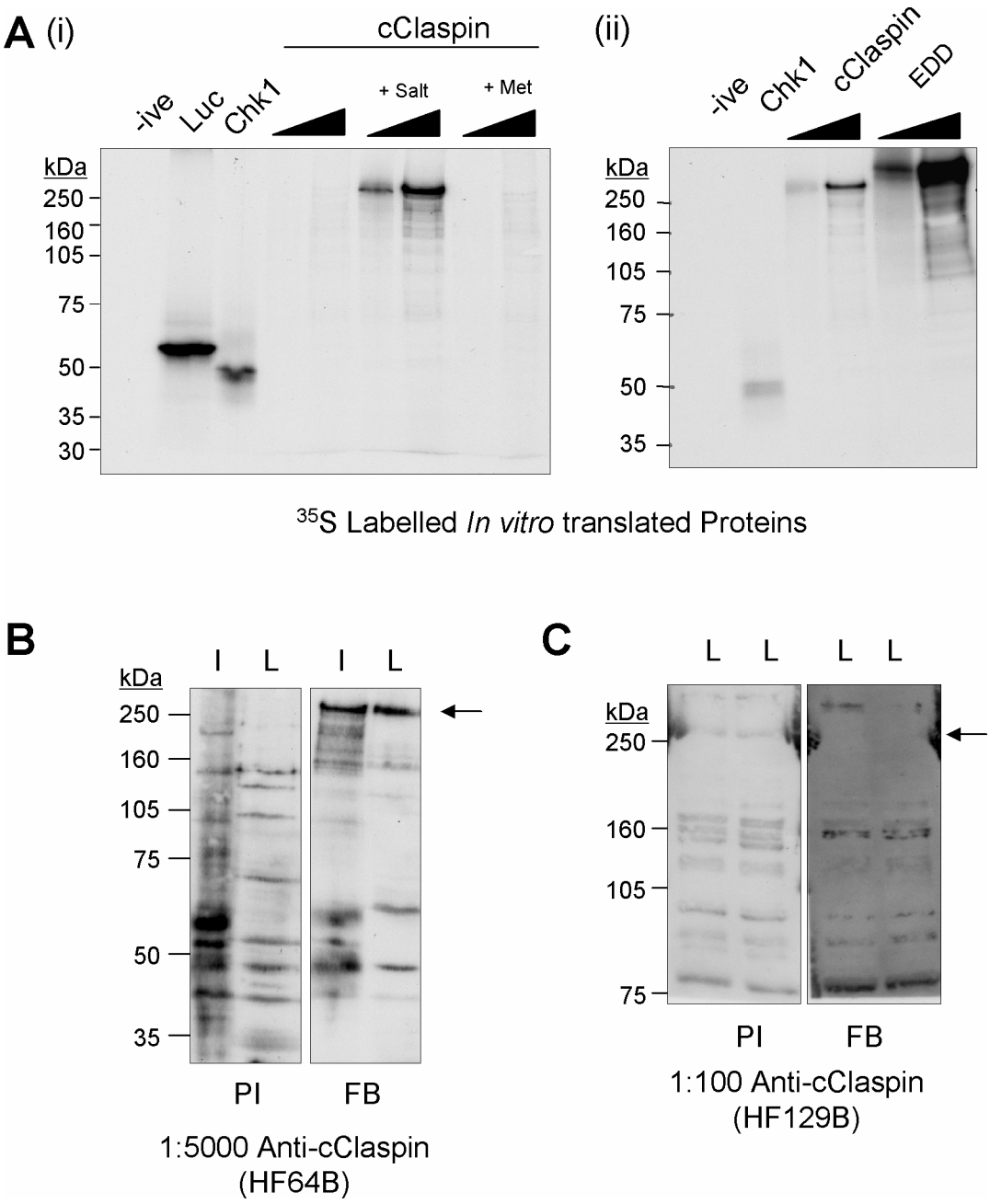


Figure 3.6 cClaspin antibody Characterisation
A) (i) Optimisation of in vitro translation of cClaspin. Standard IVT reaction facilitates the translation of Luciferase and Chk1 (Lanes 2-3) but not of cClaspin, even with increasing template (Lanes 4-5). Adding increasing amounts of Salt (PCR Enhancer, Promega) (Lanes 6-7) but not extra Methionine (Lanes 8-9) facilitates the translation of cClaspin. (ii) Optimised cClaspin in vitro translation reaction. High molecular weight proteins (including EDD, a protein twice the size of Claspin) are efficiently transcribed under these conditions. (1µg template, 1µl PCR Enhancer and a 3 hour incubation) B) Western blot characterisation of HF64B antibody using pre-immune serum (PI) and serum from the final bleed (FB) post immunisation. I = in vitro translated protein, L= DT40 cell lysate. Arrow indicates the cClaspin band. C) Western blot characterisation of HF129B antibody using pre-immune serum (PI) and serum from the final bleed (FB) post immunisation. L= DT40 cell lysate. Arrow indicates where the cClaspin band should be.

the protein migrates above 200kDa (Kumagai & Dunphy, 2000). This phenomenon was also seen with avian Claspin.

3.5. Generation and characterisation of cClaspin Antibodies

An antibody that detects cClaspin would be a useful reagent in order to study the function of the protein. All commercial antibodies available are raised against the human Claspin sequence. The commercially available antibodies (N-20 and Q-20 goat polyclonal antibodies from Santa Cruz and the BL45, BL73 and BL91 rabbit polyclonal antibodies from Bethyl Laboratories) were tested for their ability to recognise cClaspin by Western blot. None of these antibodies could detect cClaspin. (Data not shown) Therefore it was decided to generate an antibody specific for cClaspin.

3.5.1. Anti-cClaspin antibody (fusion protein)

A region of cClaspin which shared high homology with human and xenopus Claspin and for which there was EST sequences available was selected as the immunogen for the antibody. This polypeptide corresponded to amino acids 675 – 944 which contains a portion of the Chk1 binding domain, with both N- and C-terminal His_6 tags. This fragment was amplified by RT-PCR and cloned into pET28a(+), which allows the expression in bacteria of a His_6 tagged polypeptide. The cClaspin fragment was expressed in BL21 *E.coli* cells and purified using Nickel affinity chromatography. The generation of the expression construct and purification of the Claspin polypeptide was carried out by Dr. Elizabeth Black. The purified immunogen was injected into 3 rabbits to elicit an immune response. (Beatson Antibody Production Facility)

One of the rabbits (HF64B) showed a good response by ELISA so this serum was tested by Western blot to see if it can detect cClaspin. Using both in vitro translated cClaspin and DT40 whole cell lysate as samples, both pre-immune serum and serum from the final bleed post-immunisation were tested. As can be seen in Figure 3.6-B the antibody detects a band at 250kDa, the same molecular mass as in vitro-translated cClaspin. This band was not detected with the pre-immune serum indicating that it indeed corresponds to cClaspin. The other

rabbits (HF64A and HF64C) gave no response by ELISA and did not detect any immune specific bands on a Western blot. (Data not shown)

3.5.2. Anti-cClaspin antibody (peptide antibody)

The antibody generated above worked well in Western blot. However the antibody also recognised many non-specific proteins also. While it is possible to affinity purify antibodies generated against larger antigens, it is easier and usually more successful with antibodies raised against peptides. Such an affinity purified antibody would be useful in techniques such as flow cytometry or immunofluorescence where the specificity of the antibody is more crucial. In addition the region to which the fusion protein antibody was raised spans the Chk1 binding domain. It is known that this region is modified by phosphorylation and also mediate protein-protein interactions. Therefore an antibody which reacts with a site other than this region would prove useful.

The extreme C-termini of proteins are usually flexible and unstructured making them suitable epitopes for the generation of anti-peptide antibodies (Lane & Harlow, 1988). Having sequenced the mRNA, the C-terminus of the chicken Claspin protein was known. The C-terminal 15 amino acids were synthesised. An N-terminal Cys residue was added to facilitate coupling of the peptide to a carrier protein. The peptide was coupled to KLH using MBS conjugation chemistry. The resulting antigen was injected into 3 rabbits and the final serum was tested for the presence of anti-claspin antibodies.

This immunogen gave a very poor responses by ELISA with only one rabbit (HF129B) giving a small response. It was tested by Western blot for its ability to detect cClaspin. (Figure 3.6-C) However this serum failed to cross react with cClaspin.

3.6. Discussion

An important issue resolved in this Chapter was the isolation of the authentic cCLSPN mRNA sequence. This has confirmed that the original cCLSPN sequence from the GenBank database (XM_425782.1) was incorrect. It is important to remember that this sequence was generated by a prediction program, Gnomon, and in this case appears to have fused the authentic cCLSPN sequence with a

pseudo gene located downstream of the cCLSPN CDS. This sequence has since been revised (XM_425782.2) and the current sequence is much more homologous to the sequence identified in this study. Some differences however remain due to the incorrect prediction of splice sites.

In addition, using the information obtained from mRNA sequence has facilitated the generation of a cClaspin antibody. This will allow functional characterisation of the cClaspin homologue and will be a valuable tool in further exploring the potential functions of Claspin.

Chapter 4. Generation of a Claspin +/- cell line

4.1. Introduction

The isolation and sequencing of the chicken Claspin homologue described in the previous chapter has allowed the generation of an accurate map of the chicken CLSPN genomic locus. Having a detailed understanding of the genomic structure of the cCLSPN gene thus allows precise control over how best to design the targeting and screening strategy in order to knock-out cCLSPN. Using this information cClaspin+/- cells were generated.

4.2. Characterisation of chicken Claspin Genomic Locus

The chicken CLSPN gene maps to the long arm of Chromosome 23, one of the microchromosomes, according to the Genbank entry for its genomic sequence (NW_001471589). A schematic representation of the cCLSPN locus is shown in Figure 4.1-A. The gene is located downstream of a cluster of Argonaut genes (EIF2C1, EIF2C3 and EIF2C4) which are involved in RNAi splicing, and upstream of PSMB2, a proteasome subunit. Close to the 3′ end of the gene is a putative hypothetical protein which may or may not be expressed, but for which EST sequences are available. The EIF2C4 gene, the gene closest to the 5’ end of CLSPN, is positioned in the opposite transcriptional orientation from the CLSPN gene. This same genomic organisation is conserved between species. The CLSNP locus on chicken chromosome 23 shows shared synteny with human chromosome 1p34-1p35 and mouse chromosome 4D2.2-4D2.3. The chicken CLSPN genomic locus is flanked on both sides by runs of NNN’s denoting unknown sequence. Consequently much of the sequence of the promoter region of CLSPN remains unknown.

Using the sequence obtained from cloning the chicken claspin cDNA (Section 3.2) and the genomic DNA sequence from the CLSPN locus from the database, an accurate intron-exon map of the CLSPN genomic locus was generated using Spidey, an mRNA to Genomic DNA alignment program from the NCBI. This program identified all the splice sites as canonical GU-AG splice sites and organised the CLSPN gene into 25 exons with 24 intervening introns. The entire gene spans 13.5kbp.

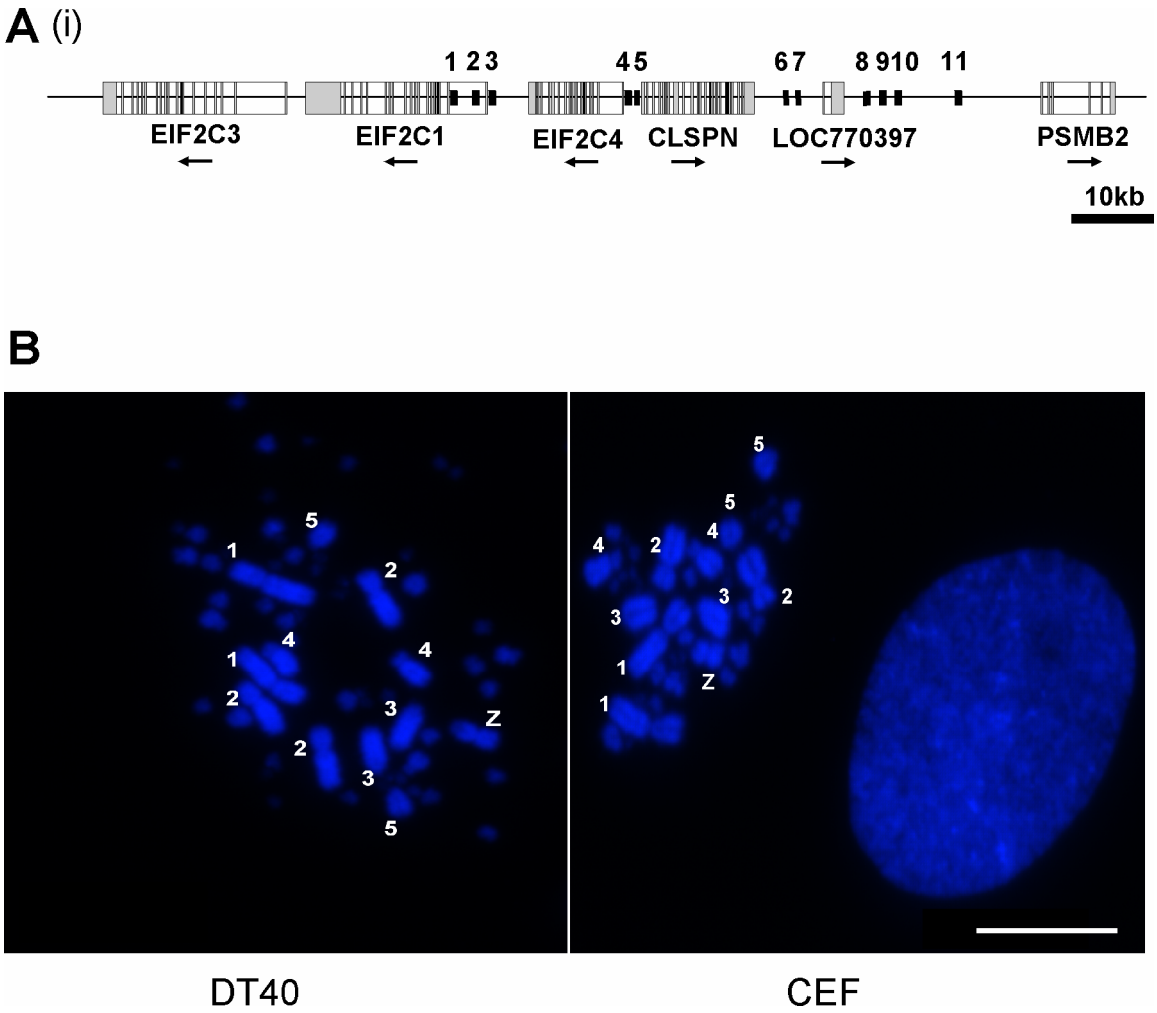


Figure 4.1 cCLSPN Genomic Locus
A) Schematic representation of the cCLSPN genomic locus located on Chromosome 23. Exons are marked by grey lines. Genes are boxed. Gaps in the sequence are represented by the black boxes labelled 1 – 11. B) DAPI stained metaphasechromosomes of DT40 and chicken embryo fibroblasts. Macrochromosomes are labelled. Note 2 copies of chromosome 2 are present in DT40 cells. Scale bar = 100µm.

The DT40 cell line is known to contain an extra copy of chromosome 2 and an extra microchromosome. Compare the normal chicken karyotype of Chicken Embryo Fibroblasts with the DT40 karyotype in Figure 4.1-B. The identity of this microchromosome is unknown. (Winding & Berchtold, 2001) An extra copy of Chromosome 23 would have implications in targeting any gene mapping on that chromosome, as 3 rounds of targeting would have to be carried out. Due to the size of the microchromosomes it is impossible to identify them on a standard karyogram. Therefore in order to determine the copy number of Chromosome 23 in DT40 cells I used FISH with a chromosome 23 specific BAC as the probe. The number of hybridisation 'spots' correlates with chromosome number (Masabanda et al, 2004). Metaphase spreads prepared from WT DT40 cells, MCM DT40[1] cells and secondary Chicken Embryo Fibroblasts were hybridised with a biotin labelled Ch 23 specific BAC. (Figure 4.2-A) After extensive washing and detection with Cy3 conjugated Streptavidin, two spots were detected in all metaphase spreads from the 3 cell lines. This confirms that two copies of Ch23 are present. Unless a gene duplication event has taken place at the CLSPN locus, this strongly indicates that there are two copies of the CLSPN gene present in the cells.

4.3. Generation of CLSPN gene targeting constructs.

There are two basic approaches to generating a knock-out DT40 cell line. In the first approach, termed gene disruption, a drug resistance cassette is inserted into an exon of the gene of interest. Transcription from the drug resistance cassette (usually under the control of a strong promoter) disrupts the transcription of the gene of interest and usually its splicing. Combined this should dramatically inhibit translation of the protein of interest resulting in a null allele. However depending on the splicing of the mutant mRNA functional proteins or fragments of proteins may still be produced. A targeting vector in for this strategy is usually made by amplifying a large region of the gene by PCR and simply inserting a drug resistance cassette into the middle of an exon sequence at an appropriate restriction enzyme site. No DNA is deleted by this method and all of the coding sequence remains in the genome, albeit disrupted.

[1] MerCreMer DT40 cells – see Section 4.4

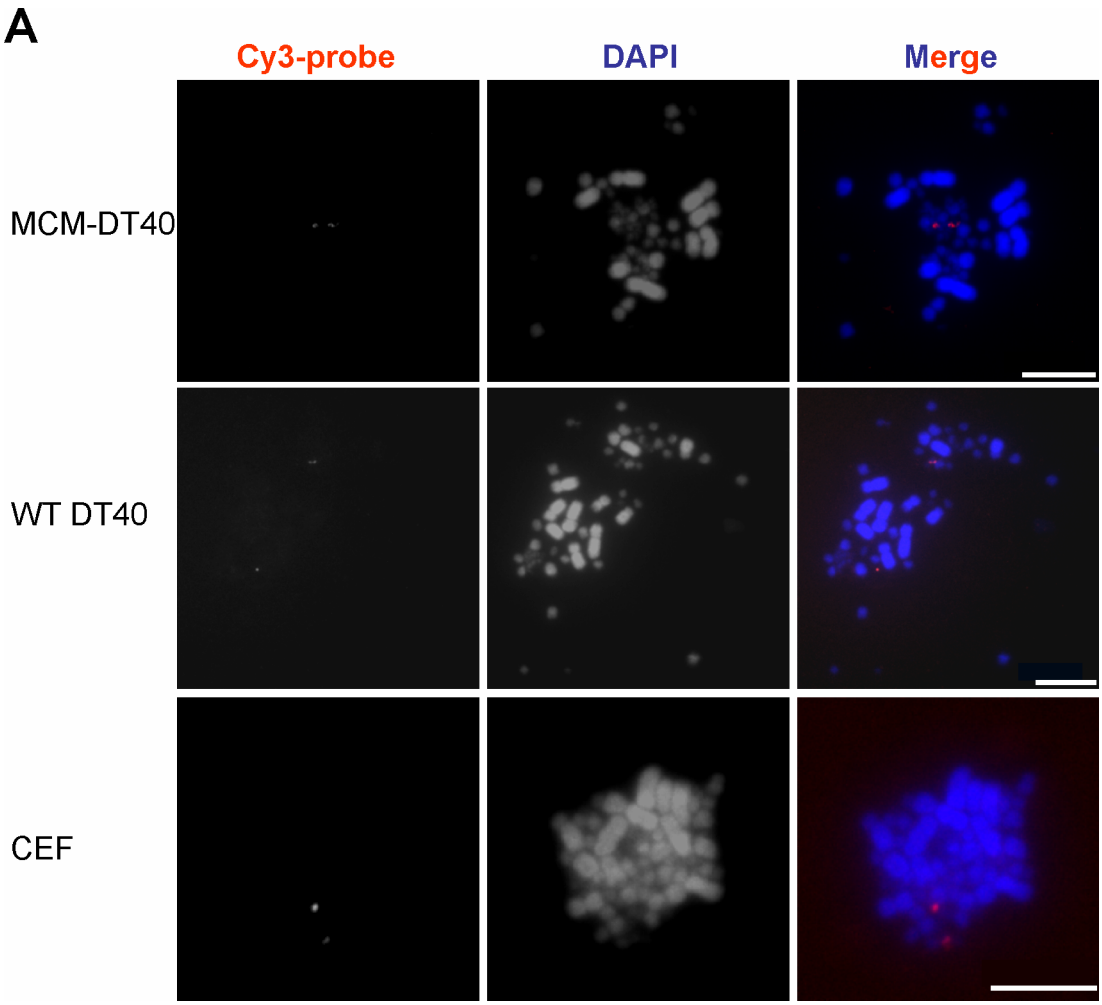


Figure 4.2 FISH with a Ch 23 specific probe
A) A biotinylated Ch23 specific BAC bound to MCM-DT40, WT-DT40 and CEF metaphase spreads is detected by Cy3-streptavidin. 2 spots are detected in each case. Chromosomes are detected with DAPI. Scale bar = 100μm

In contrast the other method, termed gene deletion, results from the physical removal of an appropriate region of DNA. The choice of how much to delete (either the entire gene or only a part of the gene) depends on the size of the gene. For small genes usually the entire gene is deleted. However for larger genes either a critical region encoding for a crucial function of the protein or the promoter region and the start codon are deleted. Removing these regions of the gene usually result in null alleles. A targeting vector for this strategy is made by amplifying 2 regions of genomic DNA, termed 'arms of homology'. The DNA fragments are designed such that they flank the region of DNA to be deleted. A drug resistance cassette replaces the intervening sequence. Both strategies rely on the process of homology directed recombination which directs the integration of the targeting vector to the appropriate region in the genome.

In order to knock-out cCLSPN this second, gene deletion, approach was taken. Ideally the entire coding region of cClaspin would be removed. However a targeting vector designed to remove such a large region would integrate at lower frequencies than one designed to delete a smaller region (Arakawa & Buerstedde, 2006). In addition it was not possible to amplify DNA from the promoter region of cCLSPN. Sequence data from this region is not present in the database and attempts at cloning this region failed probably due to the repetitive sequence and high GC content present. (data not shown)

A more conservative approach was therefore taken. A schematic diagram of the targeting strategy is shown in Figure 4.3-A. For the 5′ arm of homology I cloned a 1.8kb fragment using primers designed just upstream from the 5′ UTR (G13F) and in exon 3 (G10R). This fragment contained the 5′ UTR, exons 1 and 2 and a small portion of exon 3 which could potentially encode for the first 105 amino acids of the protein. To minimise the possible translation of this truncated polypeptide the ATG start codon, contained within exon 1, was deleted by site directed mutagenesis. Deletion of this codon also deletes an NcoI site which can be used for screening at a later stage. For the 3′ arm of homology primers were designed in the 3′ UTR (G9F) and down stream of the CLSPN gene (G9R). The resulting 3.3kb fragment was cloned adjacent to the 5′ arm and in the same orientation. Upon targeted integration exons 4-24 (which spans 11.5kbp) would be deleted only leaving exons 1-3 remaining.

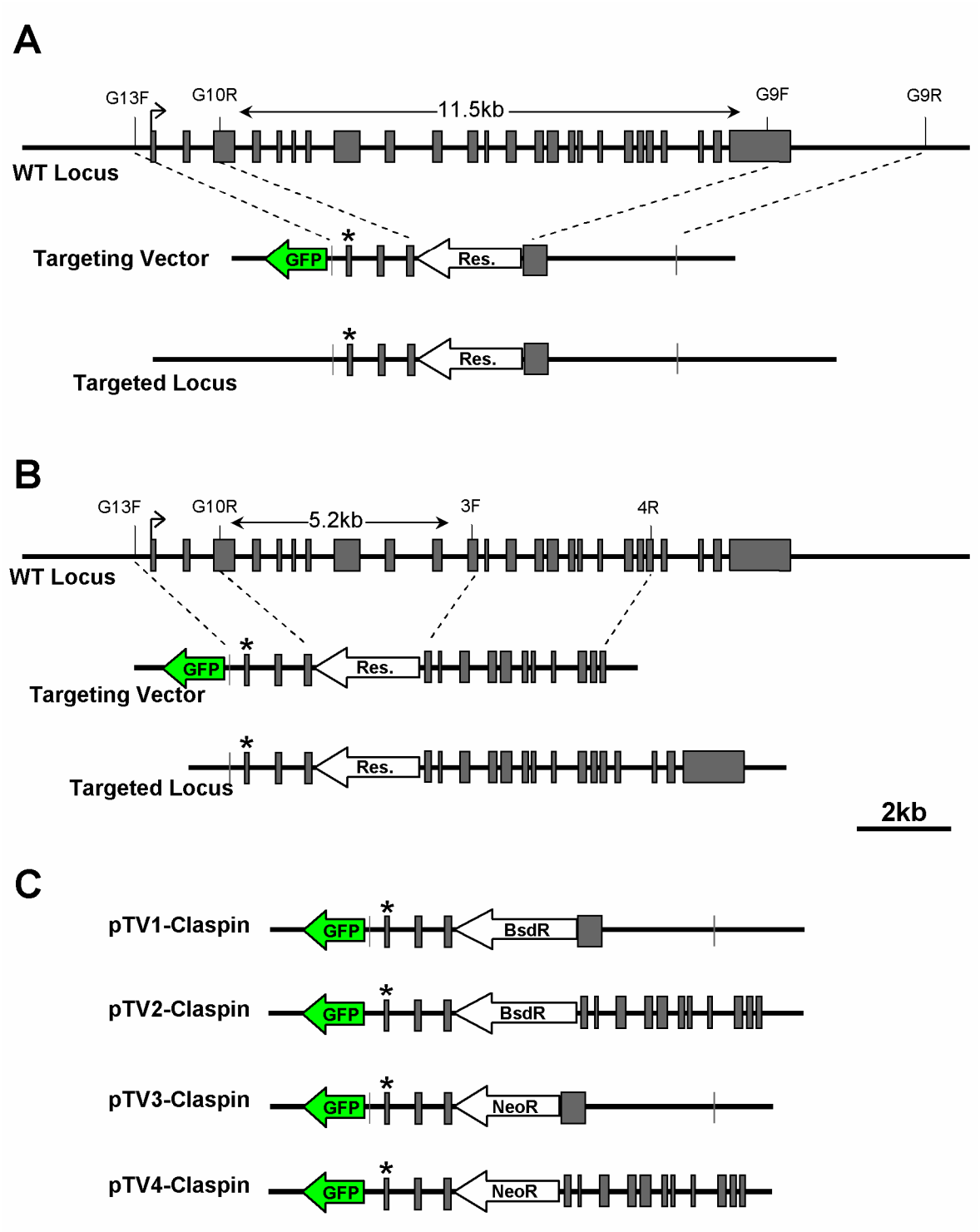


Figure 4.3 cCLSPN Targeting Vector Design
A) Targeting Strategy 1 – deletes amino acids 106 - 1337. B) Targeting Strategy 2 – deletes amino acids 106 – 675. WT cCLSPN Locus is shown with the exons represented by grey boxes. The primers used to amplify the arms of homology are indicated. The arms are cloned such that they flank the Drug resistance cassette (Res.). The mutated start codon is represented by the asterisk. The GFP cassette outside the arms of homology is shown. C) The 4 different targeting vectors constructed.

As the above approach is quite ambitious a second more conservative approach was also taken. (Figure 4.3-B) For this targeting vector a 3.8kb genomic fragment was generated using primers designed in exons 11 (3F) and 21 (4R), was cloned adjacent to the same 5′ arm as was used for the first approach. This second approach was taken in order to maximise targeting efficiency by deleting a smaller region (5.2kb) of DNA. This approach however leaves much more of the coding region of cCLSPN intact in the genome essentially only deleting amino acids 106 – 675 (570 amino acids) in the protein. The region deleted is contained within the DNA binding/replication fork interacting domain. Again, however, as the initiating Methionine is deleted from the 5′ arm of homology the probability that any mutant polypeptide would be produced is low.

In both cases both arms of homology were cloned into a modified pLox-Neo or pLox-Bsr vector (Arakawa, 2001). These vectors contain within their multi-cloning sites either the Neomycin phosphotransferase gene (confers resistance to G418) or the Blastcidin-S-deaminase gene (confers resistance to Blasticidin) which will be used as positive selection during the targeting process. Unique restriction sites are present on both the 5′ and 3′ sides of the resistance genes which facilitate the insertion of the arms of homology by conventional cloning strategies. The expression of both resistance genes is driven by the chicken ß-actin promoter. Both G418 and Blasticidin are inhibitors of protein synthesis.

The pLox-Neo and the pLox-Bsr vectors described above were modified to contain a GFP expression cassette (from pEGFP-N1, Clontech). The cassette was ligated with KpnI adaptors and cloned into the KpnI site of the multi-cloning site of the plasmid. In the final targeting vector this cassette is outside the arms of homology therefore serving as a negative selection marker in a manner similar to the Thymidine Kinase/Gancyclovir or diphtheria toxin systems for negative selection (Mansour et al, 1988; McCarrick et al, 1993). Upon targeted integration of the targeting vector the GFP cassette will be deleted and the clones derived from such integrations will be GFP negative. However in clones where random integration of the targeting vector has taken place the GFP cassette will remain and the clones will express GFP. The presence or absence of GFP was used as the initial screening.

To construct the final vector the arms of homology were then inserted into this vector such that they flanked the drug resistance cassette. The arms of homology for the 3′ arms (either G9F,G9R or 3F,4R (modified to remove the SalI site present in the 3F primer) were amplified by high-fidelity PCR using DT40 genomic DNA as template. The PCR fragments were then cloned by TA cloning into the pCR4-TOPO TA vector for sequencing. Following sequence verification the fragments were sub-cloned directionally into the NotI/SpeI sites in the MCS. The NotI/SpeI sites in the pCR4 vector were used to facilitate this. The 5′ arm was then inserted into SalI/ClaI sites in the MCS using SalI and ClaI sites engineered into the G13F and G10R primer sequences. This resulted in the final targeting vectors; pTV1-Claspin deleting the larger region and pTV2-Clasin deleting the smaller region – both with the BsdR resistance cassettes; the pTV3-Claspin deleting the larger region and pTV4-Claspin deleting the smaller region – both with NeoR resistance cassettes. (Figure 4.3-C) A ScaI site located in the Ampicillin resistance gene was used to linearise this final targeting vector.

In summary the targeted loci should not produce any functional, stable mRNA due to the deletion of large portions of the coding region. Any transcript produced is likely to be degraded by nonsense mediated decay. The transcription of any mutant transcript will be reduced by interference from the drug resistance cassette which is under the control of the strong ß-actin promoter, which yields high levels of gene transcription. Finally even if a stable, functional mRNA is produced the deleted start codon will inhibit translation of protein from this locus.

The drug resistance cassettes I chose are flanked by mutant LoxP sites (Arakawa, 2001). Upon Cre mediated recombination the resistance cassettes are deleted yielding a single non-functional LoxP site and a cell line that is now sensitive to the drug of interest. The advantage of this is two fold – firstly by removing the resistance cassette the cell line now becomes re-sensitised to the drug allowing the same targeting vector to be used to target the second allele. In addition it also opens up the possibilities of generating compound mutant cell lines by allowing more choice in which selection marker is available for use. In practice this is the most important advantage.

4.4. Characterisation of MCM-DT40 and Cre1-DT40 cells

To take full advantage of this system I wanted to use a cell line that expressed an inducible Cre recombinase which could easily facilitate the removal of the drug resistance cassettes. Two such cell lines were available DT40-Cre1 (Arakawa, 2001) and DT40-MCM (Brummer et al, 2003). Both cell lines constitutively express a CreER fusion protein under the control of the modified oestrogen receptor (Zhang et al, 1996). The modification makes the receptor more responsive to the synthetic oestrogen analogue, tamoxifen, than endogenous oestrogen. Upon tamoxifen addition, the CreER protein is released from an inhibitory complex with heat shock proteins and translocates to the nucleus as a fully functional protein which mediates recombination between 2 adjacent LoxP sites. The LoxP sites flanking the drug resistance cassettes are orientated such that recombination deletes rather than inverts the sequence. The LoxP sites have also been mutated such that once recombination has taken place the resulting single LoxP site is no longer functional (Arakawa, 2001).

As one of the main defects expected with my Claspin$^{-/-}$ cells would be defective DNA damage signalling I characterised both cell lines for their ability to initiate both the G_2-M DNA damage checkpoint and the S-M mitotic checkpoint. In the G_2-M checkpoint the ability of cells to inhibit entry into mitosis in the presence of DNA damage is assessed. From Figure 4.4-A it is evident that the DT40-Cre1 cells, even though they display a block in cells entering mitosis it is clear that the cells do not mainly arrest in G2 like the WT and MCM cells. They are also more sensitive to killing by γ-IR than the WT-DT40 cells. From the PI plots it can be seen that whereas the WT-DT40 and MCM-DT40 cells maintain the G_2-M arrest the DT40-Cre1 cells are distributed through-out the cell cycle or are dying. It is probable that the Cre1 cells treated with γ-IR with S/G1 DNA content represent G2-M cells that are dying. The MCM cells while they do not have as a robust a checkpoint as WT cells (≈ 20% of the cells enter mitosis compared with 7% for WT) they do arrest in G2. The second checkpoint that was assessed was the ability of cells to halt entry into mitosis in the presence of unreplicated DNA. (Figure 4.4-B) It has previously been reported that in the absence of Chk1 cells can enter mitosis in the presence of unreplicated DNA (Zachos et al, 2005).

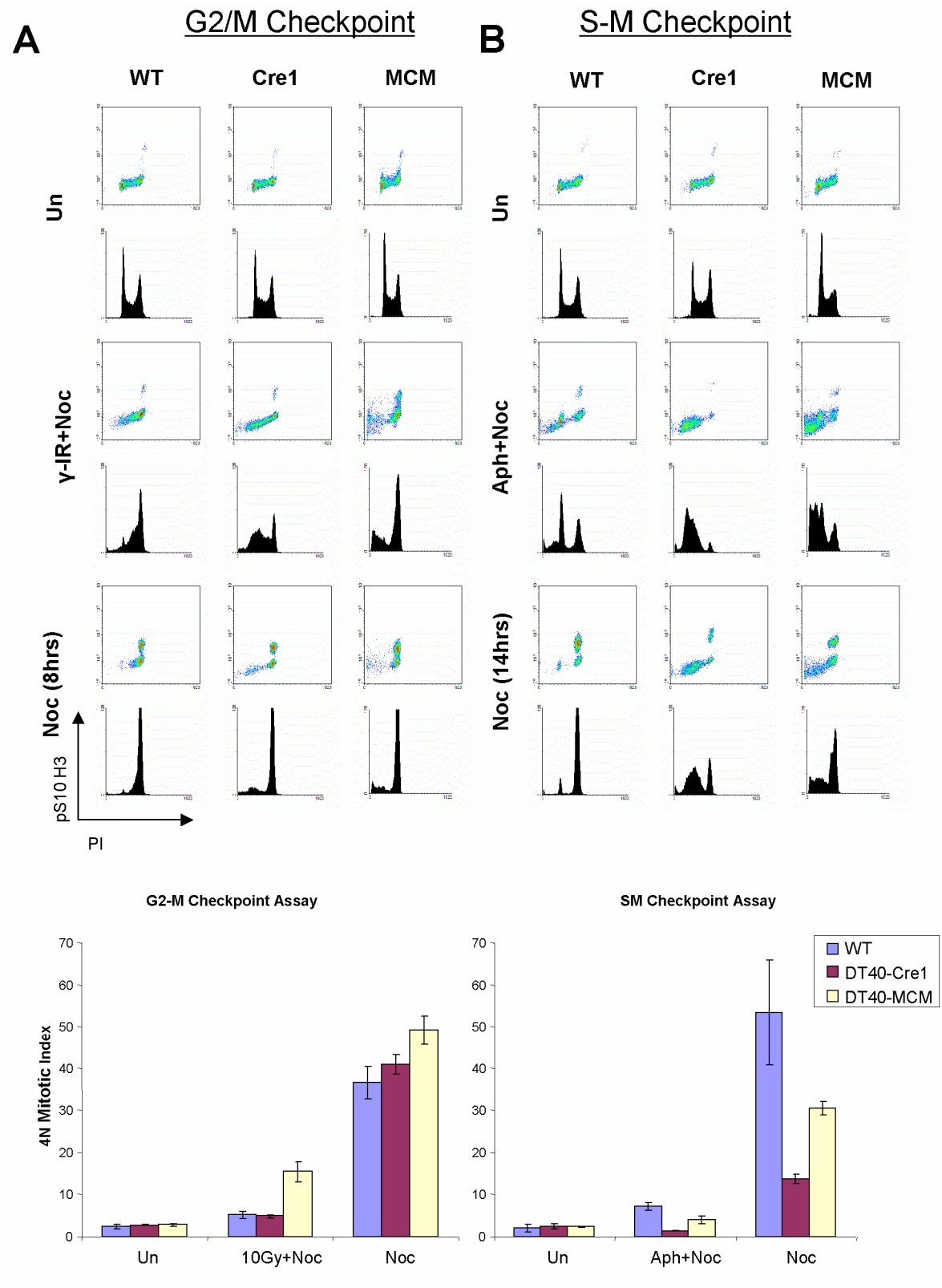


Figure 4.4 Characterisation of MCM-DT40 and DT40-Cre1 Cells
A) G2-M Checkpoint Assay. Cells were treated for 8 hours with Nocodazole with or without 10Gy of γ IR. The number of cells in mitosis (pS10 H3 positive cells) after 8 hours was assessed by flow cytometry. Density plots and the DNA histogram are shown. The quantification of the number of mitotic cells for each cell line and each treatment is shown below the FACS plots. n=3 B) S-M Checkpoint Assay. Cells were treated for 14 hours with Nocodazole with or without 60uM Aphidicolin. The number of cells in mitosis (pS10 H3 positive cells) after 14 hours was assessed by flow cytometry. Density plots and the DNA histogram are shown. The quantification of the number of mitotic cells for each cell line and each treatment is shown below the FACS plots. n=3

Treating the cells with a high dose of aphidicolin results in the persistence of unreplicated DNA and inhibits entry into mitosis. Cells with an impaired S-M checkpoint will display 2N cells which are pS10 H3 positive. From Figure 4.4-B it can be seen that both MCM-DT40 and DT40-Cre1 cells while they both show an increased sensitivity to Aphidicolin treatment compared with WT cells, they do not show any signs of cells entering mitosis in the presence of unreplicated DNA. However DT40-Cre1 cells appear to be sensitive to prolonged mitotic arrest. After the 14 hour incubation only approximately 15% of the cells are trapped in mitosis compared with nearly 60% for WT-DT40 cells. It is clear from the above results that while the DT40-Cre1 cell line appears to have functional checkpoints it is hyper-sensitive to DNA damage, replication stress and mitotic arrest. These and other defects (including defective spindle checkpoint and abnormal centrosome number) have also been observed else where. (Bree et al, 2007) The MCM-DT40 cell line while not behaving exactly the same as WT-DT40 cells is more similar than the DT40Cre1 cells. It was therefore decided to use the MCM-DT40 cell line as the background for the generation of the Claspin$^{-/-}$ cell line.

4.5. Generation of Claspin+/- cells in MCM-DT40 Background

An outline of the targeting process is presented in Figure 4.5. Briefly MCM-DT40 cells were transfected linearised pTV1-Claspin targeting vector. After recovering the cells were plated into 96 well plates under selection of Blasticidin. After 2 weeks drug resistant clones were screened for the presence or absence of GFP using fluorescence microscopy. Most clones expressing GFP were discarded at this stage but some were routinely expanded as controls. GFP negative clones were expanded and screened by southern blotting.

A summary of the results obtained with the MCM-DT40 cells is shown in Figure 4.6. From the results it is clear that many problems were observed when using the MCM-DT40 cells.

A big problem observed was the failure of clones to expand. It is usual in and DT40 knock-out project, even just when making stable cell lines, that a portion of clones die during the expansion process. However, the number of clones that failed to expand with the MCM-DT40 cells was far higher than would reasonably

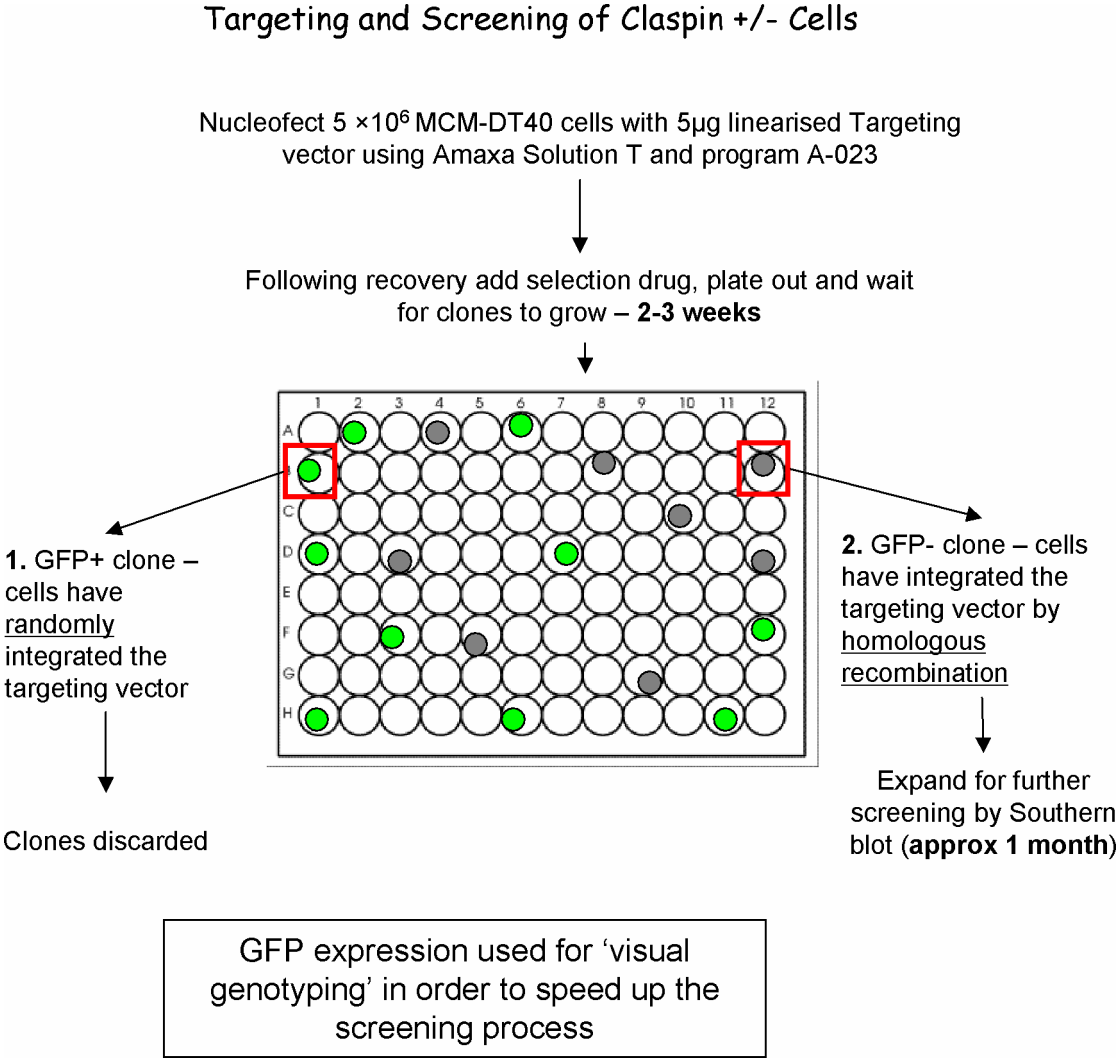


Figure 4.5 Overview of the Claspin Targeting Methodology
This schematic outlines the steps involved in targeting 1 allele of a gene in DT40 cells. Cells transfected with the targeting vector are cloned by limiting dilution to ensure each clone arises from 1 transfected cell. Only wells with one clone growing are picked. GFP is used as a negative selection marker.

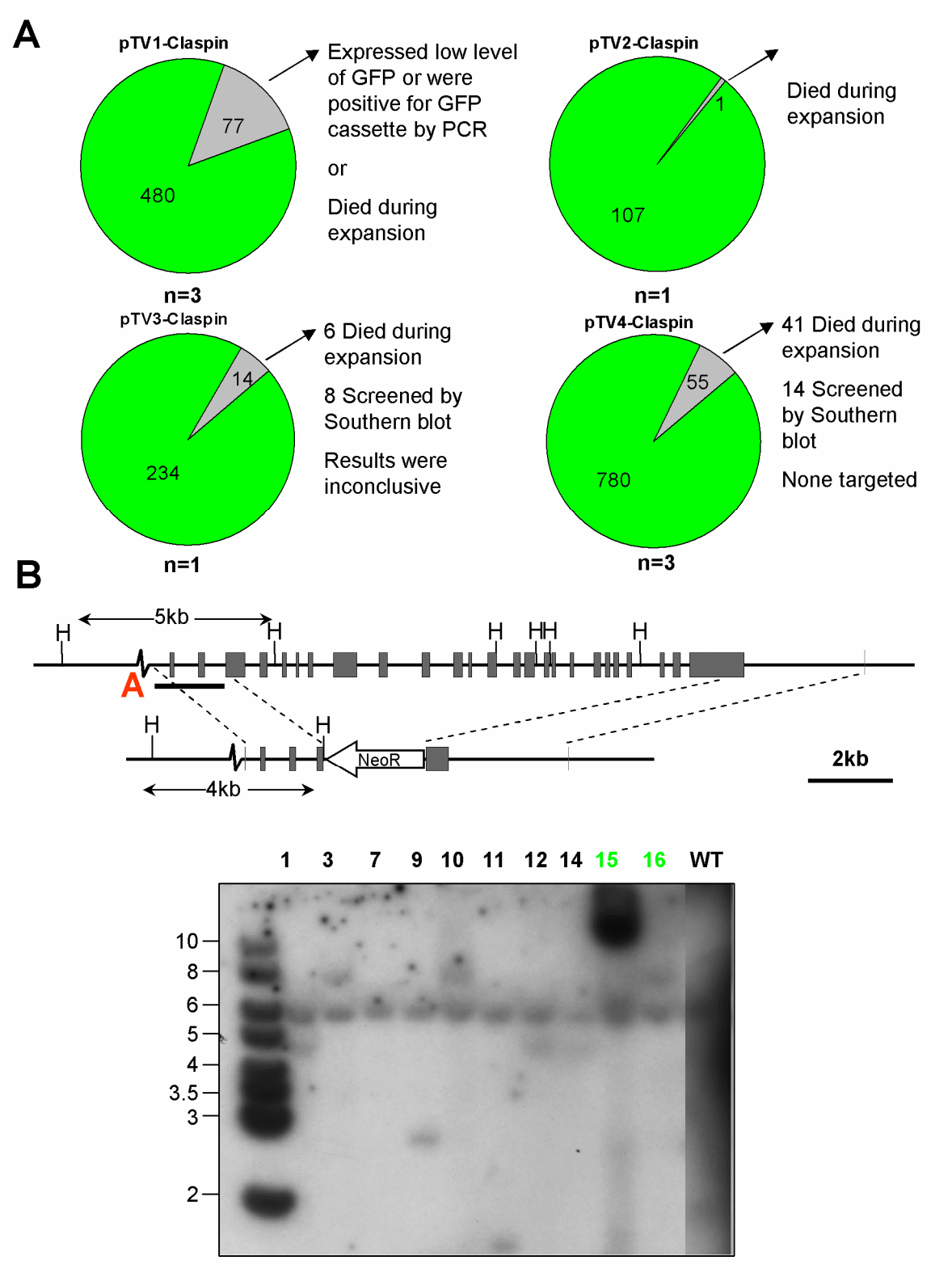


Figure 4.6 Summary of Screening for Claspin +/- MCM-DT40 Cells
A) Pie charts show the portion of GFP negative clones obtained with each targeting vector. (GFP positive in green and negative in grey) The fate of the GFP negative clones is indicated. The 77 GFP –ve clones obtained with pTV1-Claspin were incorrectly classified as GFP –ve. When expanded these clones were in fact expressing GFP. Numbers in the pie charts represent the total number of clones obtained following transfection of each targeting vector. The number of transfections per targeting vector is indicated below each pie chart. B) HindIII Southern Blot of pTV3-Claspin targeted clones. Clones 15 and 16 are GFP positive clones for comparison.

be expected. In conjunction with this there was a possible issue with the stability of the Blasticidin used in these experiments. With clones taking 2-3 weeks to appear it is possible that some of the clones that grew were actually not resistant to Blasticidin. During the expansion process Blasticidin is added back to the growth media to kill off any such cells. This may have contributed significantly to the high attrition rate of clones observed during expansion.

The first vector that was used in order to target cCLSPN was the pTV1-Claspin vector. Three separate transfections were set up and 557 clones grew. Of these only 77 were GFP negative. However during the expansion process it was evident that most of the clones that been classed as GFP negative were actually GFP positive. In addition the cells that remained GFP negative died during the expansion process. No viable GFP negative clones were obtained either using this strategy or with the pTV2-Claspin vector. For this reason the BsdR versions of the targeting vectors were abandoned in favour of the NeoR versions. G418 is much more stable in culture and therefore drug stability issues should not be as problematic with G418.

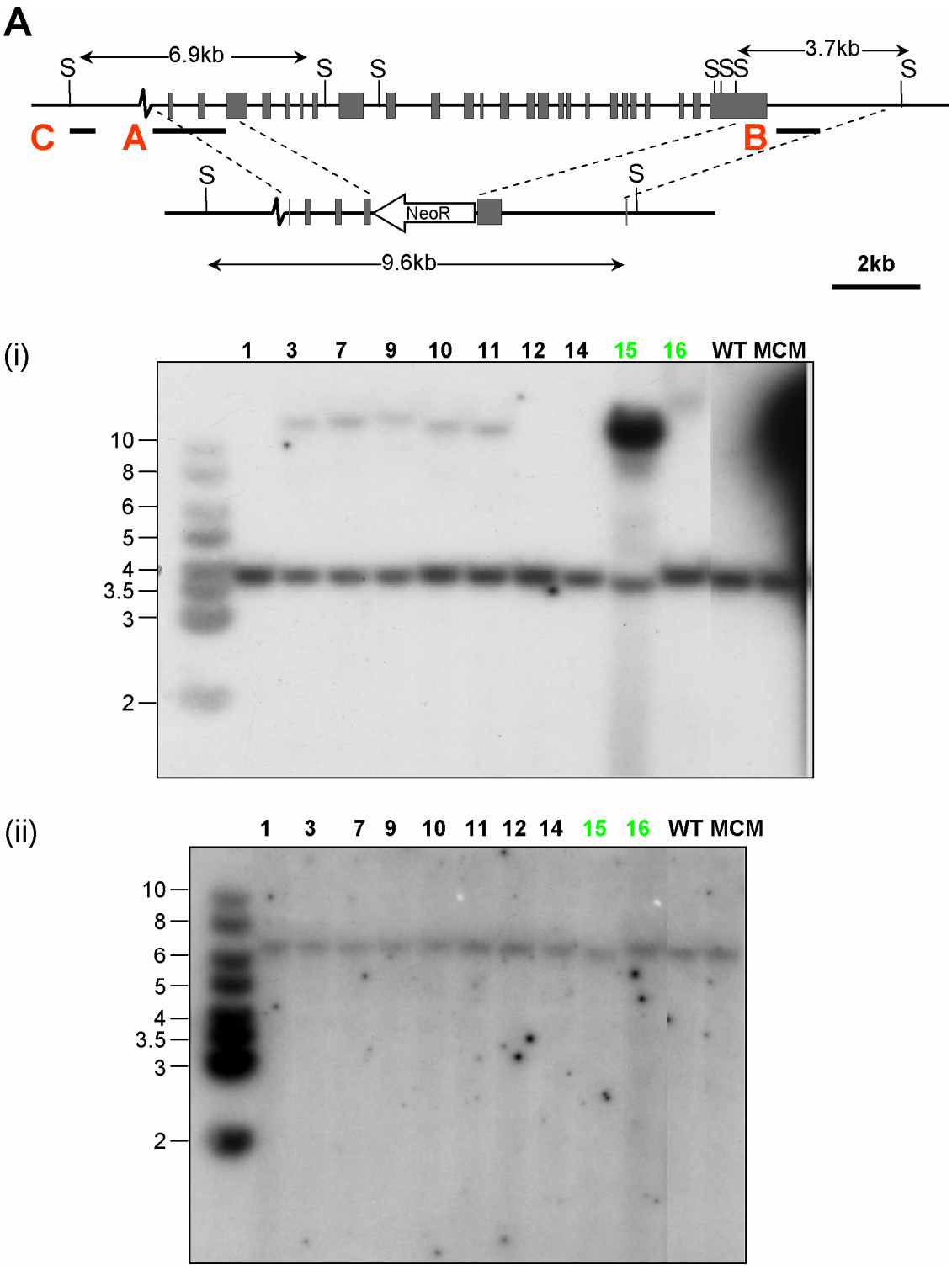
The first attempt using the NeoR vectors was made using the pTV3-Claspin targeting vector. This vector is designed to remove most of the coding region of the cCLSPN gene. Using this targeting vector 248 clones were screened initially for GFP expression. 14 of these were GFP negative and therefore possibly targeted. 8 of these survived expansion and all were negative for GFP by PCR. (data not shown) Again 50% of these clones died during the expansion process which may suggest that the Blasticidin is not to blame for the failure of cells to expand. The viable GFP negative clones were screened by Southern blot for targeted integration.

A major drawback to knocking out the entire coding region of the cCLSPN gene is that in order to design the targeting vector virtually the entire known 5′ and 3′ flanking regions of DNA were included in the targeting vector. As mentioned earlier, at both the 5′ and 3′ end of the gene there are regions of unknown sequence. Attempts at cloning and sequencing these regions proved unsuccessful. Therefore this made selecting an appropriate location for an external probe very difficult. A compromise was reached where it was decided to screen the clones using only internal probes but using multiple restriction

enzyme combinations in order to confirm targeting. If the predicted bands appeared using multiple enzymes then this should reliably indicate a targeting event.

The first enzyme chosen for Southern Blot was HindIII in conjunction with a probe contained within 5′ arm (Probe A). This is predicted to detect a WT band of approximately 5kb. As is evident from the targeting diagram the 'external' HindIII site is outside at region of unknown sequence so it is possible that there is a HindII site within this region, which would alter the length of the WT band. However as the HindIII site in the targeted locus is rearranged within the known sequence it will always be 1kb smaller than the WT allele regardless of its length. As can be seen from Figure 4.6-B this probe detects a WT band of approximately 6kb in length. Three of the clones screened showed a pattern that would be consistent with correct targeting – clones #1, 12 and 14 all showed 'targeted' band running at ≈5kb. It is interesting to note however that the intensity of the 'targeted' band is not equal to the WT band which would be the case if targeted integration had taken place and only two copies of the gene were present.

Following this potentially successful result it was necessary confirm this with more enzyme-probe combinations. The next Southern preformed was on ScaI digested DNA. (Figure 4.7-A) The WT band in this case is recognised by an internal probe located in the 3′ arm of homology (Probe B). Upon integration all the ScaI sites present within the gene are deleted and the restriction band to which the probe hybridises is generated by 2 ScaI sites which flank the remainder of the cCLSPN gene. Again it is worth noticing that the 5′ ScaI site is external to the region of unknown sequence. As expected Probe B hybridises to the correct WT band. (Figure 4.7-A(i)) Assuming the HindIII result is correct then it would be reasonable to assume that clones #1, 12 and 14 would show a targeted band with ScaI as well. However, with ScaI it appeared to be opposite way around. This time clones # 3, 7, 9, 10 and 11 showed bands which may be consistent with targeting and clones #1, 12 and 14 showed no extra DNA. Again the 'targeted' bands were not the same intensity as the WT bands and it was slightly bigger than expected. This however could be explained by the fact that the length of DNA in the gap region is unknown – therefore the increase in size of the expected fragment could be simply due to this gap being larger than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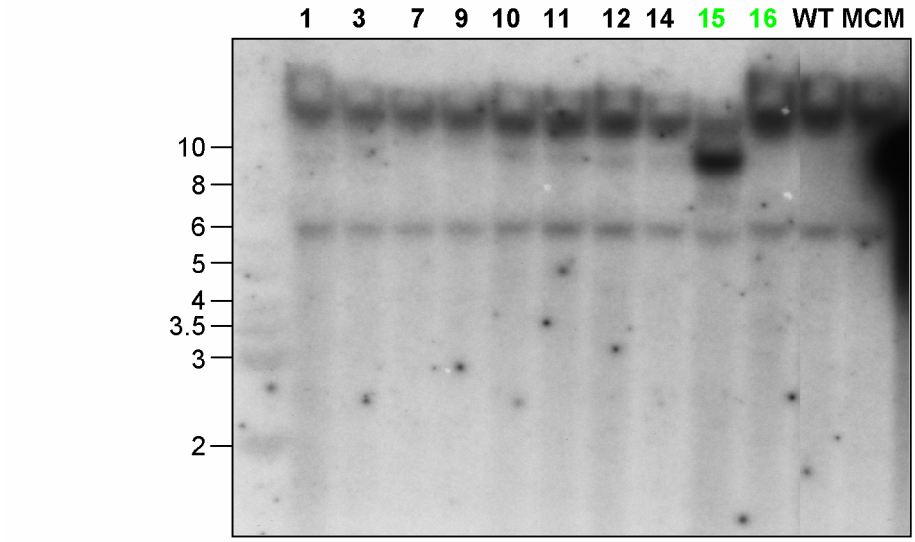
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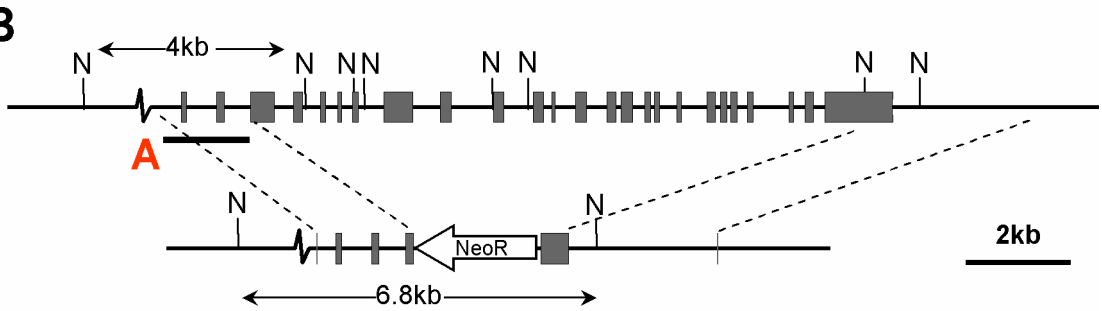
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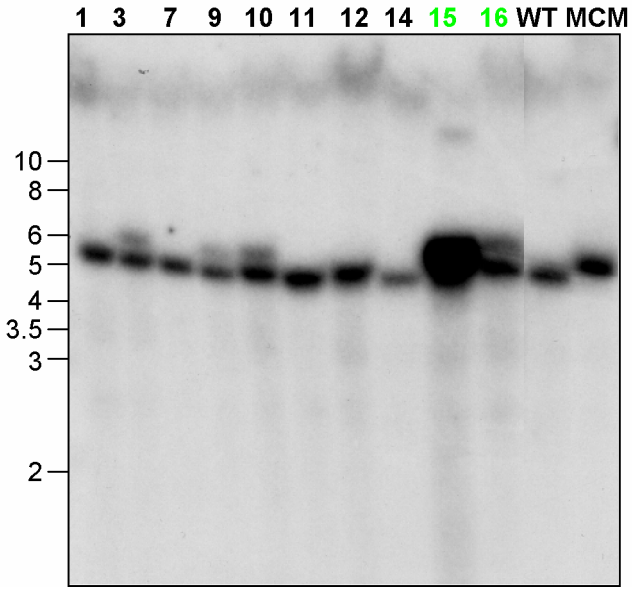
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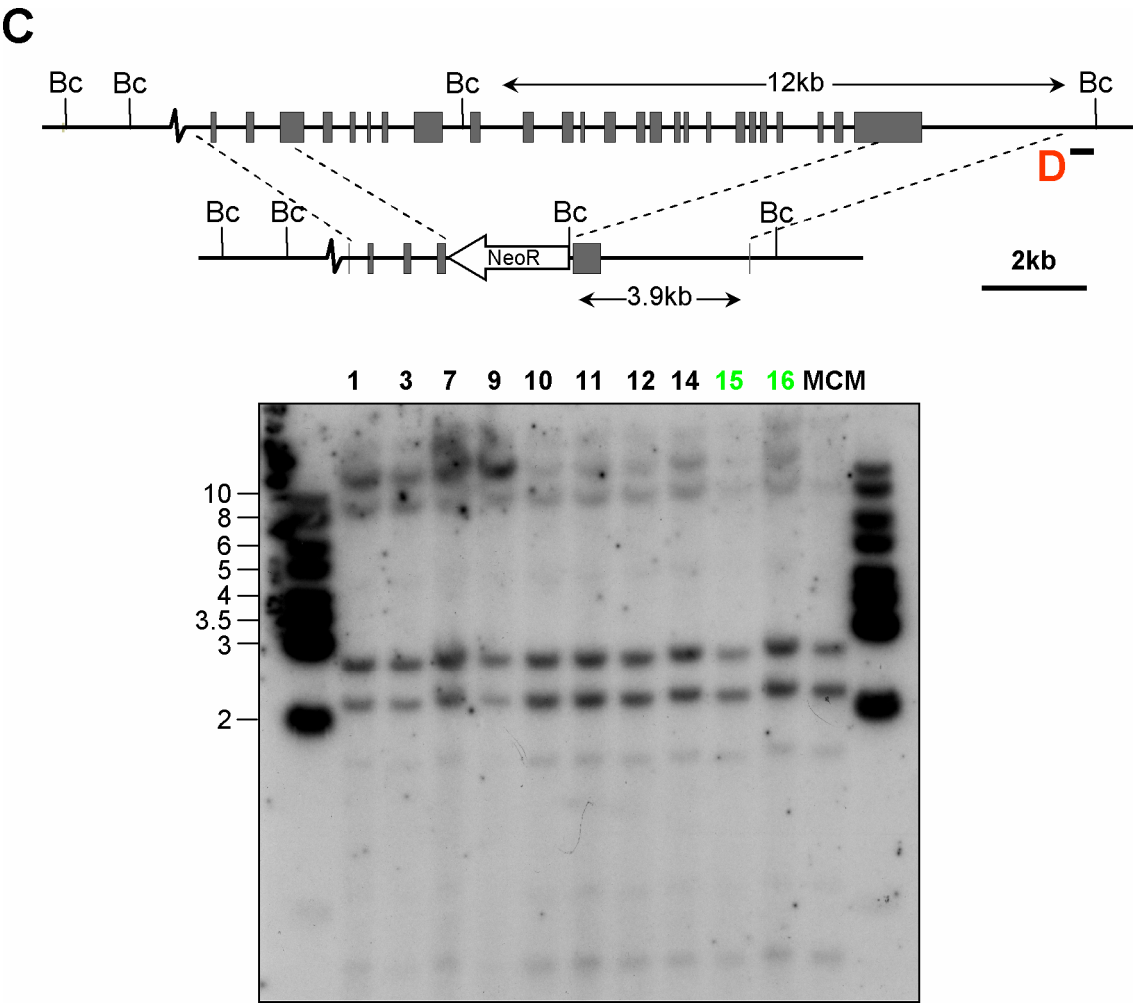

Figure 4.7 Southern Blot Screening of pTV3-Claspin Transfected MCM-DT40 clones
A) ScaI Southern Blot with pTV3-Claspin targeted clones. (i) Southern using Probe B (ii) Southern using Probe C (iii) Southern using Probe A B) NdeI Southern Blot with pTV3-Claspin targeted clones. Probe A should detect a 4kb band in WT clones and a 6.8kb band in targeted clones. A WT band of 5.5kb is detected with extra bands at 6kb detected in some lanes including the GFP +ive controls C) BclI Southern Blot with pTV3-Claspin targeted clones. Probe D is an external probe but failed due to non-specific binding. No WT band at 3.9kb could be detected.

expected. It was curious why there was such a disparity between the ScaI result and the HindIII result. In order to try and resolve the situation a 5′ External probe was generated and hybridised to the membrane (Probe C). This gave a single band in all cases of ≈7kb which would be entirely consistent with predicted gap size. (Figure 4.7-A(ii)) The absence of any other bands with this probe would indicate that none of the clones are targeted. However it is possible that this band was generated by the 5′ external ScaI site and an unknown ScaI site within the gap region. To clarify this, the membrane was probed for a third time with Probe A. This returned the exact same pattern as that obtained with Probe C indicating that they are infact detecting the same restriction fragment. (Figure 4.7-A(iii)) The only other, but unlikely, possibility for this to occur would be that an unknown ScaI site lies directly in the middle of the two known ScaI sites – where upon ScaI cleavage two fragments of equal length are produced. Therefore hybridisation with my probes would actually generate the observed pattern.

The uncertainty with the HindIII and ScaI results led me to try and find another screening method to identify targeted integration. However Southerns with NdeI and BclI digested DNA did not shed any more light on the situation. (Figure 4.7-B and 1.7-C) The major flaw with this approach was the lack of a reliable external probe with which to genotype the clones. Also the very low frequency of GFP negative clones growing was worrying. Therefore it was decided to abandon this targeting strategy in favour of the second targeting strategy, where the targeting vector should, in theory, integrate with better frequencies and for which a reliable Southern blot screening strategy exists.

Using the pTV4-Claspin targeting vector, designed to knockout the smaller region of the cCLSPN gene, in total 835 clones were screened from 3 separate nucleofections. Of these 55 were GFP negative, but of these only 14 survived the expansion process and remained GFP negative by PCR. These clones were screened by southern blot but none showed the presence of the targeted band. (Figure 4.8)

Again the percentage of GFP negative cells was very low with this targeting vector. In theory the targeting efficiency with the cells should be approximately 50%. Assuming that all the GFP negative cells are not going to be targeted it

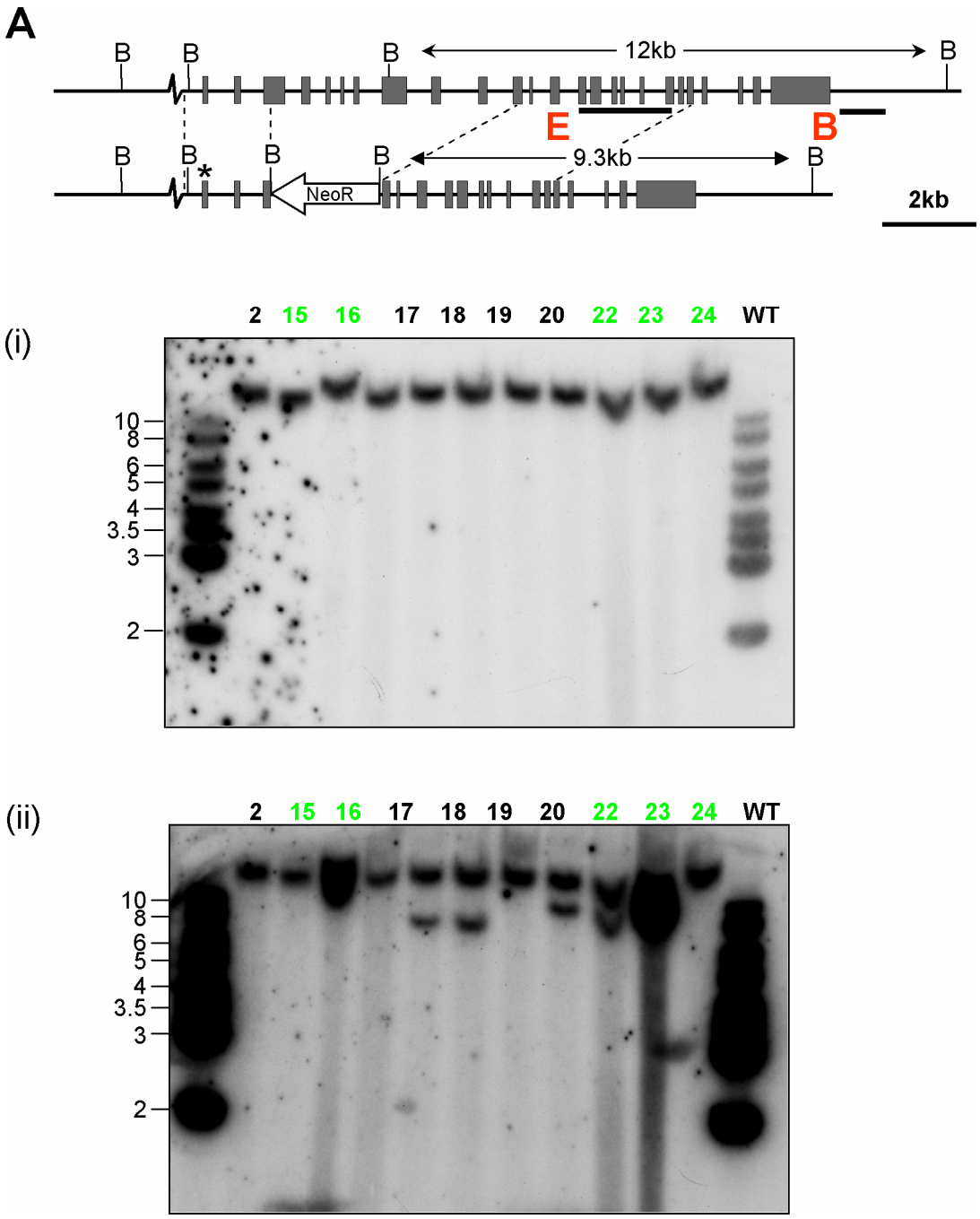

Figure 4.8 Screening pTV4-Claspin transfected MCM-DT40 Clones
A) Map of WT and targeted locus indicating the location of the probes and the BamHI restriction sites. (i) BamHI digested DNA from pTV4-Claspin transfected MCM-DT40 cells probed with the External Probe (Probe B) (ii) The same membrane stripped and re-probed with the Internal Probe (Probe E) GFP positive clones are indicated in green.

would therefore be reasonable to assume that an even greater number of clones should be GFP negative. These results therefore might call into question the capacity of these cells for homologous recombination.

To test the capacity of the MCM DT40 cells for their ability to undergo targeted recombination of targeting vectors at a high frequency I obtained the Ovalbumin targeting construct (PuroR version) which integrates into the ovalbumin locus at high frequencies (≈80%) into the DT40 genome (Buerstedde & Takeda, 1991). Upon transfection of this construct into MCM DT40 cells and selection of clones, 18 clones were screened for the presence of a correctly targeted Ovalbumin locus. Of the 18 screened none of the clones had integrated the targeting vector in the correct location (0/18 = 0% targeting efficiency). This confirms the suspicion that this cell line has lost its capacity for homologous recombination and explains much of the difficulties observed in trying to obtain a knock-out cell line. Upon realising this it was decided to revert to the 'gold standard' high efficiency DT40 cell line variant, Cl18.

The main reason for choosing the MCM-DT40 cell line was to use the inducible Cre to delete the resistance cassette. This can also be achieved in Cl18-DT40 cells by transient transfection of Cre recombinase and screening for clones where recombination has taken place.

4.6. Generation of Claspin+/- cell in Cl18 DT40 Background

Due to the difficulties encountered with using the MCM-DT40 cell line I obtained a fresh aliquot of Cl18 DT40 cells from the Lowndes group (NUI Galway, Ireland) who were using the cells with success to generate knock-out DT40 cell lines. In order to confirm that these cells did indeed show high levels of targeted recombination the control targeting experiment was repeated with these cells. Of 20 clones picked 6 were targeted indicating that these cells did have the capacity for targeted recombination. This targeting efficiency of 30% was still lower than that originally observed so it was also reasoned that the method of transfecting our cells (Amaxa Nucleofection) may also be impacting on the targeting efficiency. It was decided to revert to using the GenePulser (BioRad) in order to transfect the cells with DNA. This allows the delivery of a defined

pulse to the cells as opposed to a complex pattern of electrical pulses delivered by Amaxa system (Lonza).

Three consecutive rounds of electroporation were set up and 254 clones were obtained in total. Of these 116 were GFP negative which were then screened by Southern blot for targeted integration. (Figure 4.9-A) 37 of these clones showed the 9kb targeted band giving an overall targeting efficiency of 32% of those screened. Overall, for all the clones, it gave a targeting efficiency of 15%. The membranes were then reprobed with the internal probe to ensure that the targeting vector had not also integrated into an ectopic site in the genome. It is worth noting that the intensity of the remaining WT allele and the targeted allele are the same, indicating that there are indeed only two copies of the cCLSPN gene present in Cl18 cells. (Figure 4.9-B)

The clones confirmed to be targeted were counter screened by PCR for the presence of the GFP cassette. 8 of the targeted clones were positive for the GFP cassette. This can arise due to correct integration of the 3′ arm (which is assayed for by the Southern Blot) but non-homologous integration of the 5′ arm nearby. These clones were discarded for further study.

An important feature of this strategy relies on the deletion of the initiating ATG start codon in order to minimise the possibility of translating a mutant cClaspin polypeptide. In order to assay for the loss of the ATG codon the region surrounding the codon was amplified and digested with NcoI. (Figure 4.10) Using the Targeting vector DNA as a template the resulting PCR product is resistant to digestion by NcoI. However using DNA isolated from WT Cl18 cells yielded an unexpected result. As expected bands at ≈1200bp and ≈400bp were obtained. However bands at ≈700bp and ≈550bp were also obtained. This suggests that there is an extra NcoI site in one allele of cClaspin – termed allele 2. Mapping these sites around the NcoI site at the ATG position shows that there must also be another NcoI site upstream of the known site. Theses sites would map to intronic sequence. A schematic representation of the predicted locations of the NcoI sites is shown in Figure 4.10-A. The location of these sites does not affect the screening strategy as it is designed to simply assay for an allele that is completely resistant to digestion. Clone #2.1 is shown to be non-

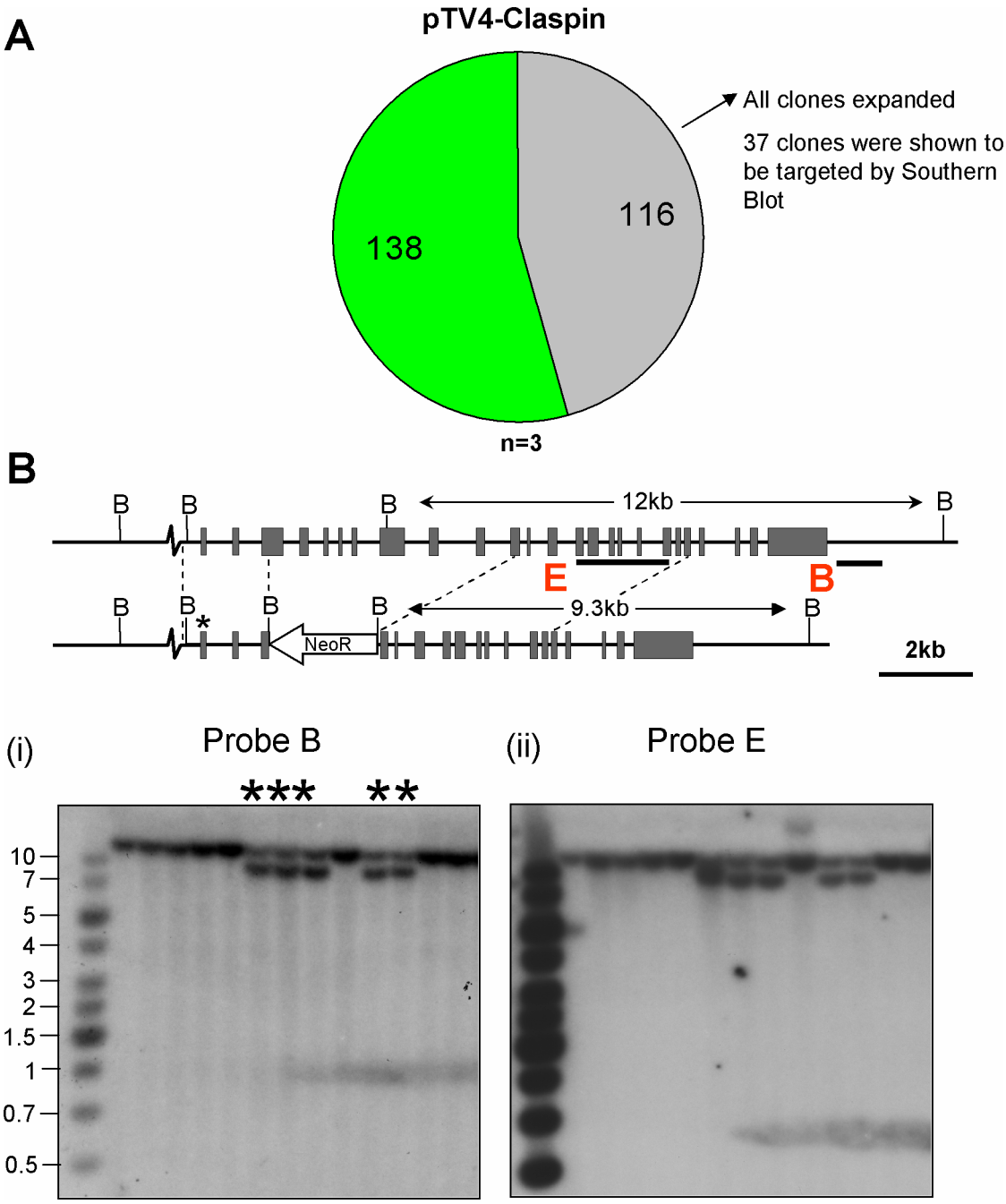

Figure 4.9 Screening of pTV4-Claspin transfected Cl18 clones
A) Summary of screening results. Pie chart shows the portion of GFP negative clones obtained. Numbers in the pie charts represent the total number of clones obtained following transfection of the targeting vector. The number of transfections is indicated below the pie chart. B) Southern Blot Screening – Location of internal and external probe with respect to the relevant BamHI sites (indicated by a B) is shown. (i) Representative blots obtained when using the External Probe, Probe B. Asterisks indicate the targeted clones. (ii) Representative blots obtained when using the Internal Probe, Probe E. No other DNA hybridises with the internal probe indicating that only one copy of the targeting vector has integrated.

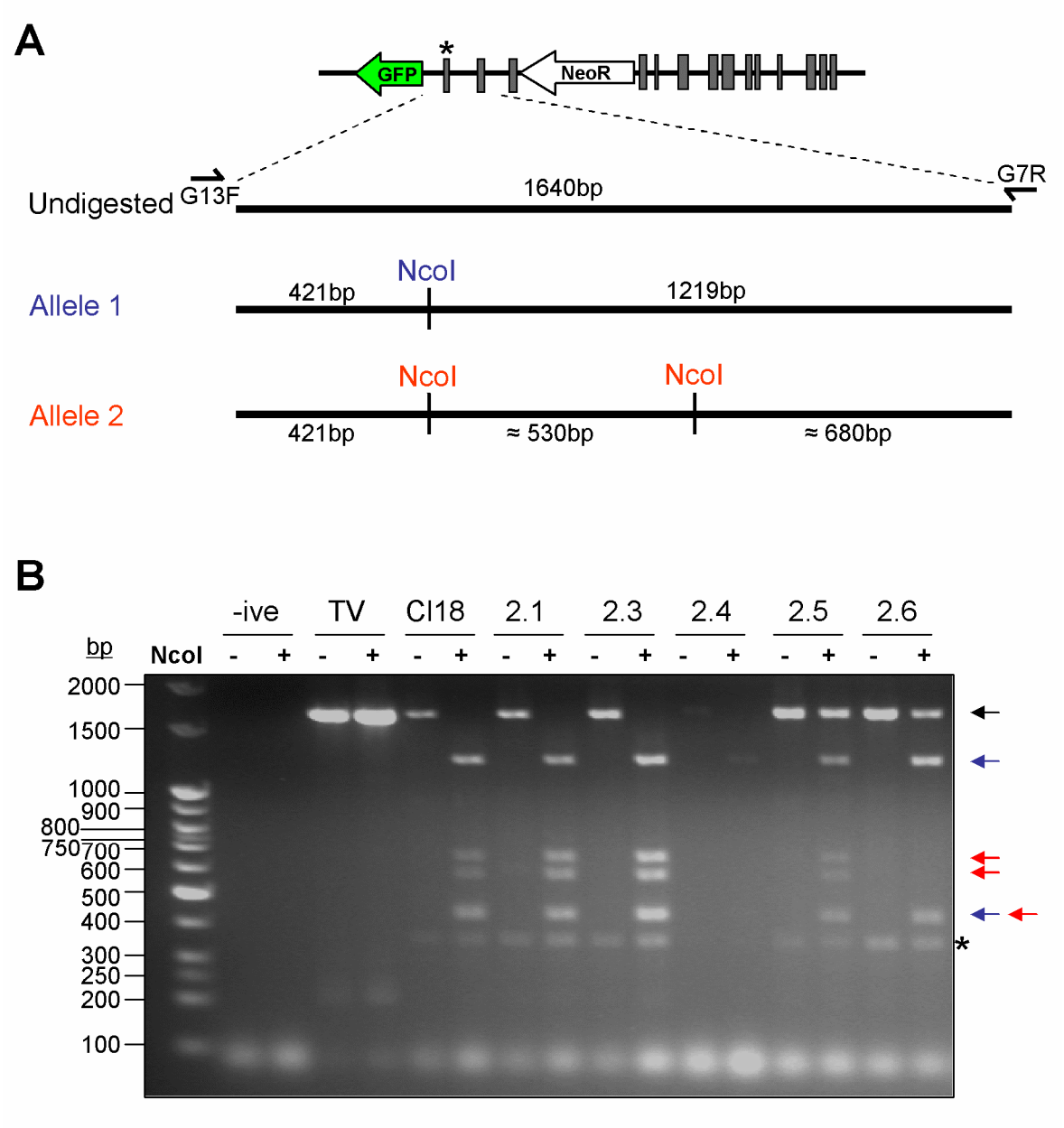

Figure 4.10 Screening for the loss of the ATG start codon
A) Schematic representation of the G13F,G7R PCR Product. Position of NcoI sites and predicted fragment sizes are indicated. B) Digested PCR Products. -ive = No template DNA, TV = Targeting vector used as template, 2.1 and 2.5 = DNA from Non-targeted clone, 2.3, 2.4 and 2.6 = DNA from targeted clones. Products from digestion of Allele 1 are shown in Blue. Products from digestion of Allele 2 are shown in Red. A non-specific band (*) is amplified by the primers in the PCR.

targeted by Southern blot. It should contain both WT alleles and the allele from the targeting vector. However only the 2 WT alleles are seen. (Figure 4.10-B) Presumably as the targeting vector in this clone integrated randomly, the DNA in the 5′ arm is disrupted such that no PCR product is generated from the integrated DNA. Clone #2.5 which is shown to be targeted by Southern blot but is also GFP positive by PCR retains both WT cCLSPN alleles and the allele from the 5′ arm of the targeting vector. Clones #2.3 and #2.4 although shown to be targeted by Southern blot and GFP negative by PCR have not recombined in such a way as to delete the endogenous start codon. However Clones #2.6 which have been confirmed as targeted by Southern blot and GFP negative by PCR has deleted the ATG start codon. WT allele 2 has been replaced by the targeting vector sequence and only allele 1 remains. This clone was selected for further analysis and for the generation of the complete Claspin$^{-/-}$ cell line. Of all the targeted clones (targeted by Southern blot and GFP negative by PCR) only 5 had completely integrated the entire targeting vector. All of these had targeted WT allele 2. In the others presumably the cross-over event took place too far 3′ relative to the ATG site to allow for its deletion.

4.7. Characterisation of Claspin+/- cells

A basic characterisation of the Claspin$^{+/-}$ clone was carried out. Firstly the Southern Blot was repeated in order to confirm the results from the screening. Figure 4.11-A shows that the diagnostic targeted band of 9.3kb is detected when using the external probe. Importantly this is also the only band detected by the internal probe showing that the targeting vector has only integrated once into the genome. The intensity of the bands is also roughly equal and half that of the signal obtained on the +/+ cells indicating that there are indeed only two copies of cCLSPN present in the DT40 genome.

But how does the loss of one copy of the cCLSPN gene impact on expression at the protein level? To address this issue +/+ and +/- cells were harvested in triplicate for Western Blot analysis using the HF64B antibody described in section 3.5.1. A representative blot is shown in Figure 4.11-B(i) with the quantitation of triplicate blots shown in Figure 4.11-B(ii). From the quantitation it is clear that only half the amount of full length cClaspin is expressed in +/- cells compared with the +/+ cells – strongly suggesting that this targeting strategy does indeed

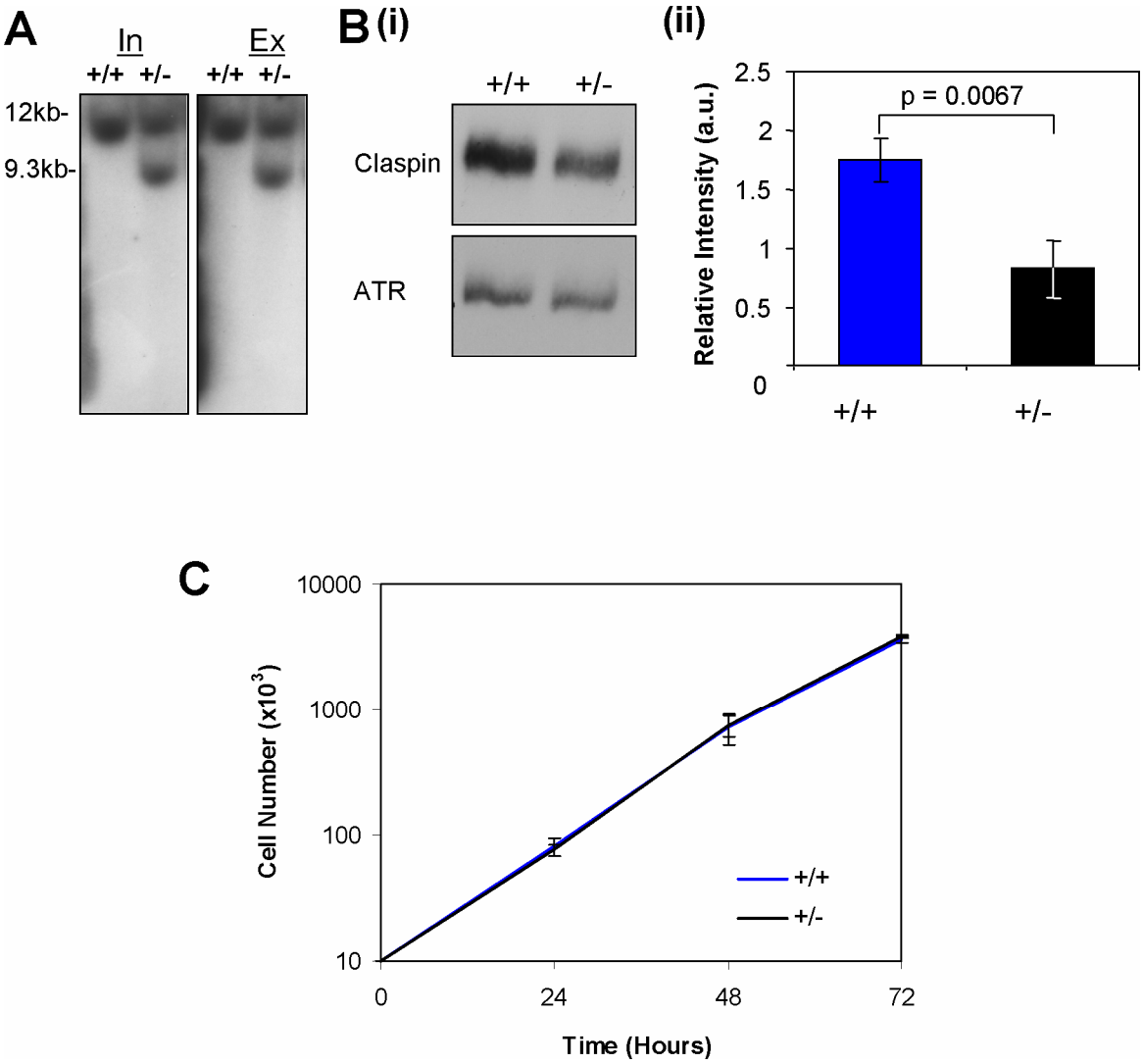

Figure 4.11 Characterisation of cClaspin+/- Cells
A) Clone #2.6 was subjected to Southern blot using internal (In) and External (Ex) Probes in order to confirm targeting. See Figure 4.9-B for targeting diagram. B)(i) Representative Western Blot using HF64B antibody showing cClaspin Levels in +/+ and +/- cells. An equal quantity of lysate as determined by Bradford Assay was loaded for +/+ and +/- cells. (ii) Quantification of cClaspin levels in +/+ and +/- cells. n=3. C) Growth curve with +/+ and +/- cells. Cells were seeded at 1 × 10^4 cells/ml and then counted using a Casy counter at the indicated times. This experiment was repeated in triplicate. The doubling time for +/+ = 9.62hrs and 9.46 hrs for +/- cells. n=3.

disrupt the expression of full length cClaspin. It is worth noting however that this reduction in protein expression has no impact on the growth of the cells. From Figure 4.11-C it can be seen that the +/- cells grow at the exactly the same rate as the +/+ cells, both doubling roughly every 9 hours.

While the a reduction in cClaspin levels does not impact on the growth of the cells under unstressed conditions I next wanted to test whether these cells would show any defects in response DNA damage. Following DNA damage DT40 cells progress through S phase and arrest in G2 in order to repair the DNA damage before entering mitosis. This arrest is usually maximal around 8 hours. A BrdU pulse experiment over an 8 hour time-course shows which cells are actively replicating their DNA at a given point. From Figure 4.12-A it can be seen that there is no difference between the +/+ and the +/- cells in their ability to progress through S phase in the presence of damaged DNA in order to arrest in G_2 phase and repair the damaged DNA. Examining cClaspin expression and the activation of Chk1 by western blot throughout this time course shows that the +/- cells behave similarly to the +/+ cells. There is no defect in Chk1 phosphorylation as the pS345 Chk1 signal is as high and maybe even slightly higher in the +/- cells. (Figure 4.12-B) Chk1 phosphorylation at S345 is rapidly induced but soon decreases only to rise again at the later time points. cClaspin is stabilised following DNA damage in the +/+ cells but this stabilisation does not occur in the +/- cells probably due to the lower amount of cClaspin present in these cells. How significant this stabilisation of cClaspin is, is not apparent given that the +/- cells can activate and maintain a block in their cell cycle just as efficiently as the +/+ cells. There is no apparent reduction in the levels of phospho-Chk1 indicating that the level of cClaspin remaining in the +/- cells is sufficient to mediate the activation of Chk1 and hence the G_2-M checkpoint. Given this data it is not surprising therefore that directly assaying the ability of the cells to activate the G_2-M checkpoint, by the nocodazole trap method, shows that the +/- cells are completely competent for this checkpoint. (Figure 4.12-C)

One of the most striking phenotypes of the $Chk1^{-/-}$ DT40 cells is their ability to enter into mitosis in the presence of incompletely replicated DNA (Zachos et al, 2005). If the level of cClaspin in the +/- cells was too low to mediate the activation of Chk1, a defective S-M checkpoint might also be observed in the +/- cells. $Chk1^{-/-}$ DT40 cells begin to enter the premature mitosis between 9 and 16

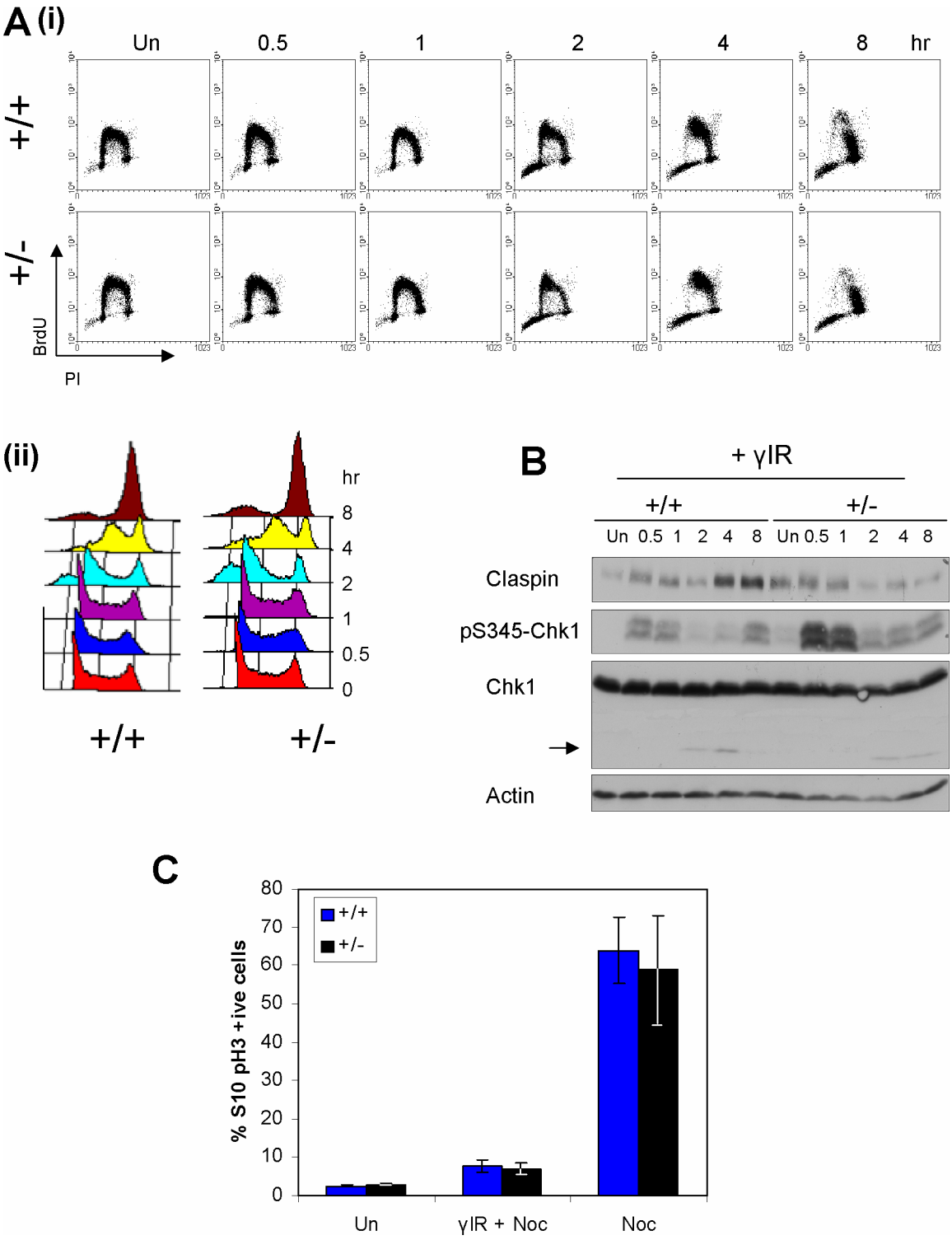

Figure 4.12 Characterisation of DNA damage response in Claspin+/- Cells
A) BrdU time-course following 10Gy γ-IR. (i) BrdU plots (ii) Corresponding PI profiles B) Western Blot analysis of cells treated with 10Gy γ-IR. Time-points are the same as for flow cytometry. Arrow indicates a fragment of Chk1 that is generated from caspase mediated cleavage. This indicates that some degree of apoptosis is taking place in the culture at this time. C) G_2-M Checkpoint Assay. Cells treated for 8 hours with 1μg/ml Noc with or without 10Gy γ-IR .

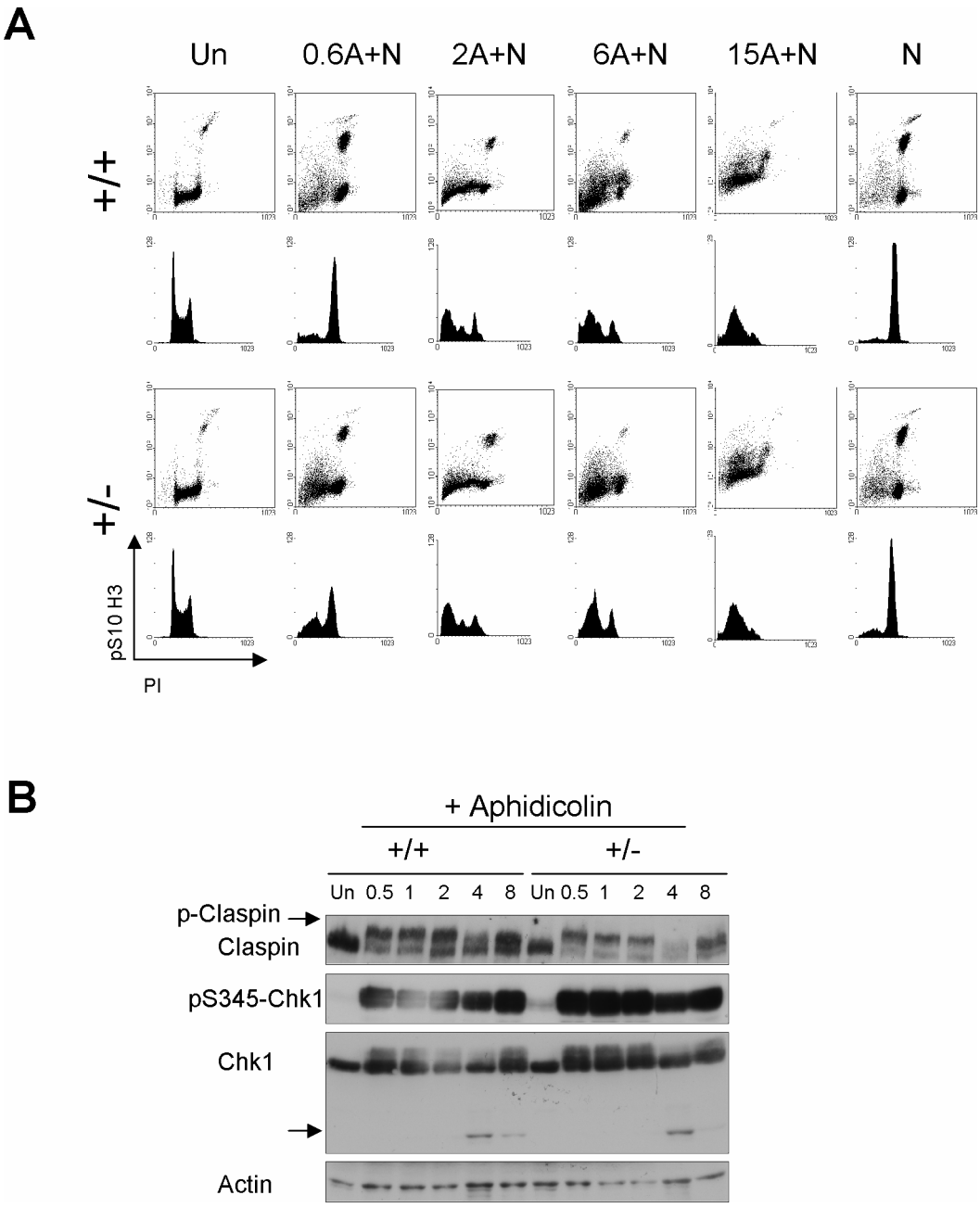

Figure 4.13 Characterisation of the Claspin+/- Cells following Replication Stress
A) +/+ and +/- cells were treated for 16 hours in the presence of 1µg/ml Nocodazole and the indicated concentration of Aphidicolin (µM) The cells were then harvested and analysed for the presence of mitotic cells (pS10 H3 positive) by flow cytometry. B) Cells were treated with 15µM aphidicolin. Cells were harvested at the indicated time and blotted for the indicated proteins. Arrow indicates a fragment of Chk1 that is generated from caspase mediated cleavage. This indicates that some degree of apoptosis is taking place in the culture at this time.

hours post inhibition of DNA replication (Oehler, 2008). Therefore a similar assay was preformed with the +/+ and +/- cells. However the Cl18 DT40 background appears to be much more sensitive to replication stress than the WT DT40 background used to generate the $Chk1^{-/-}$ cells. (Figure 4.13-A) I was unable to identify a concentration of aphidicolin (a DNA polymerase inhibitor) that was sufficient to inhibit DNA replication but not so toxic that it would kill the cells. A concentration of 0.6μM Aphidicolin was not toxic to the cells but not sufficient to block DNA replication. Even a concentration of 2μM Aphidicolin was sufficient to induce significant cell death. Therefore it was not possible to assay the +/- cell for their ability to mediate the S-M checkpoint. Further optimisation of this assay, such as analysing the cells at an earlier timepoint e.g. 9 hours or using a less toxic but equally effective DNA replication inhibitor e.g. thymidine, will have to be carried out.

In addition to the flow cytometry data, the cells were also analysed for Chk1 activation by western blot. (Figure 4.13-B) Following replication stress Chk1 kinase activity is much more strongly induced than following γ-IR (Walker et al, 2009). This is also seen here – with both Chk1 S345 phosphorylation and the level of Chk1 autophosphorylation (autophosphorylated Chk1 migrates slower on SDS-PAGE) being much stronger than compared with γ-IR. There is no defect in Chk1 phosphorylation in the +/- cells. Claspin is also phosphorylated following replication stress leading to an alteration in its electrophoretic mobility by SDS-PAGE. Even though the total levels of cClaspin are lower in the +/- cells it appears that, to compensate, comparatively more of the cClaspin in the +/- cells becomes phosphorylated than in the +/+ cells. This level of phosphorylation is sufficient to maintain the high levels of S345 Chk1 phosphorylation.

In summary the +/- cells even though they express approximately half of the amount of cClaspin compared with +/+ cells show no obvious defect in unperturbed DNA replication, in Chk1 activation following replication stress or in the activation of the G_2-M DNA damage checkpoint.

4.8. Discussion

This chapter described the successful generation of a cClaspin$^{+/-}$ cell line. However it is evident that some parts of the overall strategy employed to generate the cell line did not work as planned.

The first of these was the design of the targeting vectors themselves. As the option for deleting the entire cCLSPN locus was not available (due to the large size of the locus and the lack of some crucial sequence data) the next best option was to delete a crucial part of the gene. As Claspin is not a kinase and the function of each domain is not fully understood choosing the region to delete proved difficult. Therefore it was decided to try and delete as much of the gene as possible using the first targeting strategy describes. However this strategy was flawed by the fact that a robust screening strategy was not thought of at the time. Even though attempts were made to genotype the clones transfected with this construct, no satisfactory screening method was found. This strategy was therefore abandoned. However the second strategy employed was more robust and even though it was designed to knock out a smaller region it generates a null allele as can be seen from the reduction in cClaspin levels in the cClaspin$^{+/-}$ cells.

The second major obstacle that was encountered arose from the use of the MCM-DT40 cells. These cells were chosen in order to make full use of the floxed resistance cassettes that had recently become available (Arakawa, 2001). A cell line expressing inducible Cre recombinase easily facilitates the recycling of the drug resistance cassettes allowing compound mutations to be made and speeding up the generation of knock-out cell lines due to the necessity to only generate one targeting vector. There was no reason to suspect that there would be a problem with these MCM DT40 cells. They had been successfully used in order to make knock-out DT40 cell lines previously.(Brummer et al, 2003; Brummer et al, 2002) However the fact that there was a problem with the cells should have been picked up earlier. Even using the GFP expression as a rough estimate as to the targeting efficiency, the GFP negative cells were never more than ≈10% of the cells that grew. It should have been clear that the cells had impaired homologous recombination. Testing this possibility directly lead to the clear

conclusion that these cells had lost capacity for homologous recombination. The reason for this is unknown.

The method of transfection used in order to generate knock-out cells also appears to be very important. The first transfection of the targeting vector into Cl18 cells was done using nucleofection. (Data not shown) Even though these cells display high ratios of targeted integration none of the 15 GFP negative clones screened were positive for correct targeting. This might also explain the reduced targeting efficiency observed when using the ovalbumin construct. A targeting efficiency of only 30% was observed compared with the reported efficiency of 70-80%. It appears that Nucleofection is not suitable for the generation of knock-out DT40 cell lines.

A novel method for negatively selecting clones was used in this strategy. The GFP cassette outside the arms of homology served to rule out some clones that were not targeted. This strategy appeared to work well with none of the clones screened that expressed GFP by visual inspection showing positive targeting. As would be expected however not all of the GFP negative clones were targeted. It is also interesting to note that some of the clones that didn't express GFP actually still contained the GFP expression cassette. Therefore combining a visual inspection with a PCR for the GFP cassette proved more useful.

Although problems were encountered during the generation of the cClaspin$^{+/-}$ cells once the targeting vector and screening strategy were optimised and the correct cells used, obtaining the hemizygous cells was very straight forward. With these cells, in which one allele of cCLSPN was deleted, it was now possible to continue with the strategy and attempt to make the complete knockout.

Chapter 5. Generation of Claspin-/- Cell Line

5.1. Introduction

Having confirmed the genetic deletion of the one cCLSPN allele in Cl18 DT40 cells the next step is to generate the complete knock-out by repeating the process. This is accomplished by targeting the remaining allele of cCLSPN in the hemizygous cells.

5.2. Targeting the second cCLSPN allele

Generating the complete knock-out cell line requires that the second allele of cCLSPN must also be disrupted. As the NeoR cassette in the targeting vector is flanked by MLoxP sites it is possible to remove the cassette by transient Cre transfection and then reuse the same targeting vector. However in order to speed up the knock-out process it was decided to switch to using the Blasticidin version of this same targeting vector. Problems already experienced with Blasticidin should not be encountered with the Cl18 cell line (section 4.5) as clones from this cell line appear within 7 days following plating as opposed to 2-3 weeks for the MCM-DT40 cells.

Hemizygous cells were transfected with the linearised targeting vector and selected in the presence of both Blasticidin and G418. It is possible that the targeting vector could integrate into the already targeted allele. In this case the cells will no longer be resistant to G418. Selecting cells in both G418 and Blasticidin will ensure that any cells which target the NeoR allele will not grow. The results from the first transfection showed that there was a dramatic reduction in the total number of clones that were growing. For this transfection only 7 clones grew. Indeed the average number of clones that grew under double selection, after repeating a further four times, was only 12. (Figure 5.1-A(i)) This was a dramatic reduction from an average of ≈80 clones obtained per transfection when targeting the first allele (See Figure 4.9).

In order to try and clarify this situation more cells were transfected with the linearised targeting vector but this time the cells were selected in parallel in either Blasticidin alone or with both Blasticidin and G418. On this occasion in the presence of Blasticidin alone 109 colonies grew (Figure 5.1-A(ii)) but again under selection with both drugs only 20 colonies grew. A possible explanation

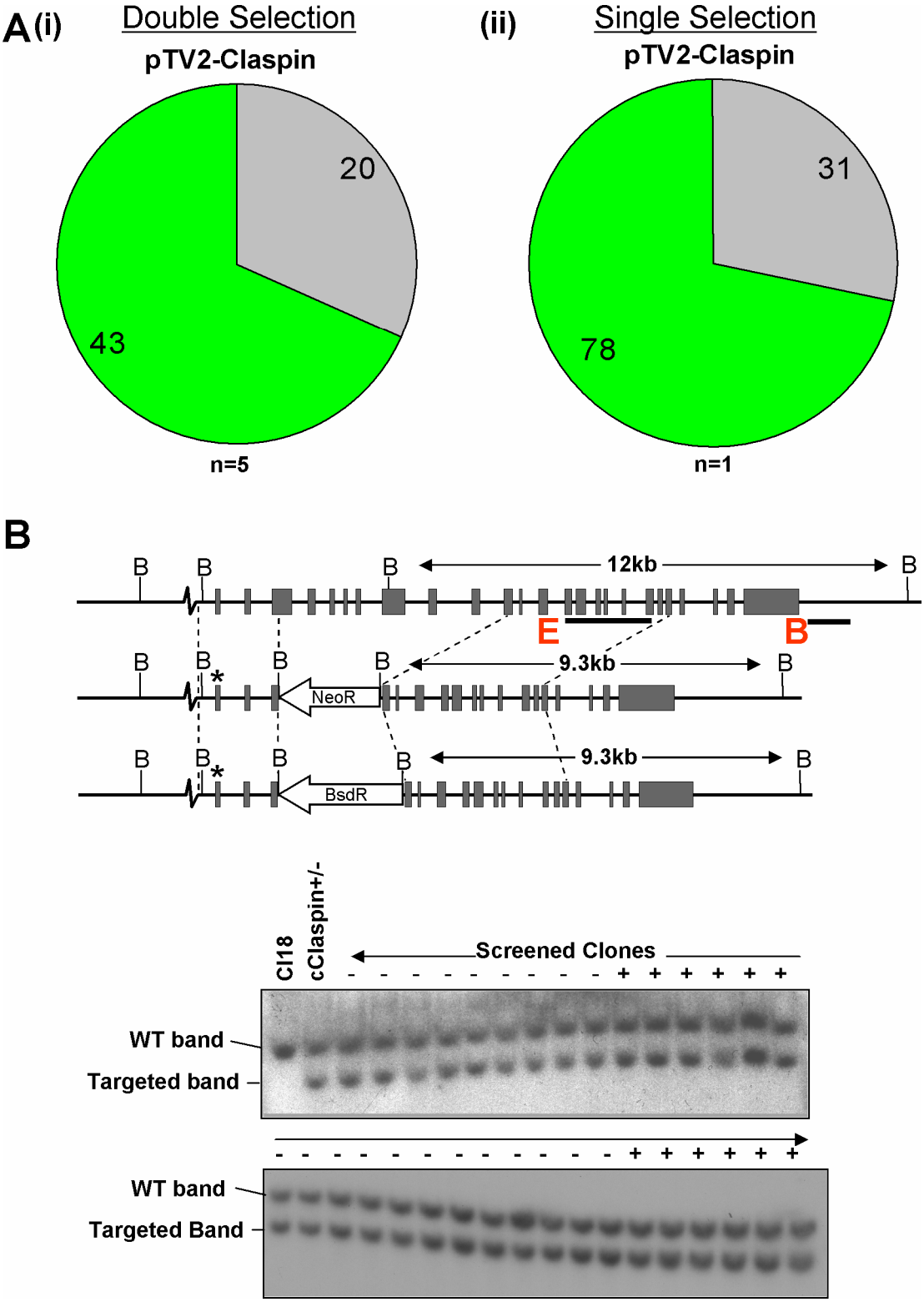

Figure 5.1 Summary of Results and Screening from Targeting the Second cCLSPN Allele
A)(i) Pie chart shows the proportion of GFP negative and positive cells obtained when transfecting cClaspin+/- cells with the pTV2-Claspin targeting vector following selection in both Blasticidin and G418. The numbers are the sum total of all transfections. The number of transfections is shown below the pie chart. (ii) Pie chart shows the proportion of GFP negative and positive cells obtained when transfecting cClaspin+/- cells with the pTV2-Claspin targeting vector following selection in Blasticidin alone. The numbers shown represent data from only one transfection. B) Southern Blot screening strategy for screening for loss of the second allele. Note that both targeting vectors generate the same pattern using this screening strategy. Therefore loss of the WT allele confirm deletion of both alleles. The + and – symbols above the screened clones indicate whether they express GFP or not.

for this is that the BsdR vector is preferentially targeting the NeoR allele – resulting in clones that are re-sensitised to G418. In this case it would be assumed that the vector would integrate faithfully and clones arising from the BsdR vector targeting the NeoR allele would be GFP negative. Therefore to account for the ≈80% reduction in viable clones it should follow that ≈80% of the clones grown under the selection of Blasticidin alone should be GFP negative. However this was not the case. In fact 70% of the clones were GFP positive indicating that they were not correctly targeted. Therefore faithful targeting of the NeoR allele by the BsdR vector cannot explain the dramatic reduction in the number of clones that appear. As noted before however it is possible to integrate the 3′ arm of homology correctly while not recombining correctly at the 5′ end. This possibility, as it was not directly tested by screening all the clones by Southern blot, cannot be ruled out.

Another possible explanation for the lower number of viable clones is that cells cannot survive in the absence of cClaspin. The targeting efficiency with the NeoR targeting vector for targeting the first allele was 30% when screening the GFP negative clones. It is reasonable to assume that the BsdR version of the targeting vector will target at approximately the same efficiency. Therefore roughly $^{1}/_{3}$ of GFP negative clones derived from targeting the second allele should be targeted. However this was not the case. In total 29 GFP negative clones were screened by Southern blot but none of them were targeted. (Figure 5.1-B)

The assumption that $^{1}/_{3}$ of GFP negative clones should be targeted would only be valid if the overall targeting efficiency of the Claspin+/- cells had not decreased. Although Claspin has not been implicated in the process of homologous recombination, if it did play a role then the reduction in cClaspin protein in the Claspin+/- cells may impact on the targeting efficiency. To directly test this possibility the Claspin+/- cells were transfected with the same Ovalbumin targeting construct used to test the efficiency of the MCM-DT40 and Cl18 cells. Southern blot analysis of the clones that grew show that there is no defect in targeting efficiency in the hemizygous cells. Of 60 clones screened 38 were targeted giving a targeting efficiency of 60%. Therefore a reduction in targeting efficiency cannot account for the inability to obtain a Claspin$^{-/-}$ clone. In fact this high targeting efficiency probably points to the fact that a combination of

targeting of the remaining allele (resulting in cell death due to lack of Claspin) combined with targeting of the NeoR allele (resulting in cell death due to sensitivity to G418) probably accounts for the dramatic reduction in viable clones. Due to the nature of the Southern blot screening strategy used to screen the clones both targeted alleles co-migrate at 9.3kb on the Southern blot. Therefore it is impossible to distinguish between them.

Although the inability to obtain a cClaspin-/- cell line by routine targeting of the second allele is not direct evidence that completely targeting Claspin in DT40 cells is incompatible with the cells viability, it is enough evidence to change strategy and generate a conditional knock-out cell line.

5.3. Generation of a conditional cClaspin expressing cell in the Claspin+/- background

The generation of conditional knock-out cells using the DT40 system is possible (Hudson et al, 2002). By far the most popular method for achieving this is the Tet On/Off system based on the Tetracycline regulated gene expression in bacteria (Hillen et al, 1982; Jorgensen & Reznikoff, 1979). The tetracycline transactivator (tTA) system provides a convenient method for the controlled expression of proteins in cells. (Figure 5.2) The regulation is achieved at the level of gene transcription by the binding of the tTA to the Tet Operator (TetO) sequences (termed Tet Responsive elements - TRE) placed immediately up-stream of the gene of interest. Binding of the tTA, and hence indirectly activation of transcription, is regulated by tetracycline. The tTA itself is composed of two parts; the Tet repressor and the transactivation domain viral VP16 protein. The Tet repressor binds the TRE in the absence of its high affinity ligand, tetracycline. Transcription is regulated by the VP16 transactivation domain which serves to recruit transcriptional co-transactivators. This system has been successfully used with the DT40 system for the generation of conditional null cell lines (Sonoda et al, 2001a; Sonoda et al, 1998; Wakasugi et al, 2007).

In order to generate the Claspin+/- + cClaspin ON/OFF cell line the first step is to generate Claspin+/- clones that stably express the tTA. (Figure 5.3) Claspin

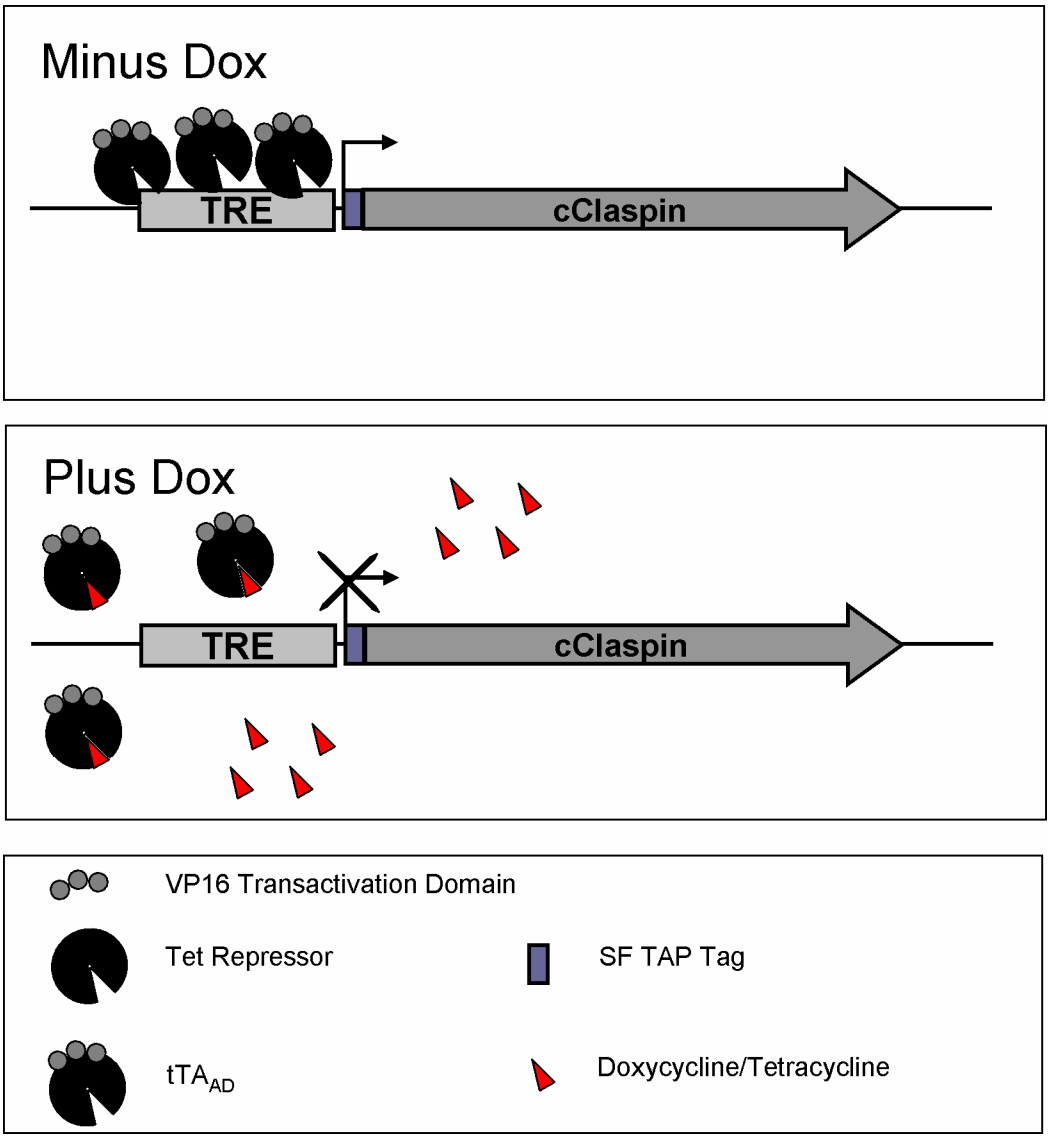

Figure 5.2 Overview of Tetracyline controlled transcriptional regulation
In the absence of Dox the tTA binds to the TRE and mediates transcription of the cClaspin mRNA. Upon addition of Dox to the system it binds to the tTA and induces an conformational change which renders the tTA incapable of binding to DNA. Consequently gene transcription is shut down.

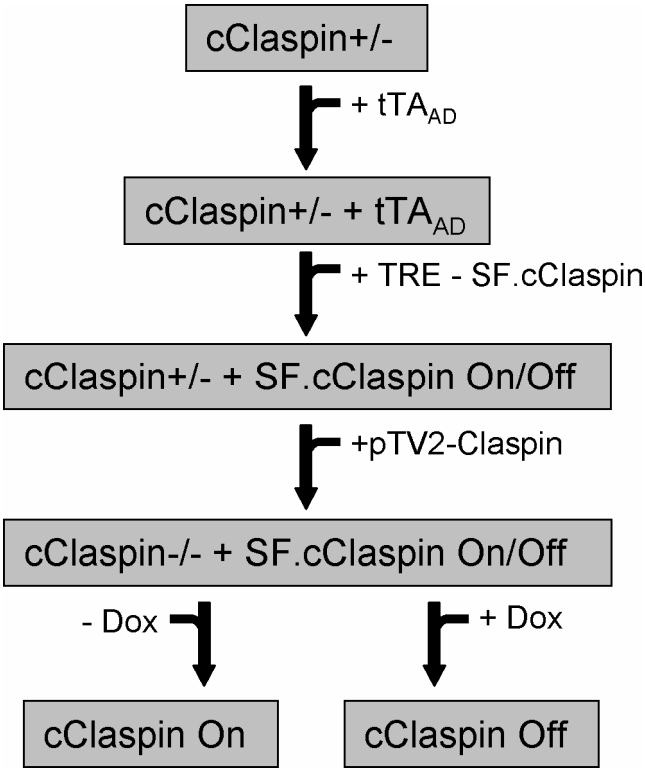

Figure 5.3 Overview of the generation of Claspin On/Off Cell line
A Claspin +/- cell line is stably transfected with the 'advanced' tet-Transcriptional Transactivator. (The advanced tTA is an improved version of the tTA available from Clontech) Once the expression and functionality of the tTA is verified, the cell line is the transfected with a tagged version of Claspin (Strep II-FLAG) under the control of a Tet Responsive Element (TRE) promoter. A cell line expressing near endogenous levels of Claspin with tight regulation by tet is selected. The second allele of Claspin is targeted in this cell line to generate an On/Off cell line.

+/- cells were transfected with the tTA_{AD} vector which had been modified to replace the NeoR cassette with a PuroR cassette. The tTA_{AD} tet transactivator is an updated version of the original tTA provided by Clontech (Resnitzky et al, 1994). The improved version is optimised for higher levels of expression and contains three repeats of the minimal VP16 transactivation domain. Clones were selected in the presence of Puromycin and then tested for tTA_{AD} expression. A luciferase assay was carried out in order to test for the presence of functional tTA_{AD}. 9 clones were transfected with the pTRE-Tight Luciferase construct (and pTK-Renilla Luciferase) and were grown in the presence or absence of doxycycline for 24 hours. A dual luciferase assay was then preformed in order to identify clones with high luciferase activity that could be repressed in the presence of dox. (Figure 5.4-A) Two clones were selected that showed either high luciferase activity (Clone #4) or very tightly controlled luciferase activity (Clone #1). To confirm this result these two clones were transfected with pTRE-d2EGFP which expresses a destabilised form of GFP under the control of a Tet responsive promoter. As can be seen from Figure 5.4-B both cell lines show both high GFP expression which is tightly controlled by the addition of dox. Together this data suggests the cell line contains functional levels of the tTA_{AD}.

Two expression cassettes were generated where either a C-terminally GFP tagged cClaspin protein (cClaspin.EGFP) or an N-terminally tagged SF (Strep II/Flag TAP tag) cClaspin protein (SF.cClaspin) were cloned down stream of a TRE promoter. The tag will distinguish the ectopically expressed protein from the endogenous protein left in the cell. Expression of the tagged versions of cClaspin from these plasmids was confirmed by transient expression in a HEK293 Tet-Off cell line (Clontech). (See Figure 5.5) Expression from the TRE-Tight plasmids was lower than from a plasmid where expression is driven by the CMV promoter. Having confirmed that the plasmids were functional, they were then co-transfected with a Histidinol Resistance (HisR) cassette into both of the tTA_{AD} expressing clones isolated above. Clones were selected in the presence of Histidinol and then were screened for expression of the transgenes. However unfortunately, following multiple transfections and rounds of screening for expression of the transgenes I was unable to identify a clone that expressed either the SF-cClaspin or cClaspin-EGFP. Due to time constraints, I could not complete this part of the project.

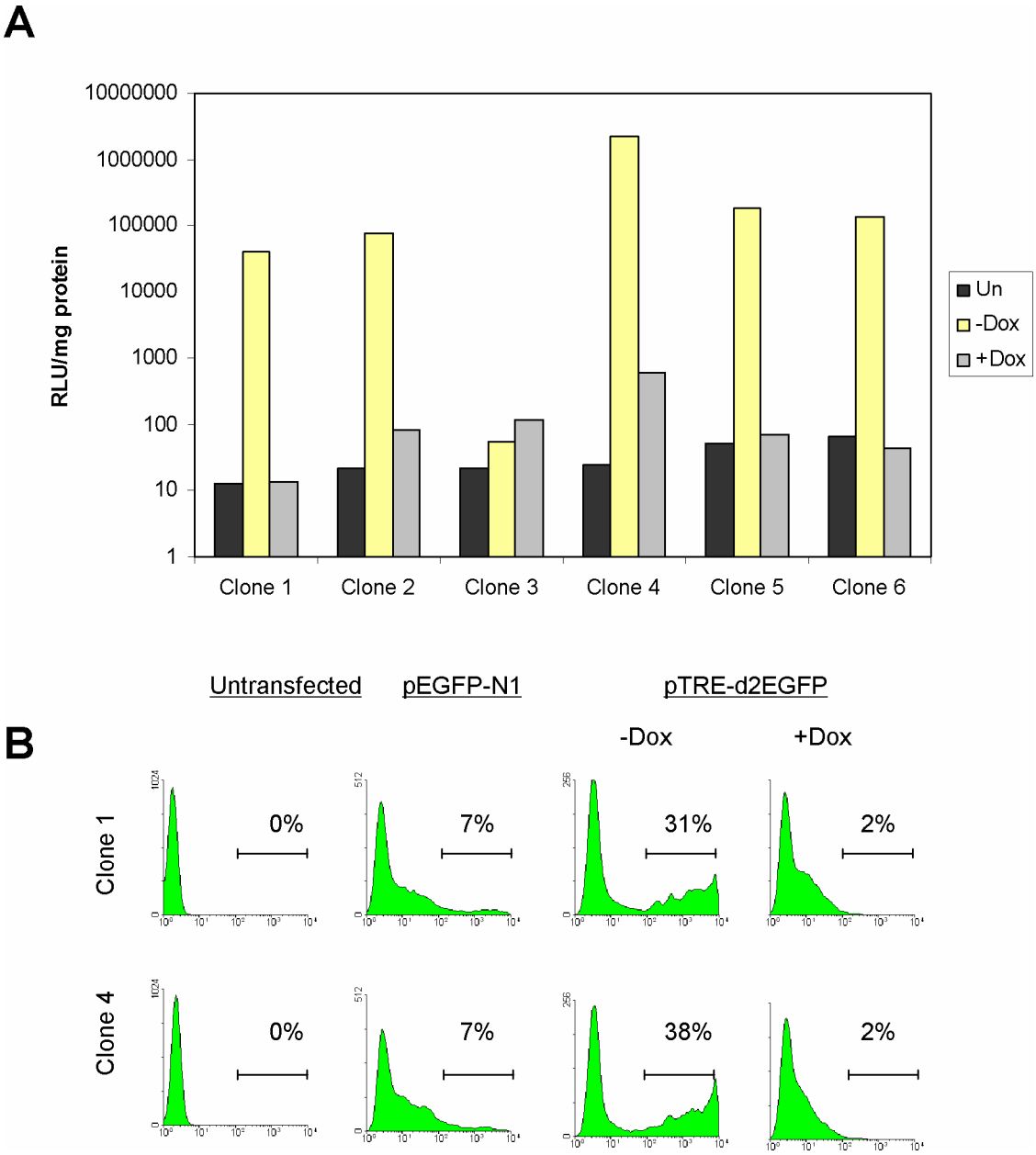

Figure 5.4 Testing the tTA_{AD} expression in Claspin+/- + tTA_{AD} Clones
A) Luciferase Assay. Claspin+/- cells transfected with the pTet-Off Advanced plasmid were selected in the presence of puromycin. The resulting clones were then transiently transfected with pTRE-Tight Luc and pTK-Renilla and were grown in the presence and absence of Dox. A Dual Luciferase Assay preformed. The data was normalised for both the amount of protein and Renilla Luciferase Expression.

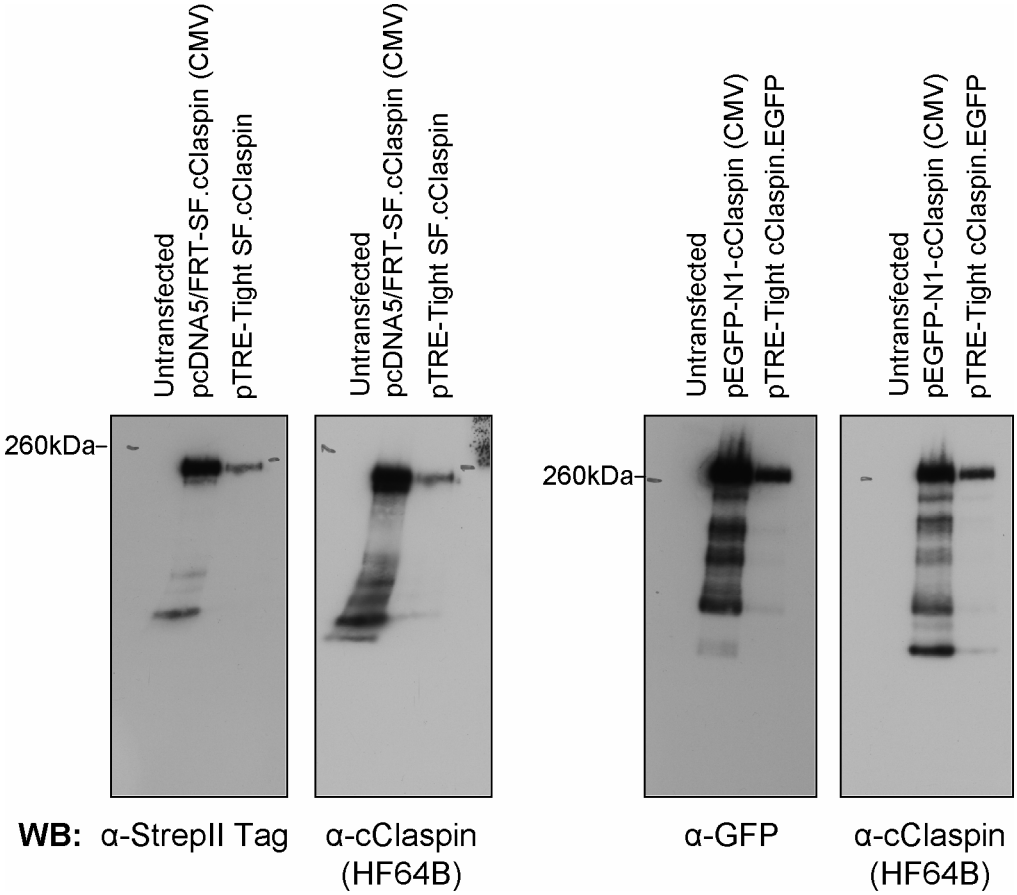

Figure 5.5 Testing pTRE-Tight SF.cClaspin and pTRE-Tight cClaspin.EGFP vectors
HEK293 Tet-Off cells were transfected with the indicated plasmids and blotted with the indicated antibodies. The HF64B antibody raised against cClaspin does not detect hClaspin.

5.4. Generation of Claspin$^{On/Off}$ cell line

Due to the difficulty in obtaining the conditional cClaspin expressing cell line the Claspin$^{On/Off}$ cell line could not be generated. In theory once this cell line is generated, the second allele of cCLSPN can be targeted and a cell line that no longer expresses cClaspin from the endogenous locus selected. The transgene that kept the cells alive during the targeting process can then be switched off in order to reveal the null phenotype.

5.5. Downregulation of cClaspin expression using siRNA and shRNA

Due to the difficulties described above in generating the cClaspin$^{-/-}$ cell line an alternative approach was also attempted. Although the main advantage of the DT40 system is the generation of a clean genetic cell line deficient for the protein of interest, this can sometimes be a difficult and lengthy process. shRNA technology has greatly improved over the past years had been shown to work in DT40 cells (Yuan et al, 2006) and can prove especially useful in the creation of conditional knock-down cell lines (Johnson et al, 2009). It was therefore decided to attempt the generation a conditional cClaspin Knock-down (cClaspin^{KD}) cell line using shRNA. Given that the major phenotype we see with the cClaspin$^{-/-}$ cells is one of lethality an inducible system would be valuable in characterising this cell death and might allow the identification of novel functions of cClaspin by titering cClaspin levels such that its function is impaired but not to the extent that it induces cell death.

The system developed in order to do this was composed of two parts – a Flp-In – T-Rex (Invitrogen) DT40 cell line and a modified pSuperior (Oligoengine) shRNA expression cassette. The Flp-In T-Rex DT40 cell line (made in the Chk1$^{-/-}$ background) was developed by Mark Walker. (Figure 5.6-A) It contains a defined locus into which expression cassettes can be integrated. The process is highly efficient and is mediated via the Flp recombinase. The cell line also expressed the Tet repressor to allow for conditional expression of the hairpin. The pSuperior vector drives expression of the shRNA from a H1 PolIII promoter. It contains a TetO site between the H1 promoter and the shRNA transcription start site which represses transcription of the hairpin in the absence of

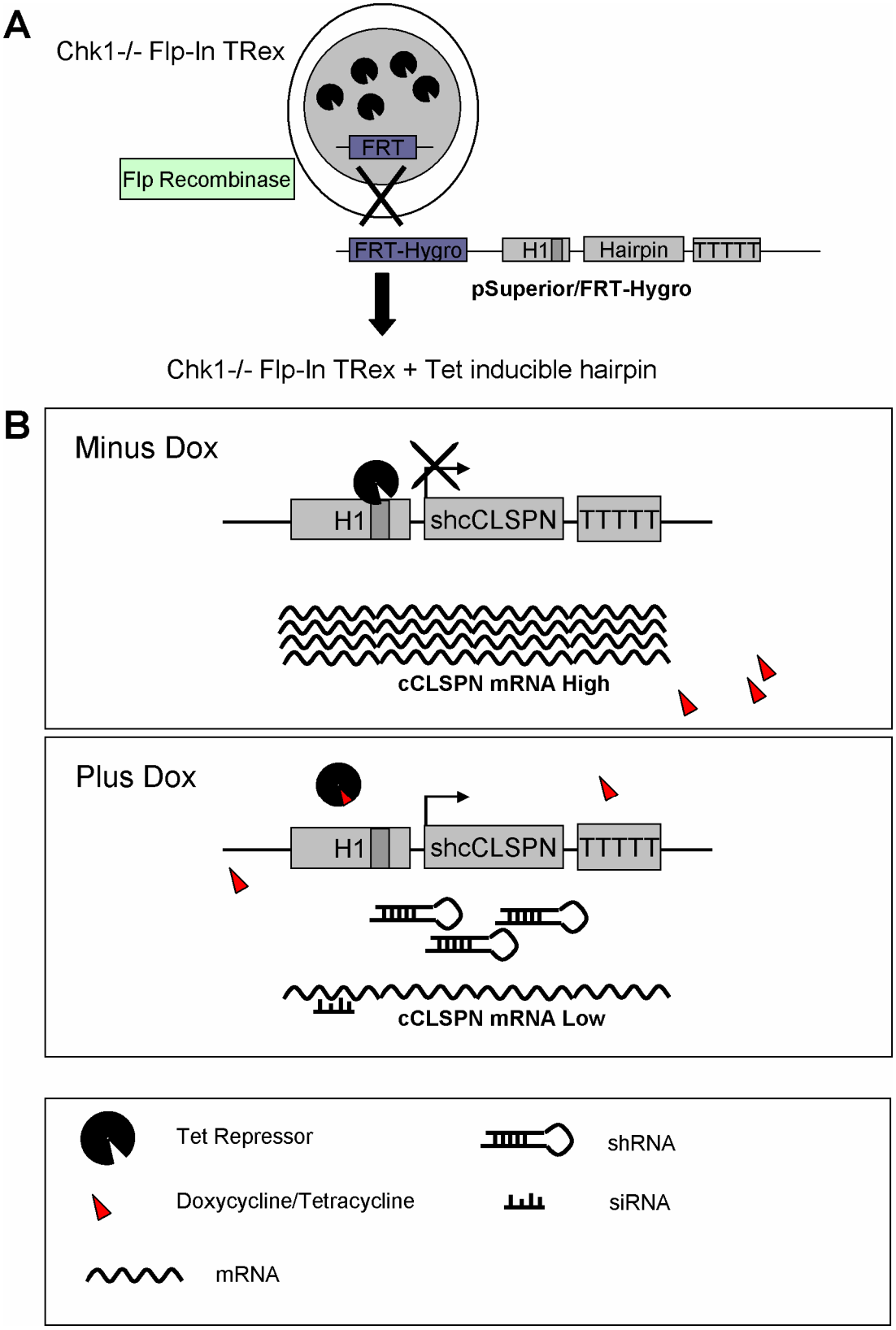

Figure 5.6 Schematic Overview of the generation of Inducible shRNA expressing cell line
A) The Chk1-/- Flp-In T Rex cell line expresses the Tet repressor and contains an FRT site. Flp Recombinase (transiently expressed) mediates recombination between the FRT site in the genome of the cell line and the FRT site in the plasmid. This results in stable incorporation of the plasmid of interest. B) In the absence of Dox the Tet Repressor binds tightly to the H1 Pol III promoter inhibiting access to the polymerase. The hairpin is not expressed and there is no effect on mRNA levels. In the presence of Dox the repressor is displaced allowing expression of the hairpin which knocks-down mRNA levels.

tetracycline. (Figure 5.6-B) Upon the addition of tetracycline the hairpin is expressed which targets the cCLSPN mRNA for degradation. This vector was modified to replace the Puromycin resistance cassette with the FRT-Hygro cassette from pcDNA5/FRT. It is this FRT site that recombines with the FRT site stably integrated into the Flp-In cell line. The resulting plasmid, pSuperior/Frt-Hygro, was used to express shRNA oligos directed against cCLSPN and cCDK2 as a control. The shRNA oligos were designed using an algorithm developed by Oligoengine. The most suitable hairpins from this prediction program were selected and cloned into the expression vector.

Clones of the Chk1-/- Flp-In T-Rex cells having stably integrated the shRNA expression cassette directed against either cCLSPN or cCDK2 were isolated. They were then grown in the presence of tetracycline to induce the expression of the shRNA and left for 72 hours to allow processing of the shRNA and knock-down of cClaspin or cCdk2. The cells were then analysed by western blot for the presence or absence of the protein of interest. (Figure 5.7-A) However after screening multiple clones for both the shcCLSPN and the shcCDK2 no clone was identified for its ability to knock-down its target protein.

Possible reasons for this are that the shRNA is not actually expressed from the cassette, there is a defect in the shRNA processing or the siRNA generated does not mediate effective knock-down it its target mRNA. To test this, the equivalent siRNA duplexes corresponding to the siRNA duplexes that would be generated from the shRNA were synthesised and transfected directly into cells. The method used had previously been shown to achieve knock-down of Chk1 in DT40 cells (Churikov & Price, 2008). Examining the level of knock-down 72 hours post transfection revealed that these oligos could not mediate knock-down of either cClaspin or cCdk2. (Figure 5.7-B) As DT40 cells are difficult to transfect the experiment was repeated with DF-1 chicken fibroblasts (Himly et al, 1998). This time the EDD positive control siRNA seems to mediate knock-down of its target indicating that the siRNAs are being effectively delivered to the cells. (Figure 5.7-C) However the cCLSPN siRNA did not reduce cClaspin levels, but the siRNA directed against cCDK2 did appear to show some efficacy. The reason this oligo does not work as a hairpin is probably due either to low expression of the hairpin or defective processing to generate functional siRNA.

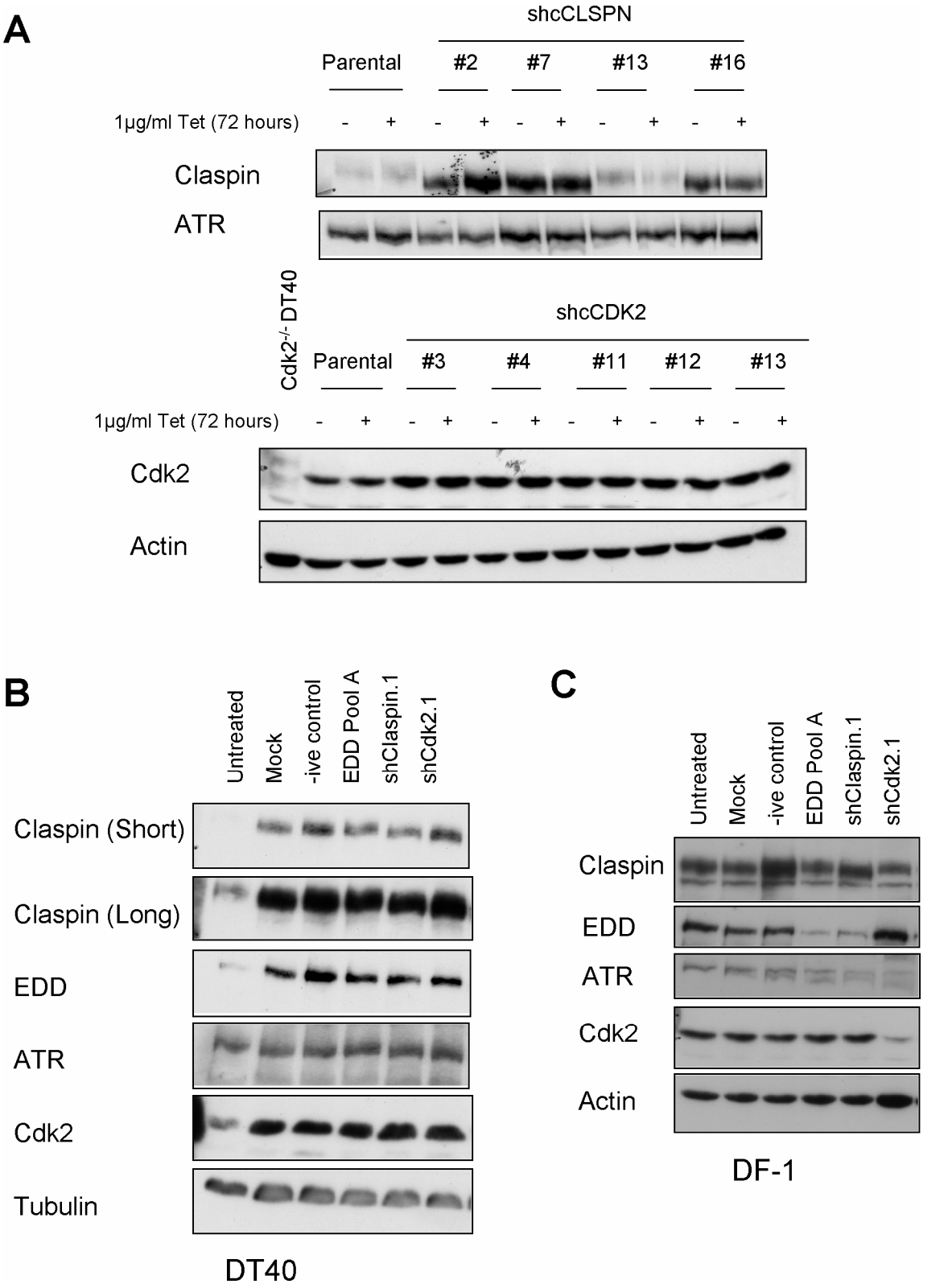

Figure 5.7 shRNA Knock down of cClaspin
A) Clones stably transfected with either shcCLSPN or shCDK2 were treated with Tetracycline for 72 hours. Clones were then analysed for the expression of cClaspin or cCdk2 by western blot. Cdk2-/- DT40 cells were obtained from Helfrid Hochegger (Hochegger et al, 2007) B) DT40 cells were transfected with the indicated siRNA duplexes. 72 hours post-transfection the cells were harvested and analysed by western blot for the indicated proteins. C) DF-1 fibroblasts cells were transfected with the indicated siRNA duplexes. 72 hours post-transfection the cells were harvested and analysed by western blot for the indicated proteins.

The oligos chosen to knock-down cClaspin are not effective. More oligos would need to be tested for their ability to first work as an siRNA and then they could be tested for their ability to work in the shRNA expression cassette.

5.6. Discussion

The major unresolved issue from this Chapter is whether knocking out cClaspin in DT40 cells is actually lethal. The inability to obtain Claspin$^{-/-}$ cells by simply targeting the second allele is a strong indication that this is the case, but is not definitive proof. To resolve this issue it is necessary to generate a conditional knock-out and characterise the cell death following repression of the rescuing transgene. This has also however proven unsuccessful. Why a cell line that conditionally expresses either the SF.cClaspin or cClaspin.EGFP cannot be obtained is currently unknown. It is not due, however, to the cells not being able to tolerate high expression of these transgenes. Stable cell lines have been made with these plasmids previously in DT40 cells but in different genetic backgrounds. The expression level in these cells is roughly 10 times above normal level. Therefore cells can tolerate high levels of cClaspin. Efforts to generate this Claspin+/- cell line conditionally expressing cClaspin will continue.

What options remain for the generation of the conditional cell line? Obviously the most straightforward option would be to continue with the current strategy and obtain a cell line in which cClaspin can be switched off by the addition tetracycline. The major hurdle in this effort is obtaining enough cell lines to screen in order to identify a cell line that is positive for both tTA_{AD} expression and that also has integrated enough copies of the TRE-cClaspin transgene. This process should be relatively straight forward as DT40 cells quite readily integrate exogenous DNA randomly into their genomes. However it may be necessary to increase the probability of obtaining the correct cell line by using enzyme mediated recombination. Systems such as the Jump In™ System from Invitrogen which uses ΦC31 integrase-mediated recombination to stably integrate DNA of choice at defined genomic regions (Groth et al, 2000) or the Tol2 transposase system which has been shown to work in chicken cells (Sato et al, 2007).

Targeting the second allele with a different targeting vector might also prove fruitful. The chicken genome sequence has been updated since the release of the first draft. In it more DNA sequence flanking the cCLSPN gene has become available. This allows the generation of external probes that could be used in order to screen clones targeted by the first strategy using SacI digested genomic DNA. Up until now this targeting vector had been abandoned due to the unreliability of screening resulting clones by Southern blot. Targeting the second allele by a different mechanism to the first reduces the possibility of targeting of the already targeted allele.

An alternative approach would be to completely redesign the entire knock-out strategy. Conditional alleles in mice are often generated by flanking exons in critical regions of genes by LoxP sites. Upon the controlled activation of Cre recombinase the intervening regions are deleted and a null allele is generated. Such an approach could also be taken with DT40 cells – but would require significantly more work.

It may also prove useful to revisit the conditional shRNA knock-down approach. This approach requires the identification of suitable oligos that effectively knock-down cClaspin levels. Once identified the hairpin could be in the cClaspin$^{+/-}$ background – which already expresses half the amount of cClaspin compared with the Cl18 cells, in order to reduce the levels of cClaspin. This would of course require in addition to the shRNA that the cells express the Tet Repressor to control expression of the hairpin.

More work will be required in order to complete the DT40 cClaspin knock-out cell line.

Chapter 6. Analysis of cClaspin Phosphorylation

6.1. Introduction

Claspin was originally identified as a mediator protein which facilitated the activation of Chk1 (Kumagai & Dunphy, 2000). It is thought that Claspin mediates this function through protein-protein interactions, presumably by bringing ATR into close enough proximity or in the correct orientation to facilitate Chk1 phosphorylation and activation. It is known that Claspin and Chk1 interact and this interaction is dependent on phosphorylation of Ser/Thr residues in the Chk1 Binding Domain of Claspin. The kinase that mediates phosphorylation of these sites remains unknown. The possibility that this kinase was actually Chk1 itself had been raised in the literature (Chini & Chen, 2006). Having generated an antibody that recognises cClaspin (Section 3.5.1) and having $Chk1^{-/-}$ DT40 cells available it was decided to try and examine this further.

6.2. Analysis of cClaspin Phosphorylation following Replication Stress

A system was developed where the phosphorylation of cClaspin could be monitored following replication stress. Claspin has been shown previously to undergo a phosphorylation induced mobility shift in response to checkpoint activation following replication stress (Chini & Chen, 2003; Clarke & Clarke, 2005; Kim et al, 2008). In order to analyse cClaspin phosphorylation following replication stress a similar system was set up. DT40 cells were treated with aphidicolin (directly inhibits DNA polymerase α, δ and ε) or Hydroxyurea (which inhibits ribonucleotide reductase thus decreasing available dNTP pools) to induce replication stress and the resulting lysates were run on different SDS-PAGE gels in order to maximise the phosphorylation induced mobility shift observed. Samples treated with 60μM aphidicolin and run on a 6% SDS-PAGE gel until only proteins greater than ≈ 150kDa remained on the gel showed the greatest mobility shift. (Figure 6.1-A) This concentration of aphidicolin also induced the strongest activation of Chk1 at S345. Agents that best induce this modification of cClaspin are those that impact on DNA replication such as aphidicolin and hydroxyurea, in addition to UV-C light which damages DNA through the introduction of cyclobutane pyrimidine dimers and 6,4-photoproducts. These treatments also induce the strongest Chk1 activation as

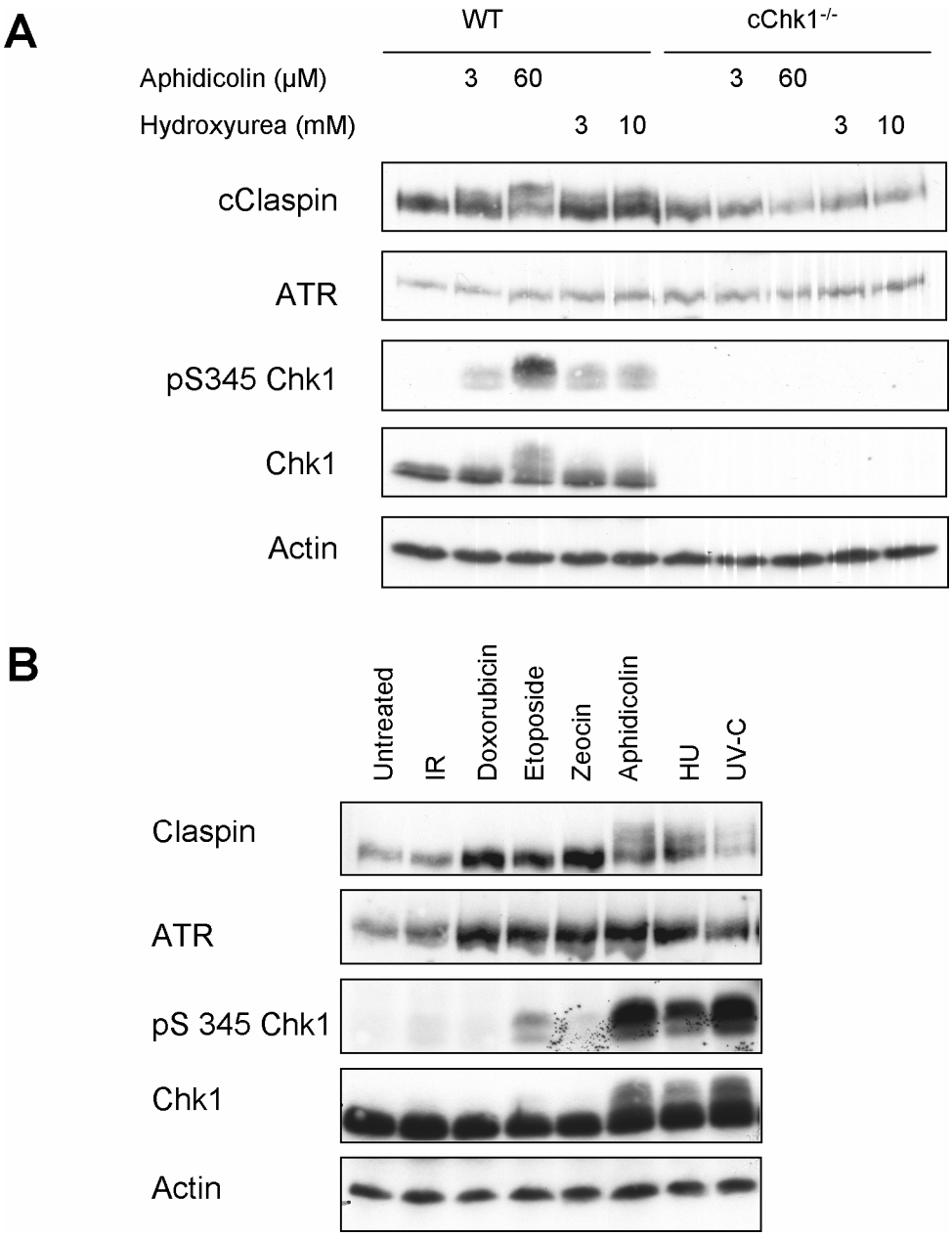

Figure 6.1 Claspin Phosphorylation following Replication Stress in WT and Chk1$^{-/-}$ DT40 cells

A) Cells were treated with the indicated drugs at the indicated concentrations for 90 minutes. The cells were then harvested and processed for western blotting. Western Blots using the indicated antibodies are shown. B) DT40 cells were treated with 10Gy γ-IR, 200ng/ml Doxorubicin, 10μM Etoposide, 800μg/ml Zeocin, 30μM Aphidicolin, 10mM Hydroxyurea or 50Jm^{-2} UV-C. Cells were harvested 2 hours later and analysed by western blotting for the indicated proteins.

judged by phosphorylation at S345. In contrast treatments which induce different DNA damage seem to lead to an accumulation of the protein. Such treatments do not lead to a very strong induction in Chk1 activity. It appears that there is a direct correlation with the mobility shift observed following replication stress and the activation of Chk1. (Figure 6.1-B) Pre-treating the WT cells with caffeine, which is known to inhibit ATM/ATR signalling (Sarkaria et al, 1999), inhibits the cClaspin mobility shift following aphidicolin treatment. (Figure 6.2-A) This indicates that the cClaspin phosphorylation induced mobility shift is dependent on the activity of the ATM/ATR kinases. In contrast to the situation observed in the WT cells no cClaspin mobility shift was observed in $Chk1^{-/-}$ cells undergoing replication stress. (Figure 6.1-A) This indicates that somehow Chk1 is involved in regulating cClaspin phosphorylation following during replication stress.

To probe this role of Chk1 in the phosphorylation of cClaspin a time course was carried out to see whether the phosphorylation mobility shift was completely absent or simply delayed in the $Chk1^{-/-}$ cells. As can be seen from Figure 6.2-B(i) cClaspin is rapidly phosphorylated in WT cells following the induction of replication stress. This phosphorylation remains up the 8 hours, the latest time point examined in this time-course. In contrast no mobility shift is observed in the $Chk1^{-/-}$ cells at any of the time points analysed. To further this observation the time-course was repeated with $Chk1^{-/-}$ cells which stably expressed either WT Chk1 (Chk1 Rev) or Kinase Dead version of Chk1 (KD Chk1) (Walker et al, 2009). Figure 6.2-B(ii) shows that stably re-expressing WT but not the KD version of Chk1 restores the cClaspin mobility shift. This implicates the Chk1 kinase activity in playing a direct role in mediating the cClaspin mobility shift induced by replication stress.

There are two possible explanations for this. Chk1 itself may directly phosphorylate or contribute to the phosphorylation of cClaspin. Alternatively a downstream function of Chk1 (which relies on its kinase activity) such as maintaining viable replication forks, is necessary to facilitate the phosphorylation. Unperturbed replication in $Chk1^{-/-}$ cells is known to progress relatively normally (Petermann et al, 2006). However Chk1 is known to be required to maintain replication forks in a competent state for replication

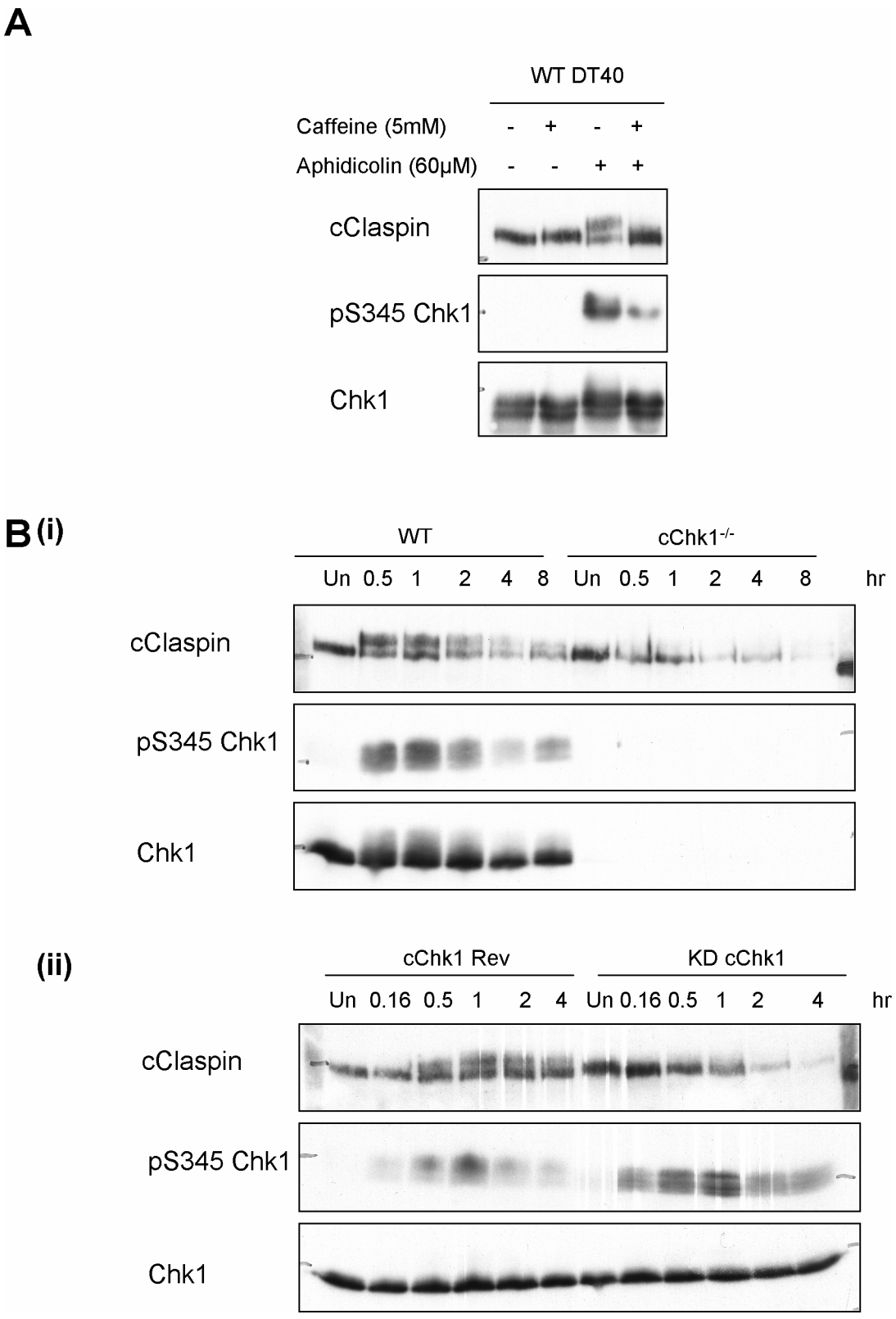

Figure 6.2 Time course of cClaspin phosphorylation following Replication Stress
A) WT DT40 cells were either pre-treated or not with 5mM Caffeine for 1 hour. Cells were then treated with 601μM aphidicolin. After 45 minutes the cells were harvested and blotted for the indicated proteins. B) (i) WT and Chk1$^{-/-}$ cells were treated with 60μM Aphidicolin. Samples were taken at the indicated times (hour). Lysates were blotted with the indicated antibodies. (ii) Chk1 Revertant (Chk1 Rev) and Kinase Dead Chk1 (KD Chk1) cells were treated as in A(i).

allowing replication to recommence once the stress has been resolved. One hypothesis of how Chk1 fulfils this function might be through keeping the required proteins at the stalled replication fork. cClaspin might be one of these proteins. Mis-localised cClaspin might not be effectively phosphorylated following replication stress. If this hypothesis were correct however it would be expected that cClaspin phosphorylation would be normal until replication fork collapse takes place. Therefore at early time-points following the addition of aphidicolin phosphorylated cClaspin would still be expected to be seen in the absence of Chk1. It is probable therefore that Chk1 plays a more direct role in promoting the phosphorylation of cClaspin following replication stress.

In order to test this directly an in-vitro kinase assay was carried out to see whether Chk1 can phosphorylate cClaspin. cClaspin tagged with a tandem StrepII-FLAG tag was expressed using the in vitro transcription-translation as described in section 3.4. The SF.cClaspin was then bound to Streptactin Beads and used as the substrate in a kinase assay using increasing amounts of recombinant active Chk1. The reaction was first carried out using 'cold' ATP and the samples were analysed by Western blotting. From Figure 6.3-A however it is clear that in an in vitro kinase assay Chk1 is not able to induce the mobility shift even using a presumed large molar excess of Chk1. Although Chk1 does not induce the cClaspin mobility shift it may still phosphorylate the protein. The kinase assay was therefore repeated in the presence of radioactive ATP in order to monitor the incorporation of phosphate into cClaspin. From Figure 6.3-B it can be seen that there is non-specific incorporation of ^{32}P into an unknown protein that binds non-specifically to the Streptactin Beads and runs with a similar molecular mass to cClaspin. This incorporation is present in the absence of recombinant Chk1 indicating that unknown kinases are also present in the kinase assay. However in addition to this background incorporation there seems to be incorporation of ^{32}P into cClaspin as well. The incorporation increases with increasing Chk1 levels but because cClaspin is only phosphorylated by Chk1 when it is in vast excess it is unlikely that cClaspin is a true substrate. Assuming that the in vitro situation fully recapitulates what is occurring in cells Chk1 appears not to be able to efficiently phosphorylate cClaspin.

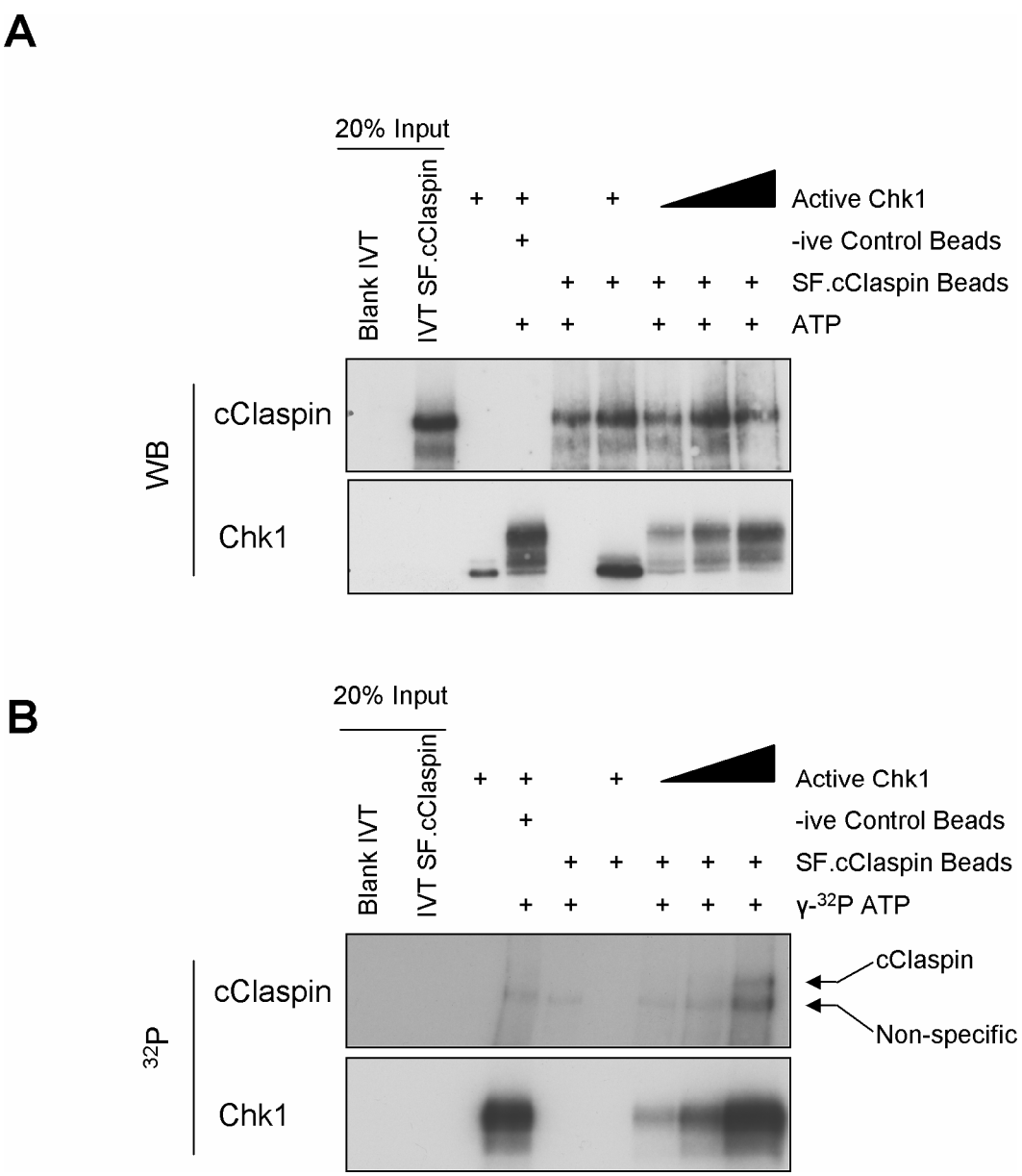

Figure 6.3 In vitro Chk1-Claspin Kinase Assay
A) In vitro Kinase assay on SF.cClaspin using recombinant active Chk1. IP'ed IVT SF.cClaspin was incubated with increasing amounts of Chk1 in the presence of ATP. The reactions were incubated for an hour at 30°C. $^{1}/_{6}$ of the reaction was run on a 10% acrylamide gel (Chk1) and the remainder was run on a 6% acrylamide gel (cClaspin). Western Blot analysis was preformed using the indicated antibodies. B) IP'ed IVT SF.cClaspin was incubated with increasing amounts of Chk1 in the presence of γ-^{32}P-ATP. The reactions were incubated for an hour at 30°C. The reactions were separated on a 6% SDS-PAGE gel then the proteins were transferred to nitrocellulose membrane and exposed to film. The resulting autoradiograms are shown.

6.3. Analysis of cClaspin Phosphorylation following Protein Synthesis Inhibition

While comparing the phosphorylation of cClaspin in both WT and Chk1$^{-/-}$ DT40 cells in response to replication stress it was apparent that cClaspin in the Chk1$^{-/-}$ cells was, in addition to not being phosphorylated, more unstable than in WT cells. It has been reported that Chk1 is required to maintain Claspin stability so it was therefore decided to examine this further (Chini et al, 2006). A cycloheximide (CHX) chase experiment was performed in order to analyse the turnover of cClaspin in WT and Chk1$^{-/-}$ cells. As can be seen in Figure 6.4-A the trend is that cClaspin is more stable in the WT cells than in the Chk1$^{-/-}$ cells indicating that Chk1 is required to stabilise cClaspin.

In addition it was also noted that cClaspin undergoes a pronounced mobility shift following treatment with CHX. In contrast with the mobility shift induced by replication stress, this shift occurs in both the WT and Chk1$^{-/-}$ cells and the entire population of cClaspin is modified indicating that cClaspin is being modified in all cells in all phases of the cell cycle.

In order to determine whether this response is specific to CHX or whether it is a more general response to protein synthesis inhibition, cells were treated with a panel of protein synthesis inhibitors. Anisomycin, Emetine, Puromycin and Blasticidin were chosen. All of these inhibitors inhibit protein synthesis at the translational elongation phase but they have distinct chemical structures and inhibit elongation by different mechanisms. As can be seen from Figure 6.4-B they all induce the mobility shift as efficiently as CHX. In addition a dose response experiment using a range of CHX concentrations shows that the modification of cClaspin only occurs when the CHX level is high enough to inhibit protein synthesis. (Figure 6.4-C) c-myc loss was used as a marker of protein synthesis inhibition in these cells since c-myc is over-expressed in DT40 cells but is turned over very rapidly and is quickly lost when protein synthesis is inhibited (Salghetti et al, 1999). This indicates that it is an effect on protein synthesis, rather than an indirect action of CHX that is leading to the modification of cClaspin.

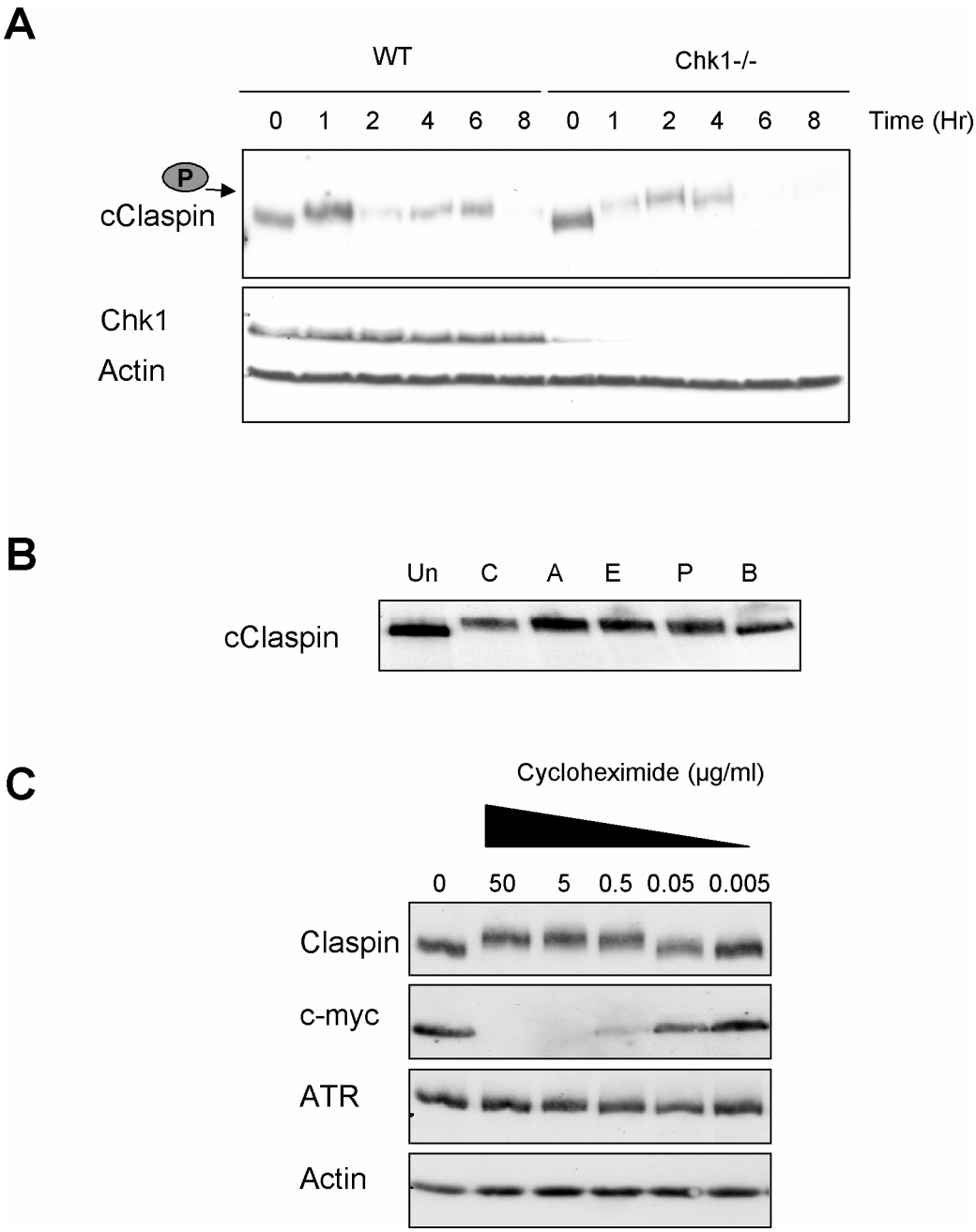

Figure 6.4 cClaspin is modified following treatment with Protein Synthesis Inhibitors
A) CHX chase experiment. WT and Chk1$^{-/-}$ cells were treated with 50µg/ml CHX. Samples were harvested at the indicated times and analysed for the indicated proteins by western blot. B) WT DT40 cells were either left untreated or treated with C (50µg/ml CHX), A (10µg/ml Anisomycin), E (20µM Emetine), P (10µg/ml Puromycin) and B (30µg/ml Blasticidin) for 90 minutes. The resulting extracts were blotted for cClaspin. C) DT40 cells were treated with the indicated concentrations of CHX for 1 hour, then blotted for the indicated proteins.

Protein synthesis can also be inhibited by using the cells own surveillance mechanisms that monitor protein biosynthesis. Initiation of cap-dependent protein synthesis requires the eIF2A initiation factor which controls the recruitment of the tRNA-iMet to the 40S ribosome in a GTP dependent manner. (Figure 6.5-A) eIF2A, now in the GDP bound state, must be converted back to the GTP bound state in order for the cycle to be repeated and the synthesis of other proteins initiated. This reaction is catalysed by eIF2B which is usually present in cells at a much lower level than eIF2. However phosphorylation of the α subunit of eIF2A results the formation of a tight complex between eIF2A and eIF2B. This sequestration of the eIF2B subunit means that it is no longer capable of re-setting eIF2A and protein synthesis is stops (Brostrom et al, 1997). Phosphorylation of eIF2A occurs in response to distinct cellular stresses which include Endoplasmic Reticulum (ER) stress and amino acid deprivation. Therefore inducing these stresses should also lead to protein synthesis inhibition but by a different mechanism to direct inhibition by chemicals.

In response to amino acid starvation protein biosynthesis is inhibited on two levels. Elongation is inhibited by the lack of amino acid precursors but initiation is also inhibited through the GCN2 mediated phosphorylation of eIF2A. As can be seen from Figure 6.5-B growing DT40 cells in the absence of amino acids (either all amino acids or just Glutamine, Methionine and Cysteine) leads to an increase in the phosphorylation of eIF2A. c-myc levels decrease in the cells grown in the absence of amino acids indicating that protein synthesis is indeed inhibited. As was seen when inhibiting protein synthesis using drugs cClaspin is also modified following protein synthesis inhibition by amino acid starvation.

Inhibition of protein synthesis can also occur in response to ER stress, which leads to the activation of PERK (PKR-like ER Kinase) which in turn phosphorylates eIF2A. In this instance protein synthesis is only inhibited through the p-eIF2 pathway. Cells were treated with drugs that cause ER stress. (Figure 6.5-C) Thapsigargin depletes ER calcium stores thus inhibiting calcium dependent ER chaperones like calreticulin leading to an immediate accumulation of unfolded proteins. Tunicamycin inhibits protein N-Glycosylation which is a crucial step in the folding of newly synthesised proteins. Inhibition of N-glycosylation will lead to a gradual accumulation of unfolded proteins. DTT causes a redox imbalance

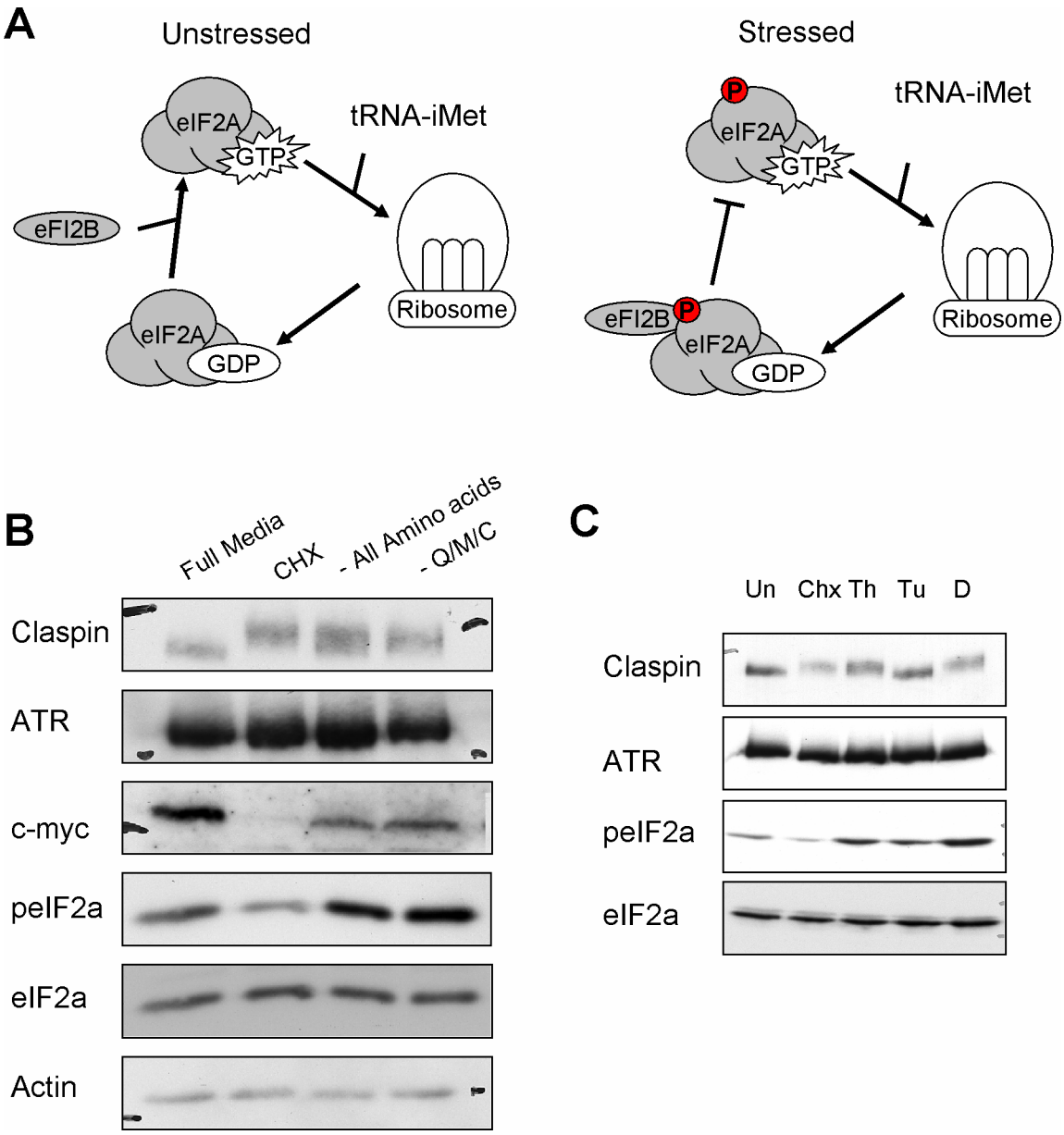

Figure 6.5 Inhibition of Protein Biosynthesis through p-eIF2A also leads to cClaspin modification

A) Schematic representation of how eIF2A controls translational initiation. Phosphorylation of eIF2A results in the formation of a complex between eIF2A and eIF2B which inhibits the initiation cycle. B) Cells were washed in pre-warmed PBS and then returned to full media with or without CHX or to media lacking all amino acids (EBSS supplemented with 10% dialysed serum and MEM Vitamins) or to full media lacking only Glutamine, Methionine and Cysteine (Q/M/C) supplemented with dialysed serum. C) Cells were treated with 50µg/ml CHX, 2µM Thapsigargin, 5µg/ml Tunicamycin or 2mM DTT for 1 hour. Western blot analysis using the indicated proteins is shown.

in the ER and thus leads to improperly formed disulphide bonds and unfolded proteins. Treating cells with these agents leads to an increase in p-eIF2A as expected. This treatment also leads to the same modification in cClaspin as seen before. No modification is seen with tunicamycin treatment but this is probably due to the fact that there is lower p-eIF2 induced with a short treatment of tunicamycin. CHX leads to a reduction in p-eFI2A levels presumably through reducing the biosynthetic load on the ER.

It has now been firmly established that cClaspin is modified following protein synthesis inhibition. The next issue that was addressed was what modification was occurring to lead to the altered electrophoretic mobility. Many post-translational modifications have been identified (Kia-Ki & Martinage, 1992). Some of these lead to an altered electrophoretic mobility by SDS-PAGE. As phosphorylation of cClaspin has already been shown to alter its electrophoretic mobility this was a reasonable candidate to investigate first. To test whether treating cells with protein synthesis inhibitors leads to the phosphorylation of cClaspin, cClaspin was immuno-precipitated from cells that had been treated with CHX in order to induce the mobility shift. The partially purified cClaspin protein was then incubated with λ Protein phosphatase in order to remove any potential phosphate groups from the protein. cClaspin was then boiled off the beads and analysed by western blot. Figure 6.6-A shows that this modified form of cClaspin can be readily purified from cells treated with CHX and that this modification can be completely reversed by phosphatase treatment providing evidence that phosphorylation of cClaspin is responsible for the mobility shift. It is interesting to note that the completely dephosphorylated form of cClaspin runs lower than the cClaspin from untreated cells indicating that even in an unstressed state cClaspin exists in a phosphorylated protein.

Having now confirmed that cClaspin is hyperphosphorylated following protein synthesis inhibition the next obvious question to address is which kinase is responsible for the phosphorylation. A candidate approach using selective kinase inhibitors was first taken in order to try and identify the relevant kinase.

Protein synthesis inhibition using drugs (especially Anisomycin) has been known for some time to activate signalling pathways, especially those mediated by the

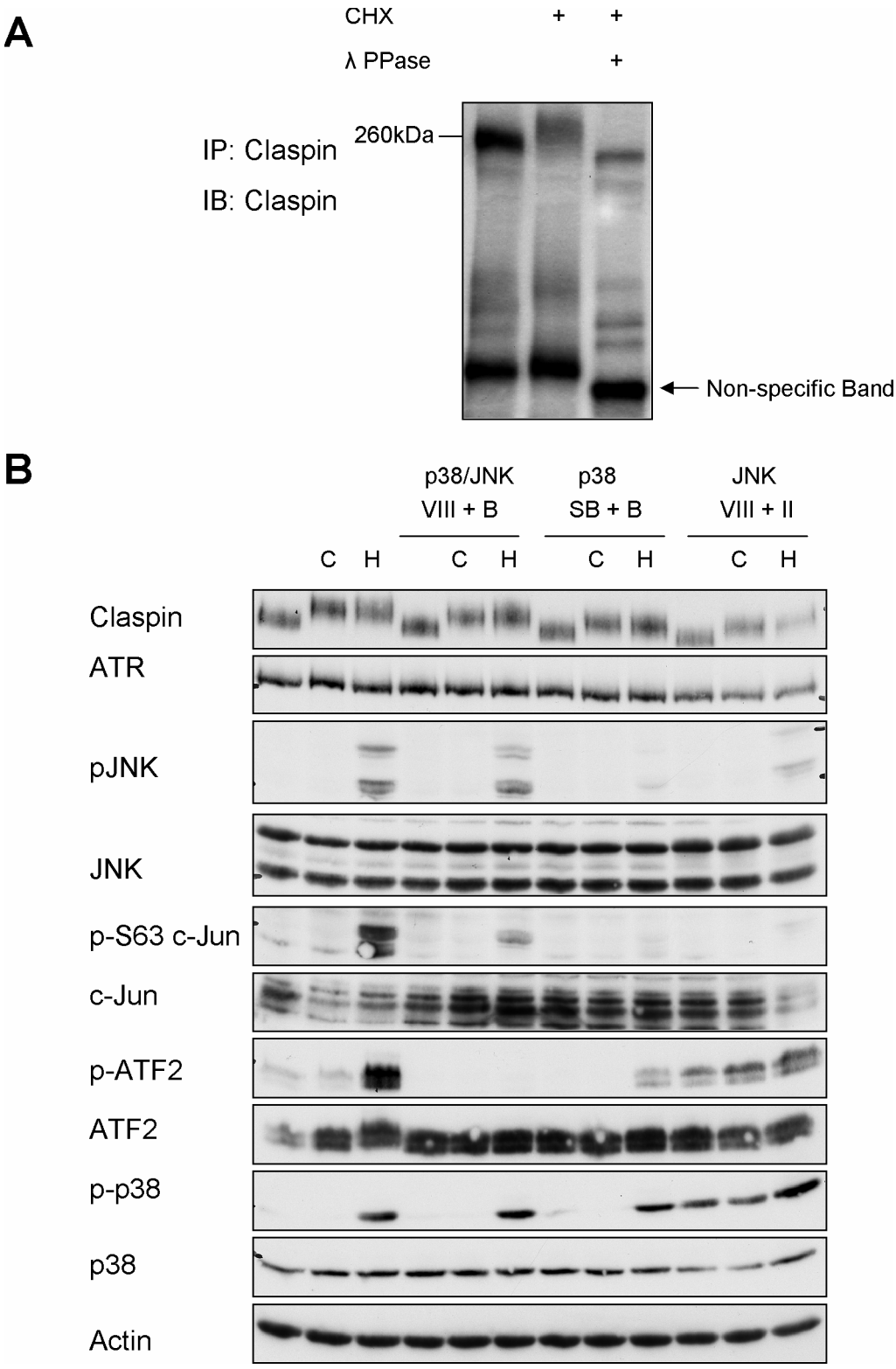

Figure 6.6 cClaspin is phosphorylated following Protein Synthesis Inhibition but not by p38/JNK

cClaspin was immunoprecipitated from cells either untreated or treated with 50µg/ml of CHX for 90 minutes. The partially purified protein was then incubated with λ phosphatase. cClaspin was then boiled off the beads and analysed by western blot. B) Cells were pre-treated with the indicated combinations of inhibitors for 15 minutes prior to treatment with either 50µg/ml CHX or 1mM H_2O_2. Cells were harvested 30 minutes later and analysed by western blot for the indicated proteins. (VIII = JNK Inhibitor - 10µM, II = JNK Inhibitor SB600125 - 10µM, SB = p38 Inhibitor SB202190 - 10µM, B = p38 Inhibitor BIRB 796 - 5µM, C = Cycloheximide, H = Hydrogen peroxide)

p38 and JNK family members (Cano et al, 1994; Hazzalin et al, 1996). Cells pre-treated with inhibitors to these kinases were then challenged for their ability to mediate cClaspin hyperphosphorylation following protein synthesis inhibition. To inhibit p38 a combination of SB202190 and BIRB-796 was used. This has been recommended to inhibit all isoforms of p38 (Bain et al, 2007). To inhibit JNK two commercially available inhibitors were used. JNK Inhibitor VII shows K_i values of = 2 nM, 4 nM and 52 nM for JNK1, 2 and 3, respectively and JNK Inhibitor II (SB600125) shows IC_{50} values of 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3. The results from these studies are shown in Figure 6.6-B. Firstly it is interesting to note that CHX does not induce phosphorylation of JNK or p38 or their down stream targets c-Jun and ATF2. Therefore these pathways are not always activated in response to protein synthesis inhibition. In addition H_2O_2, which activates p38 and JNK, also induces hyperphosphorylation of cClaspin. H_2O_2 exerts multiple effects on the cell including DNA damage but it will most likely also disrupt protein synthesis. Using p-ATF2 as a readout of p38 activity and p-c-Jun as a readout for JNK activity it can be seen that the combination of inhibitors used effectively inhibits the target kinases. The combination of JNK inhibitors leads to a complete loss of c-Jun phosphorylation in response to H_2O_2 treatment and inhibiting p38 leads to a reduction in ATF2 signalling. However even though these kinases have been inhibited effectively there is no effect on cClaspin hyperphosphorylation even when both pathways are inhibited simultaneously. This strongly indicates that the p38/JNK pathway is not involved.

The next pathway that was examined was the mTOR pathway. (Figure 6.7-A) mTOR belongs to the same family of kinases as ATM and ATR, the PI3 Kinase-like Kinases. mTOR has also been shown to be activated by protein synthesis inhibition through the loss of a short-lived repressor protein REDD1 (Kimball et al, 2008). Inhibition of protein synthesis also leads to an increase in intracellular amino acid levels which presumably also activates mTOR. Using two chemical inhibitors of mTOR, rapamycin and Torin1, the possible role of mTOR in cClaspin hyperphosphorylation was examined (Thoreen et al, 2009; Thoreen & Sabatini, 2009). Rapamycin effectively blocks the phosphorylation of the ribosomal subunit S6 used here as an indirect readout of mTOR activity.

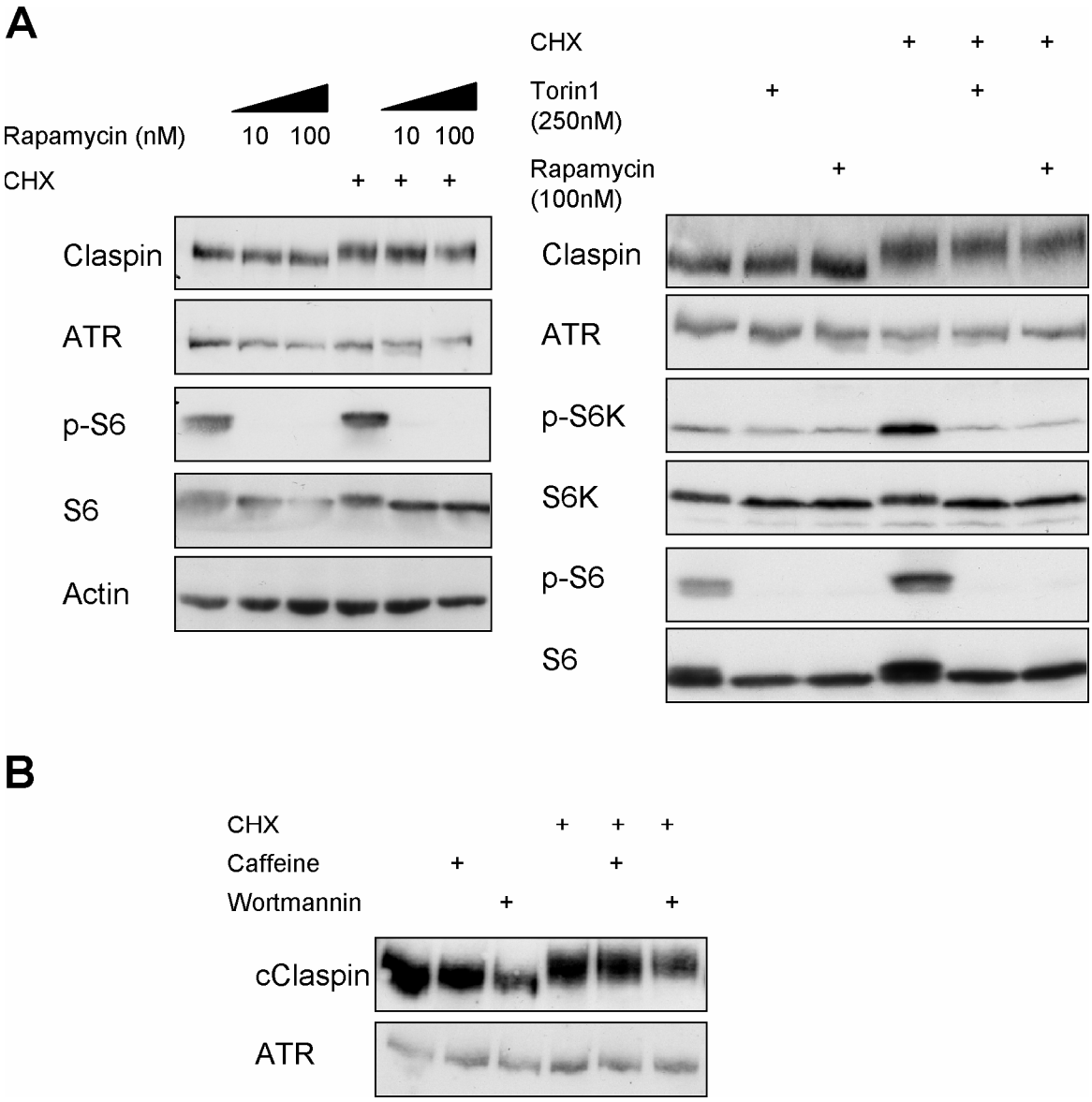

Figure 6.7 Testing mTOR and DNA damage signalling inhibitors for their ability to inhibit cClaspin hyperphosphorylation

A) Cells were treated with the indicated drugs for 1 hour prior to treatment with 50µg/ml of CHX for a further hour. Western blot analysis with the indicated antibodies is shown. B) Cells were treated with either 5mM Caffeine or 10µM Wortmannin for 1 hour prior to the addition of 50µg/ml CHX. Cells were harvested 1 hour later and blotted for the indicated proteins.

However there is no effect on cClaspin hyperphosphorylation. Torin1, an ATP competitive inhibitor of mTOR itself also effectively blocks both S6 Kinase and S6 phosphorylation but is unable to inhibit cClaspin hyperphosphorylation either. This excludes the possibility that both mTOR complexes (mTORC1 and mTORC2) are involved in the pathway that leads to cClaspin hyperphosphorylation.

Although cClaspin hyperphosphorylation occurs in the absence of Chk1 it is still possible that DNA damage signalling might be responsible for cClaspin hyperphosphorylation following protein synthesis inhibition. To test this directly cells were treated with the ATM/ATR inhibitor caffeine and Wortmannin which inhibits not only PI3 Kinase but also Plk1 and 3 and DNAPKcs which are involved in DNA damage signalling (Liu et al, 2007; Sarkaria et al, 1998). (Figure 6.7-B) However pre-treatment with either drugs could not inhibit the mobility shift.

The phosphorylation status of a protein is regulated by two opposing enzymes; kinases which phosphorylate proteins and phosphatases which dephosphorylate proteins. Activation of a kinase or inhibition of a phosphatase will both lead to an increase in the phosphorylation of a protein. It has been assumed thus far that the cClaspin hyperphosphorylation observed is due to the action of a kinase but it is equally plausible that inhibition of protein synthesis may lead to the loss of a phosphatase that normally keeps cClaspin dephosphorylated. In order to examine this possibility, protein degradation was blocked prior to inhibition of protein synthesis. Blocking protein degradation via the proteasome (MG132) or lysosome (leupeptin and bafilomycin A1) should ensure that the phosphatase or negative regulator of a kinase would not be degraded. However as can be seen from Figure 6.8-A inhibiting protein degradation has no impact on cClaspin phosphorylation following protein synthesis inhibition. This again points to the fact that protein synthesis inhibition leads to the activation of an, as yet, unknown signalling pathway that leads to the phosphorylation of cClaspin.

The last issue that was examined was whether inhibition of protein synthesis had any impact on DNA replication, a process that cClaspin may be involved. It has been shown in the past that inhibitors of protein synthesis also inhibit DNA synthesis to some extent (Grollman, 1967; Grollman, 1968). DT40 cells were treated with CHX, Anisomycin, Emetine, DTT and Thapsigargin and DNA replication was monitored by the cells ability to incorporate BrdU. As can be

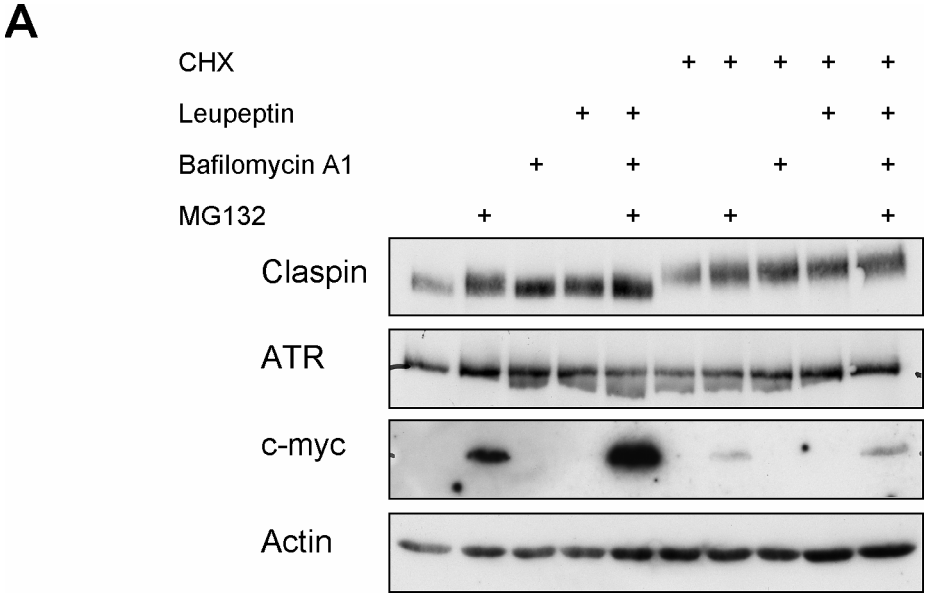

Figure 6.8 cClaspin hyperphosphorylation is not due to the loss of a short-lived protein
A) DT40 cells were treated with 10μM MG132, 10μg/ml Leupeptin or 10nM Bafilomycin A1 for 1 hour to inhibit protein degradation. They were then treated with CHX for a further hour and blotted for the indicated proteins.

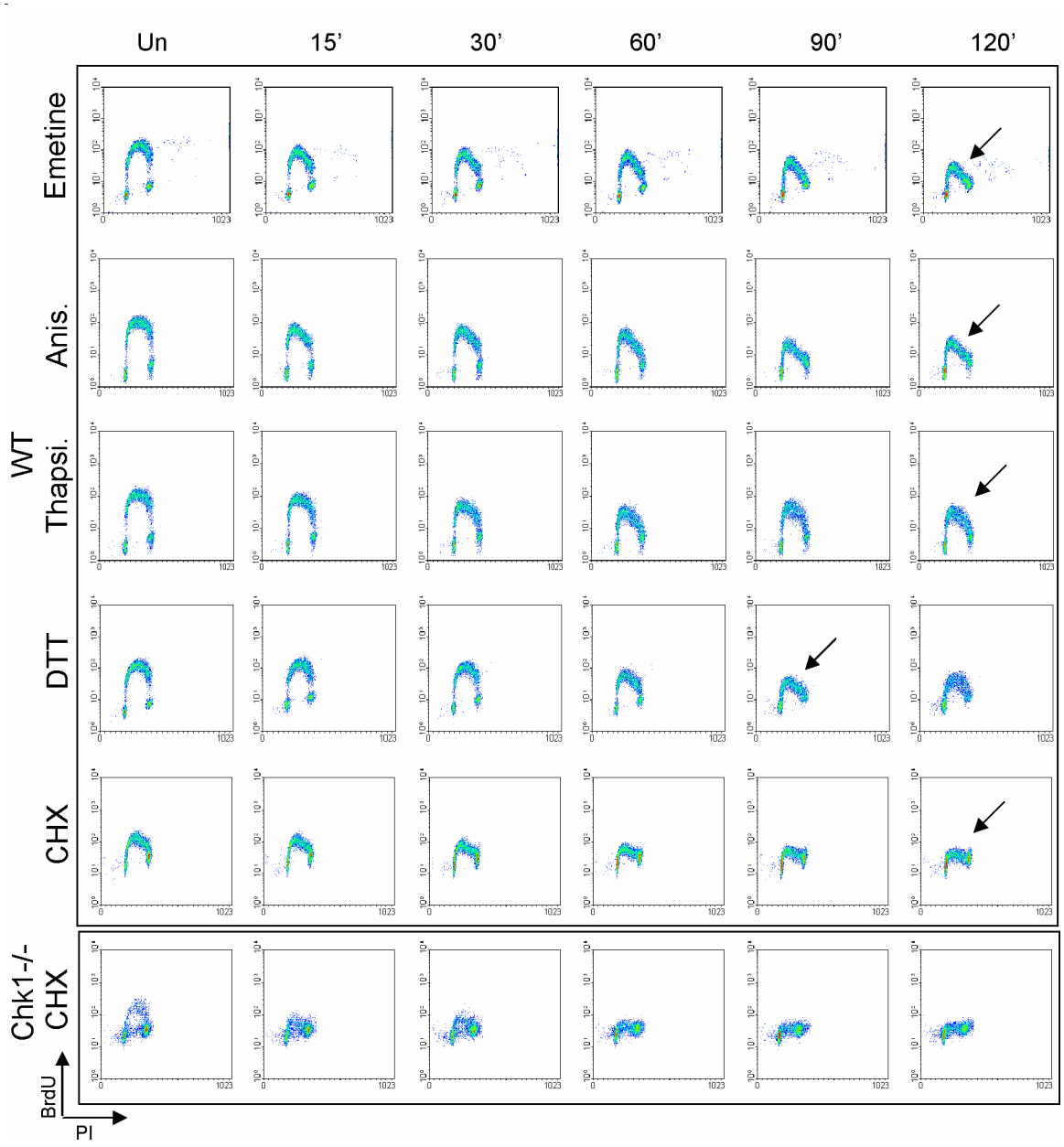

Figure 6.9 Effect of Protein synthesis inhibition of DNA replication
Cells were treated with 20μM Emetine, 10μg/ml Anisomycin, 2μM Thapsigargin, 2mM DTT of 50μg/ml CHX and samples were harvested at the indicated times (mins). Cells were pulsed with 25μM BrdU 15 minutes prior to harvest and processing for flow cytometry. The resulting density plots are shown.

seen from Figure 6.9 all inhibitors have an effect on DNA replication. There is an overall effect on the rate of DNA replication as can be seen by a reduction in the intensity of BrdU incorporation but there seems to also be a more specific effect on mid-late S phase cells (see arrows in Figure 6.9). Interestingly $Chk1^{-/-}$ cells are particularly sensitive to protein synthesis inhibition as by 2 hours post treatment with CHX the cells have completely shut down replication. This is in contrast with DNA damage where $Chk1^{-/-}$ cells continue to replicate their DNA following γ-IR (data not shown). In $Chk1^{-/-}$ cells however, cClaspin is phosphorylated to the same extent as it is in WT cells which indicates that this phosphorylation of cClaspin is not, at least in the context of Chk1 loss, able to facilitate active DNA replication.

6.4. Discussion

6.4.1. cClaspin Phosphorylation following replication stress

When examining the phosphorylation of cClaspin in the $Chk1^{-/-}$ cells it was observed that there was a defect in cClaspin phosphorylation in the absence of Chk1. A phosphorylation induced mobility shift could not be detected in the $Chk1^{-/-}$ cells. This mobility shift was never induced in the absence of Chk1 and could only be rescued by the reintroducing WT Chk1 but not KD Chk1 into the $Chk1^{-/-}$ cells. It is known that Claspin is phosphorylated in an ATR dependent manner and Chk1 kinase activity is activated by ATR. Therefore it was reasonable to hypothesise that Chk1 itself might actually phosphorylate cClaspin. However when this possibility was tested directly, by doing an in vitro kinase assay, it was seen that cClaspin was not phosphorylated efficiently under the assay conditions used. In addition it is worth noting that a panel of kinase inhibitors has previously been shown inhibit the mobility shift induced by replication stress (Clarke & Clarke, 2005). Here it was observed that UCN-01, Staurosporine, Ro318220 and Bim1 could inhibit the phosphorylation induced mobility shift induced by replication stress. These kinase inhibitors, while they do show some selectivity towards some kinases over others, are generally classed as general kinase inhibitors and inhibit a wide variety of kinases (Davies et al, 2000; Flavio et al, 1995). These kinase inhibitors also inhibit Chk1 to some

extent with UCN-01 being the most potent. However Gö6976 which is also a potent inhibitor of Chk1 amongst other kinases only partially inhibits the phosphorylation mobility shift of Claspin.

The sites that are phosphorylated following replication stress that contribute to the mobility shift are unknown. However it is likely that many phosphorylation sites are involved. Therefore it is reasonable to assume that a single kinase might not be solely responsible for mobility shift. Chk1 may act as a priming kinase which facilitates the phosphorylation of cClaspin once it has been phosphorylated by Chk1. Alternatively kinases downstream of Chk1 might also contribute to the mobility shift. Interestingly while the phosphorylation induced mobility shift is dependent on the presence of catalytically active Chk1, phosphorylation at S911 which lies within the SG motif in the Chk1 binding domain, is not compromised in $Chk1^{-/-}$ cells (Bennett et al, 2008). Previously Chk1 itself had been implicated in phosphorylating this site (Chini & Chen, 2006). It is clear therefore that both the phosphorylation state of Claspin and the kinases that mediate this phosphorylation are very complex.

6.4.2. cClaspin phosphorylation following Protein Synthesis Inhibition

This study has identified a novel phosphorylation pattern of cClaspin following inhibition of protein synthesis. This phosphorylation is induced by drugs that directly inhibit the process of translational elongation but also through the inhibition of the initiation of protein synthesis protein medicated by p-eIF2A. Experiments carried out have shown that it is the activation of a kinase directly rather than the loss of a repressor or phosphatase that is responsible for the phosphorylation. However attempts at identifying this kinase, using a candidate approach, have failed.

How could the identity of this kinase be elucidated? It is clear from my initial studies that a more global approach will need to be taken in order to identify the kinase. One possibility is to carry out in vitro kinase assays using fractionated cell lysates in order to try and try and identify a fraction that can induce the cClaspin mobility shift. Once this fraction has been identified the proteins it contains can be identified by mass spectrometry. Another approach

would be the use of a siRNA library that targets the human kinome. By removing each kinase individually would allow the identification of the kinase responsible. Finally instead of using very specific kinase inhibitors as I have used here it is also possible to use very unspecific inhibitors. Once an inhibitor has been identified, technology is now available to couple the inhibitor to beads to allow the purification of the kinases inhibited by the compound (Daub, 2005). The kinases identified could then be tested for their ability to phosphorylate cClaspin. Identifying the kinase would also probably provide an insight into how and why protein synthesis inhibition leads to the phosphorylation of cClaspin.

One possibility not discussed thus far is that protein synthesis inhibition leads to the activation of multiple signalling pathways that contribute to the phosphorylation of cClaspin. In this instance multiple kinases are likely to be involved. Therefore simply assaying every kinase for its ability to phosphorylate cClaspin will never result in the recapitulation of the cClaspin mobility shift.

An alternative approach would be to try and identify the sites on cClaspin that are phosphorylated in response to protein synthesis inhibition. In this way by examining the amino acids surrounding the phosphorylated site may give a clue as to which kinase is responsible. To this end some preliminary experiments have been carried out in order to try and map phosphosites on cClaspin. A system had been generated in which cClaspin can be readily purified from cells following stimulation to facilitate analysis by mass spectrometry. This approach also allows the identification of binding partners under different conditions. Unfortunately the data obtained thus far has only been obtained from one experiment and is too preliminary to report. A list of potential binding partners has been included in Appendix G for reference purposes. In trying to maximise the coverage of peptides from the cClaspin protein two phosphosites were identified on cClaspin purified from untreated cells (at Ser143 and Ser803) indicating that this approach is indeed feasible. The first of these sites is a non-conserved S residue but the S803 residue is contained with in a well conserved SP motif indicating a potential role for Cdk's in phosphorylation of Claspin.

Without knowing the kinase responsible it is difficult to speculate as to what role phosphorylated cClaspin is playing following protein synthesis inhibition. DNA replication is inhibited following protein synthesis inhibition suggesting that DNA

damage signalling might be involved – however testing this directly has ruled this out. It is reasonable to suggest that inhibition of protein translation would affect the translation of histones during S phase and lead to a perturbation of chromatin formation during S phase. However in this case it would be reasonable to assume that only cClaspin in S phase cell would be modified. However as the entire cClaspin band shifts it is reasonable to assume that cClaspin is modified in every cell no matter what phase of the cell cycle it is in.

Another issue that remains to be resolved is what the cell is actually responding to? It doesn't appear to be the loss of a specific protein as the modification still occurs when protein degradation is inhibited. It also does not appear to be amino acid levels as the modification is apparent either when amino acid levels are increased (by treating with CHX for example) or when they are removed. Protein synthesis inhibitors may affect the structure of the ribosome but inhibiting protein synthesis by removing amino acids or inducing ER stress also induces the cClaspin modification arguing against this as a mechanism. Finally by inhibiting protein synthesis in multiple ways rules out that a particular protein synthesis inhibitor is also capable of activating a signalling pathway independent of its role in inhibiting protein synthesis.

In summary a novel modification of a cell cycle checkpoint protein has been identified. However the fundamental significance of this and the mechanism behind it remains to be elucidated.

Chapter 7. Conclusions and Future Directions

7.1. Chicken Claspin Homologue

The chicken orthologue of Claspin has been cloned and characterised during this study. At the amino acid level cClaspin share significant similarity across its full length with human and xenopus Claspin but especially in the C-terminus which has been shown to mediate Chk1 activation through direct binding (Lee et al, 2005). The residues of Claspin that are important for this function are the phospho S/T sites located within the Chk1 binding domain (Clarke & Clarke, 2005; Kumagai & Dunphy, 2003) and these sites are conserved in the chicken Claspin orthologue.

Although the N-terminus of Claspin is not as highly conserved as the C-terminus the overall structure appears similar. Lee et al have described that there are basic patches in the N-terminus of the protein that mediate binding of cClaspin to chromatin (Lee et al, 2005). We have identified another conserved basic patch in the N-terminus of the protein which could also play a role in this process.

Claspin has also been shown to interact with another mediator protein – Brca1 in order to facilitate Chk1 activation. Claspin and Brca1 appear to work together in the activation of Chk1 (Lin et al, 2004; Yoo et al, 2006). Nothing is known about where Claspin and Brca1 interact. As discussed previously it is interesting to note that the Chk1 binding motif itself provides the perfect BRCT consensus interaction motif once the Ser residues are phosphorylated. However Chk1 has been shown to bind to this region and the binding is dependent on phosphorylation which leads to the speculation that Chk1 itself binds these phospho-residues (Jeong et al, 2003). It would be interesting to see whether the Claspin-Brca1 interaction is also compromised upon mutation of the critical S/T residues.

Some confusion exists in the literature about where Claspin migrates in SDS-PAGE. The predicted molecular mass of Claspin is approximately 150kDa but it has been reported previously to run above 200kDa, possibly due to its low pI (Kumagai & Dunphy, 2000; Sar et al, 2004). Despite this some authors refer to a band migrating at 150-160kDa as Claspin (Brondello et al, 2007; Collis et al, 2007). Whether this band is a non-specific cross reaction with an unrelated

protein or a Claspin related protein is unknown. However from the in-vitro translation experiments carried out using the cClaspin cDNA isolated, cClaspin clearly migrates with an apparent molecular mass of >250kDa. In addition the antibody raised against cClaspin also recognises a band at >250kDa in cell extracts. Interestingly it also recognises a band around 150kDa. The identity of this protein was not determined.

7.2. cClaspin plays an essential role in DT40 cells

Although heritable mutations in many proteins involved in the DNA damage response have been identified and implicated in human disease, such mutations in the ATR-Chk1 pathway are relatively very uncommon (Marsh et al, 2007; Zhang et al, 2009). Any mutations that do arise are generally not complete loss of function mutations. Coupled with this information is the fact that both ATR knock-out and Chk1 knock-out mice are embryonic lethal showing that this pathway is critical for cell survival (Brown & Baltimore, 2000; Takai et al, 2000). DT40 cells however tolerate loss of Chk1 but not of ATR (Zachos et al, 2003), N Lowndes, Personal Communication). Work presented in this thesis strongly indicates that DT40 cells do not tolerate loss of Claspin either. Given this, it is reasonable to assume that Claspin plays role in the normal cell cycle which is distinct from its role as a facilitator of Chk1 activation. What this role is has yet to be determined but given that the yeast homologue of Claspin plays a role during replication is it reasonable to speculate that vertebrate Claspin also plays an intrinsic role in DNA replication.

Due to time constraints and technical difficulties it was not possible to generate a conditional knock-out of cClaspin. However a cClaspin$^{On/Off}$ cell line would be a useful tool to study the essential function of Claspin. Such a cell line would permit characterisation of how cells die following removal of cClaspin. If, as might be expected, cClaspin plays an essential role in DNA replication then one might expect that the cells would accumulate in S phase before they die. cClaspin depletion could also be carried out simultaneously with cell cycle synchronisation in either early S phase or in mitosis. Releasing the cells into back into cycle in the absence of cClaspin might reveal unknown functions of cClaspin in other phases of the cell cycle.

A cClaspin$^{On/Off}$ cell line can also be used to carry out structure-function studies on the protein. The cells could be stably transfected with portions of the protein expressed from a constitutive promoter. Once the WT cClaspin protein is repressed the function of the fragment could be assessed. Indeed taking this approach a screen could be carried out in order to determine the regions of Claspin that are essential for cell survival. It could also allow the identification of separation of function mutants that can support cell viability but may be defective in checkpoint activation for example.

One disadvantage of the Tet-system is that repression of the protein expression depends both on the stability of the transgene mRNA and on the stability of the final protein. This means that it may take up to 24 hours for the protein levels to be reduced enough in order to reveal the null phenotype. A way around this would be to express an N-degron tagged version of cClaspin in the cells (Labib et al, 2000). This degron is temperature sensitive and once the cells are shifted to a higher temperature the protein is actively degraded in the cells. In this manner the protein can be cleared from the cells in between 60-90 minutes allowing characterisation of the consequences of immediate loss of the protein from the cell. This has successfully been used in DT40 cells to further probe the functions of Rad51, knock-out of which also proves lethal (Su et al, 2008). Coupling this rapid loss with cell cycle synchronisation could reveal cell cycle phase specific functions of the protein.

7.3. Phosphorylation of cClaspin is complex

It is clear from the literature that Claspin is a highly phosphorylated protein. Large scale proteomics analyses frequently report the identification of phosphorylated Claspin residues (Bodenmiller et al, 2007; Dephoure et al, 2008; Gauci et al, 2009; Olsen et al, 2006; Zhai et al, 2008). Indeed simply examining the primary structure of the protein reveals that 15% of the protein consists of potentially phosphorylatable residues (S/T/Y). Given this information it is reasonable to expect that many kinases and phosphatases are involved in regulating its function. (See Figure 7.1)

Phosphorylation of Claspin following two different stresses was examined in this thesis, by examining phosphorylation induced mobility shifts. In agreement with

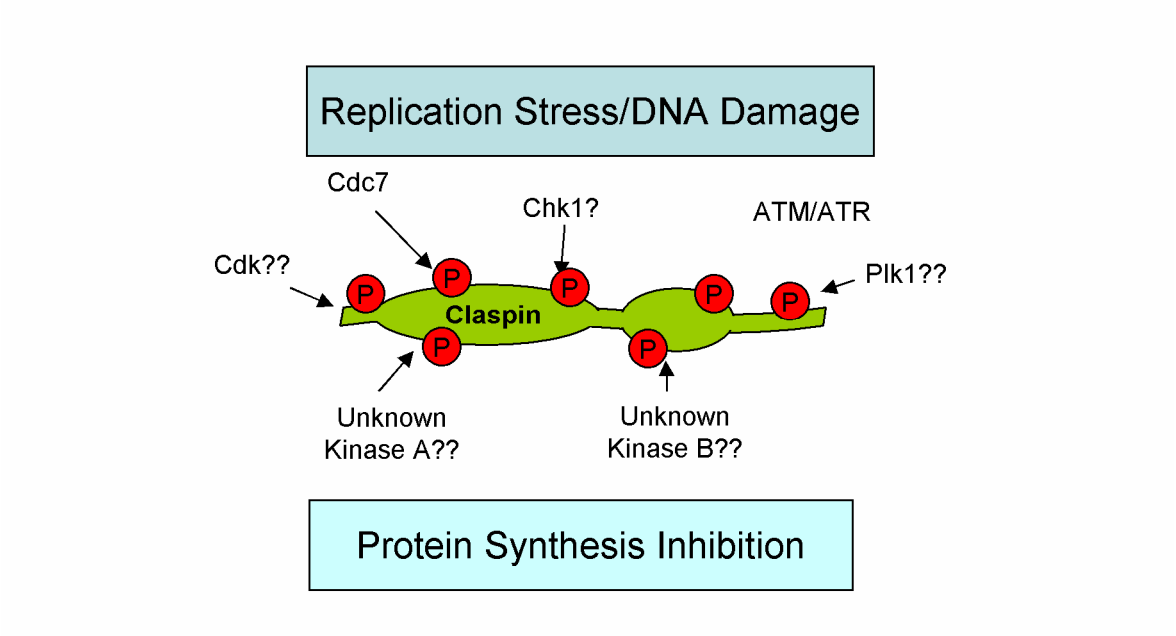

Figure 7.1 Claspin Phosphorylation is regulated by multiple kinases
Claspin has been shown to be phosphorylated by multiple kinases, however the kinases that phosphorylate Claspin following replication stress remain unknown.

work already published, cClaspin was shown to be phosphorylated in an ATM/ATR dependent manner during replication stress. Chk1 was also shown to be required for this phosphorylation. An important issue not addressed in this study is which sites on cClaspin are required for the phosphorylation induced mobility shift. A reasonable assumption would be that cClaspin, like xClaspin and hClaspin, would also be phosphorylated on S911 and S930 (which correspond to T916 and S945 in hClaspin). But given the nature of a phosphorylation induced mobility shift other sites of phosphorylation are probably also going to be involved.

The regulation of cClaspin phosphorylation by Chk1 appears complex. Work presented here has shown that Chk1 kinase activity is required for cClaspin phosphorylation during replication stress however when tested directly it appears that cClaspin is not a direct substrate of Chk1, at least under the in vitro conditions tested. Work reported by Bennett et al expanded on this observation and showed that in the absence of Chk1 only a partial inhibition of Claspin phosphorylation was seen; in the absence of Chk1, cClaspin could still be phosphorylated on S911 (T916 in hClaspin), one of the SG sites in the CKBD (Bennett et al, 2008). Further work will need to be carried out in order to clarify why Chk1 is required for certain cClaspin phosphorylation events. The

Chk1-/- DT40 cells provide the ideal system in which this can be analysed. Once optimised the TAP purification method for cClaspin coupled with mass spectrometry should provide a valuable insight into the cClaspin phosphorylation pattern regulated by Chk1. Indeed by using this approach 2 phosphorylation sites were identified at S143 and S803 by mass spectrometry, showing that this approach is feasible. An important issue that remains unresolved is why Chk1 would be required to phosphorylate its own upstream activator. Whether this forms part of a positive amplification loop is unknown. Phosphosite mapping will be the next critical step in understanding this phenomenon.

Furthermore a novel cClaspin phosphorylation pattern was discovered following protein synthesis inhibition. Inhibiting protein synthesis through multiple mechanisms led to the phosphorylation of cClaspin, potentially uncovering an undiscovered link between the protein synthesis and DNA replication. The next step in this project will be to identify which sites are being phosphorylated and by which kinases. With this knowledge intervention studies, where only the modification in response to protein synthesis inhibition is inhibited, will be possible. This should provide a valuable insight into what the functional significance of this phosphorylation is. Inhibition of protein synthesis only has a mild effect on DNA replication, at least at early time points. Perhaps Claspin is required to allow replication to proceed under these conditions. The key to further investigation of this novel modification will be identifying a way to inhibit it in order to address its functional significance.

Appendices

Appendix A cCLSPN mRNA Sequence Isoform 1

```
   1 gagggcgggt ctagggcagc gcgcgaaggc tcgaaggttt cttacgcggc
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 151 agcgactcag acagcgggca gggcagtgct gagcccccct caccgggccg
 201 accacccact ggctccacgt ccccacagga tggagattca gaagaagaga
 251 tttttgtaag tagaaaagca aaaggcaaga aggtgcttca ggacagtgaa
 301 agtgaagatg gagaggacgg tgactcttct gtgcacaatg acactctggg
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1751 acatgagcat tattcgtaag gagaccacat ctgaagggaa ggaggaacta
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NCBI Accession No - GU248360

Appendix B cCLSPN mRNA Sequence Isoform 2

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4651 aaaaaaaaaa
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NCBI Accession No - GU248361

Appendix C cClaspin Amino Acid Sequence

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 101 EKVTAQRNKK SRIRQGLLDS DDSDTGDHLQ IENLDTSRKS VLSENEVEEG
 151 RPLKSGKKSR KHKHSFEDEA AEKAVGKPRR RKERERRAES IKQLIKGKKP
 201 SSEGQQVDGG EGYPFNDSGC LLDDKELFDN GLEEENNSFP GDEESIESIR
 251 AAVKEKIKKY KNKERFSEDE GYKHVFDDDN EESALKEPKR KERKAARLSK
 301 EAIKQLHSET QRLIRESSVS LPYHMPEAKS VHEFFKRRSR PVYEGNAMAL
 351 LKSTKYEFTL NEEAAGTKTS STDCKDGPTE GGQSAANEPE ANLGGHTDPA
 401 AKDPLLGEGE NLTEDSAEKS RKNNDDSHSA VTVTTASETE EQQSVLNTDC
 451 SEQKESEIPL PVGGNALEQR DETAPGLENS QQVGPGLAAQ PEKVRKSKLD
 501 KLRELGIDLT IKPRICSGNE SFINLDESDS NKELEALKAR FLKHTLQTSK
 551 PKLERTINMS IIRKETTSEG KEELKADVVP AVLAAESLDE AVHTKPGEKL
 601 QVLKAKLQEA MKLRRTEERQ KRQALFKLDN EELLEEEEEE EEEEEMTDES
 651 EEEEEGDHEA VESLLDEAEE DNEDLEEKQV EDGDKETDRE SIDGEKVEQA
 701 VDCASVPKPP STESTLMLFK DSSSKMGYSL PDEKHESEEA ANKEATKLED
 751 DDSFSLPTPA KENSHNSSFE LIGSMIPSYQ PCNKQMSRGG NFLPAAGGFR
 801 SPSPGFFKTS FISSASKSSG RMSEPSLPIE DSQDLYNASP EPKSLFPGAG
 851 ESQFQFSLED DTQSQLLDAD GFLNVGQHRN KYQSSKHGLT LASMDENAMD
 901 ANMDELLDLC SGQFSSQAEH VPSTSSNKKQ NMEELLNLCS GKFVTQNSPT
 951 WASSVSSKAE KDSDIEDPMA EALALCSGSF PTDREEEDEE HEEHEELGDF
1001 QLVTDDNAFD SEEDEKSGDS DGEEAEVSDE EELLRRREGS KKKLKLEDFM
1051 EDEAELSGSD VGSEDEYDGE DLNEYEEEII DEELPNEAEL ESQVQKLHMK
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1151 DSDNEDENES FDETEVKWRK ERFEREQWLR EQKEKNKEQE EEEEEDIGED
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1251 SLLNRPKDIL QKLAAMSDLN PNAPRNSRNF VFHTLSPDKN EEAKEKSKHQ
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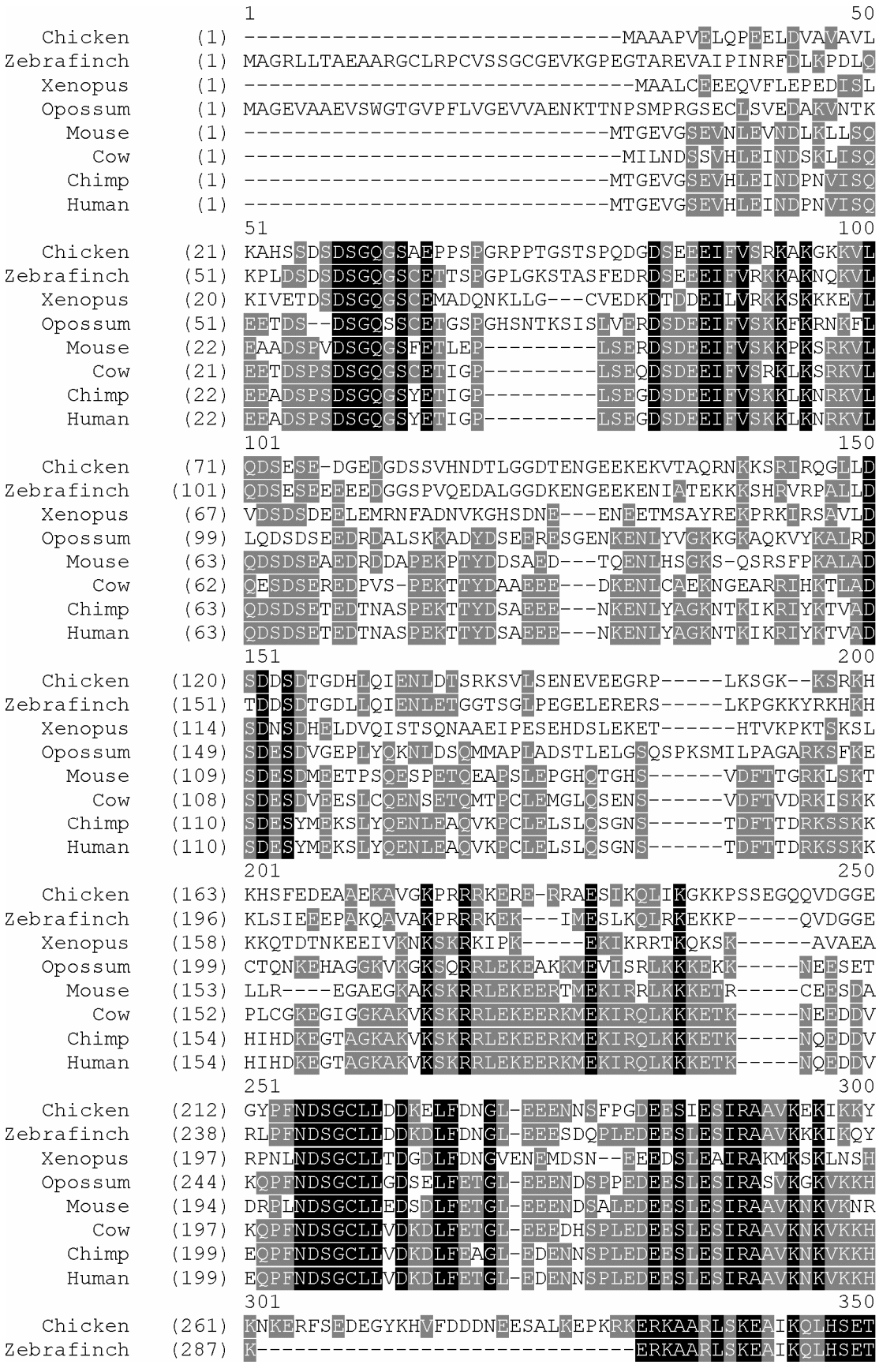
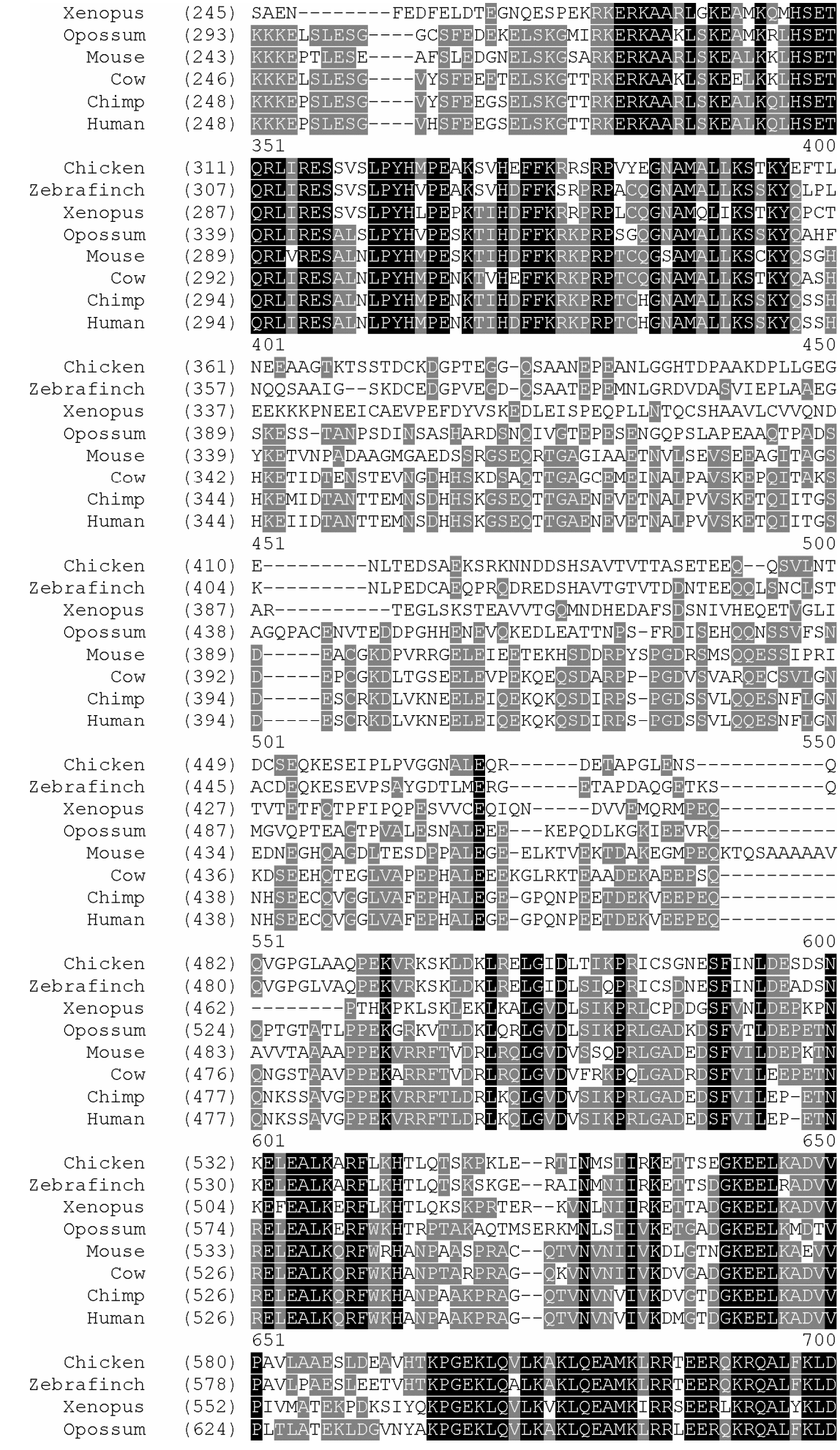
Appendix D Multiple Sequence Alignment of Claspin Orthologues

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Mouse (243) KKKEPTLESE----AFSLEDGNELSKGSARKERKAARLSKEALKKLHSET
Cow (246) KKKELSLESG----VYSFEEETELSKGTTRKERKAAKLSKEELKKLHSET
Chimp (248) KKKEPSLESG----VYSFEEGSELSKGTTRKERKAARLSKEALKQLHSET
Human (248) KKKEPSLESG----VHSFEEGSELSKGTTRKERKAARLSKEALKQLHSET
351 400
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Zebrafinch (307) QRLIRESSVSLPYHVPEAKSVHDFFKSRPRPACQGNAMALLKSSKYQLPL
Xenopus (287) QRLIRESSVSLPYHLPEPKTIHDFFKRRPRPLCQGNAMQLIKSTKYQPCT
Opossum (339) QRLIRESALSLPYHVPESKTIHDFFKRKPRPSGQGNAMALLKSSKYQAHF
Mouse (289) QRLVRESALNLPYHMPESKTIHDFFKRKPRPTCQGSAMALLKSCKYQSGH
Cow (292) QRLIRESALNLPYHMPENKTVHEFFKRKPRPTCQGNAMALLKSTKYQASH
Chimp (294) QRLIRESALNLPYHMPENKTIHDFFKRKPRPTCHGNAMALLKSSKYQSSH
Human (294) QRLIRESALNLPYHMPENKTIHDFFKRKPRPTCHGNAMALLKSSKYQSSH
401 450
Chicken (361) NEEAAGTKTSSTDCKDGPTEGG-QSAANEPEANLGGHTDPAAKDPLLGEG
Zebrafinch (357) NQQSAAIG--SKDCEDGPVEGD-QSAATEPEMNLGRDVDASVIEPLAAEG
Xenopus (337) EEKKKPNEEICAEVPEFDYVSKEDLEISPEQPLLNTQCSHAAVLCVVQND
Opossum (389) SKESS-TANPSDINSASHARDSNQIVGTEPESENGQPSLAPEAAQTPADS
Mouse (339) YKETVNPADAAGMGAEDSSRGSEQRTGAGIAAETNVLSEVSEEAGITAGS
Cow (342) HKETIDTENSTEVNGDHHSKDSAQTTGAGCEMEINALPAVSKEPQITAKS
Chimp (344) HKEMIDTANTTEMNSDHHSKGSEQTTGAENEVETNALPVVSKETQIITGS
Human (344) HKEIIDTANTTEMNSDHHSKGSEQTTGAENEVETNALPVVSKETQIITGS
451 500
Chicken (410) E---------NLTEDSAFKSRKNNDDSHSAVTVTTASETEEQ--QSVLNT
Zebrafinch (404) K---------NLPEDCAFQPRQDREDSHAVTGTVTDDNTEEQQLSNCLST
Xenopus (387) AR----------TEGLSKSTEAVVTGQMNDHEDAFSDSNIVHEQETVGLI
Opossum (438) AGQPACENVTEDDPGHHENEVQKEDLEATTNPS-FRDISEHQQNSSVFSN
Mouse (389) D-----EACGKDPVRRGELEIEETEKHSDDRPYSPGDRSMSQQESSIPRI
Cow (392) D-----EPCGKDLTGSEELEVPEKQEQSDARPP-PGDVSVARQECSVLGN
Chimp (394) D-----ESCRKDLVKNEELEIQEKQKQSDIRPS-PGDSSVLQQESNFLGN
Human (394) D-----ESCRKDLVKNEELEIQEKQKQSDIRPS-PGDSSVLQQESNFLGN
501 550
Chicken (449) DCSEQKESEIPLPVGGNALEQR------DETAPGLENS----------Q
Zebrafinch (445) ACDEQKESEVPSAYGDTLMERG------ETAPDAQGETKS---------Q
Xenopus (427) TVTETFQTPFIPQPESVVCEQIQN-----DVVEMQRMPEQ----------
Opossum (487) MGVQPTEAGTPVALESNALEEE---KEPQDLKGKIEEVRQ----------
Mouse (434) EDNFGHQAGDLTESDPPALEGE-ELKTVEKTDAKEGMPEQKTQSAAAAAV
Cow (436) KDSEEHQTEGLVAPEPHALEEEKGLRKTEAADEKAEEPSQ----------
Chimp (438) NHSEECQVGGLVAFEPHALEGE-GPQNPEETDEKVEEPEQ----------
Human (438) NHSEECQVGGLVAFEPHALEGE-GPQNPEETDEKVEEPEQ----------
551 600
Chicken (482) QVGPGLAAQPEKVRKSKLDKLRELGIDLTIKPRICSGNESFINLDESDSN
Zebrafinch (480) QVGPGLVAQPEKVRKSKLDKLRELGIDLSIQPRICSDNESFINLDEADSN
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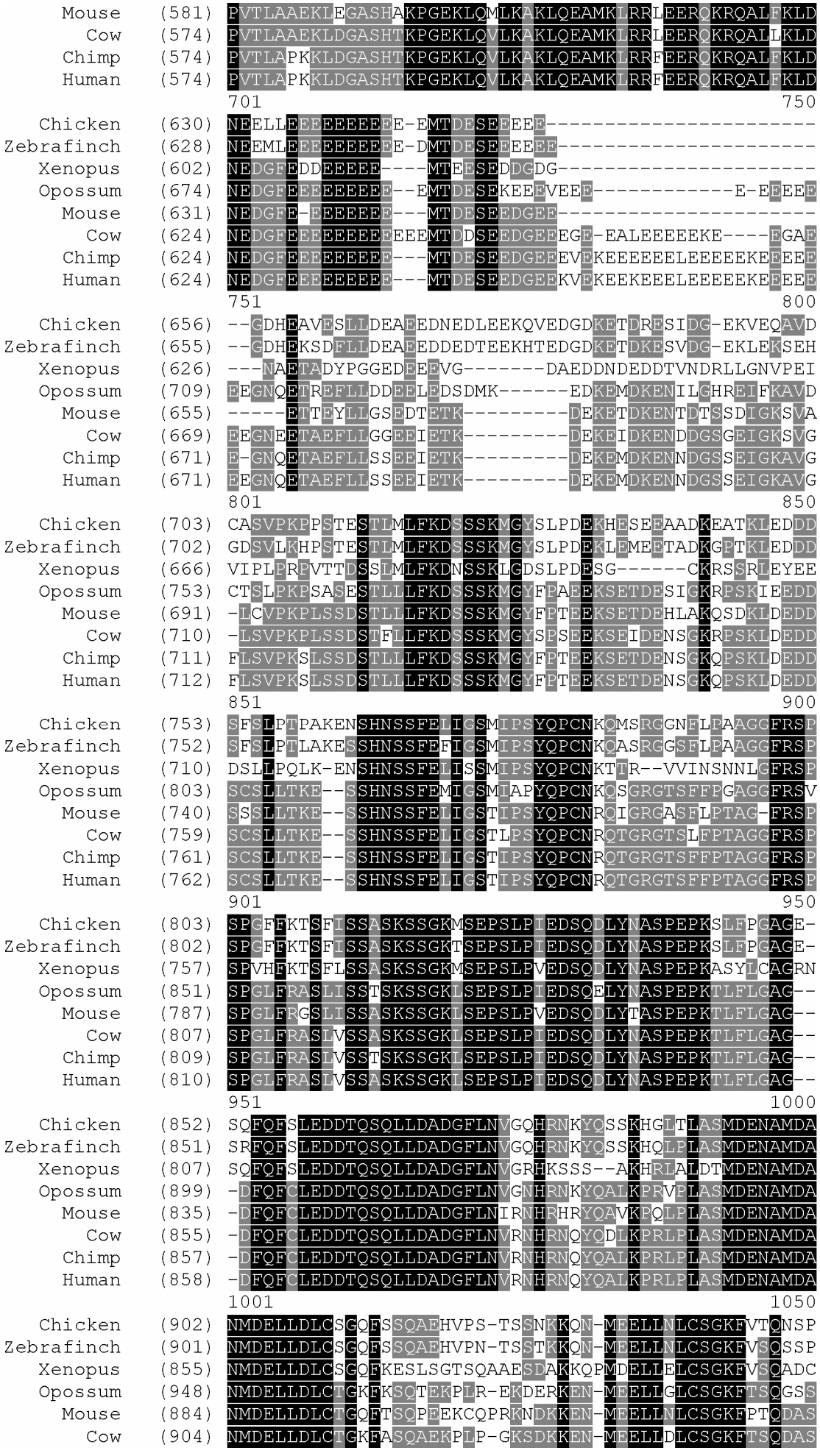
Opossum (524) QPTGTATLPPEKGRKVTLDKLQRLGVDLSIKPRLGADKDSFVTLDEPETN
Mouse (483) AVVTAAAAPPEKVRRFTVDRLRQLGVDVSSQPRLGADEDSFVILDEPKTN
Cow (476) QNGSTAAVPPEKARRFTVDRLRQLGVDVFRKPQLGADRDSFVILEEPETN
Chimp (477) QNKSSAVGPPEKVRRFTLDRLKQLGVDVSIKPRLGADEDSFVILEP-ETN
Human (477) QNKSSAVGPPEKVRRFTLDRLKQLGVDVSIKPRLGADEDSFVILEP-ETN
601 650
Chicken (532) KELEALKARFLKHTLQTSKPKLE--RTINMSIIRKETTSEGKEELKADVV
Zebrafinch (530) KELEALKARFLKHTLQTSKSKGE--RAINMNIIRKETTSDGKEELRADVV
Xenopus (504) KEFEALKERFLKHTLQKSKPRTER--KVNLNIIRKETTADGKEELKADVV
Opossum (574) RELEALKERFWKHTRPTAKAQTMSERKMNLSIIVKETGADGKEELKMDTV
Mouse (533) RELEALKQRFWRHANPAASPRAC--QTVNVNIIVKDLGTNGKEELKAEVV
Cow (526) RELEALKQRFWKHANPTARPRAG--QKVNVNIIVKDVGADGKEELKADVV
Chimp (526) RELEALKQRFWKHANPAAKPRAG--QTVNVNVIVKDVGTDGKEELKADVV
Human (526) RELEALKQRFWKHANPAAKPRAG--QTVNVNVIVKDMGTDGKEELKADVV
651 700
Chicken (580) PAVLAAESLDEAVHTKPGEKLQVLKAKLQEAMKLRRTEERQKRQALFKLD
Zebrafinch (578) PAVLPAESLEETVHTKPGEKLQALKAKLQEAMKLRRTEERQKRQALFKLD
Xenopus (552) PIVMATEKPDKSIYQKPGEKLQVLKVKLQEAMKIRRSEERLKRQALYKLD
Opossum (624) PLTLATEKLDGVNYAKPGEKLQVLKAKLQEAMKLRRLEERQKRQALFKLD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Chimp (574) PVTLAPKKLDGASHTKPGEKLQVLKAKLQEAMKLRRFEERQKRQALFKLD
Human (574) PVTLAPKKLDGASHTKPGEKLQVLKAKLQEAMKLRRFEERQKRQALFKLD
701 750
Chicken (630) NEELLEEEEEEEEEE-EMTDESEEEEE----------
Zebrafinch (628) NEEMLEEEEEEEEEE-DMTDESEEEEEEE----------
Xenopus (602) NEDGFEDDEEEEE----MTEESEDDGDG----------
Opossum (674) NEDGFEEEEEEEEE--EMTDESEKEEEVEEE----------E-EEEEE
Mouse (631) NEDGFE-EEEEEEE---MTDESEEDGEE----------
Cow (624) NEDGFEEEEEEEEEEEEMTDDSEEDGEEEGE-EALEEEEEKE----EGAE
Chimp (624) NEDGFEEEEEEEEE---MTDESEEDGEEEVEKEEEEEELEEEEEKEEEEE
Human (624) NEDGFEEEEEEEEE---MTDESEEDGEEKVEKEEKEEELEEEEEKEEEEE
751 800
Chicken (656) --GDHEAVESLLDEAEEDNEDLEEKQVEDGDKETDRESIDG-EKVEQAVD
Zebrafinch (655) --GDHEKSDFLLDEAEEDDEDTEEKHTEDGDKETDKESVDG-EKLEKSEH
Xenopus (626) ---NAETADYPGGEDEEEVG-------DAEDDNDEDDTVNDRLLGNVPEI
Opossum (709) EEGNQETREFLLDDEELEDSDMK------EDKEMDKENILGHREIFKAVD
Mouse (655) -----ETTEYLLGSEDTETK---------DEKETDKENTDTSSDIGKSVA
Cow (669) EEGNEETAEFLLGGEEIETK---------DEKEIDKENDDGSGEIGKSVG
Chimp (671) E-GNQETAEFLLSSEEIETK---------DEKEMDKENNDGSSEIGKAVG
Human (671) EEGNQETAEFLLSSEEIETK---------DEKEMDKENNDGSSEIGKAVG
801 850
Chicken (703) CASVPKPPSTESTLMLFKDSSSKMGYSLPDEKHESEEAADKEATKLEDDD
Zebrafinch (702) GDSVLKHPSTESTLMLFKDSSSKMGYSLPDEKLFMEETADKGPTKLEDDD
Xenopus (666) VIPLPRPVTTDSSLMLFKDNSSKLGDSLPDESG------CKRSSRLEYEE
Opossum (753) CTSLPKPSASESTLLLFKDSSSKMGYFPAEEKSETDESIGKRPSKIEEDD
Mouse (691) -LCVPKPLSSDSTLLLFKDSSSKMGYFPTEEKSETDEHLAKQSDKLDEDD
Cow (710) -LSVPKPLSSDSTFLLFKDSSSKMGYSPSEEKSEIDENSGKRPSKLDEDD
Chimp (711) FLSVPKSLSSDSTLLLFKDSSSKMGYFPTEEKSETDENSGKQPSKLDEDD
Human (712) FLSVPKSLSSDSTLLLFKDSSSKMGYFPTEEKSETDENSGKQPSKLDEDD
851 900
Chicken (753) SFSLPTPAKENSHNSSFELIGSMIPSYQPCNKQMSRGGNFLPAAGGFRSP
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Xenopus (710) DSLLPQLK-ENSHNSSFELISSMIPSYQPCNKTTR--VVINSNNLGFRSP
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Cow (759) SCSLLTKE--SSHNSSFELIGSTLPSYQPCNRQTGRGTSLFPTAGGFRSP
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Human (762) SCSLLTKE--SSHNSSFELIGSTIPSYQPCNRQTGRGTSFFPTAGGFRSP
901 950
Chicken (803) SPGFFKTSFISSASKSSGKMSEPSLPIEDSQDLYNASPEPKSLFPGAGE-
Zebrafinch (802) SPGFFKTSFISSASKSSGKTSEPSLPIEDSQDLYNASPEPKSLFPGAGE-
Xenopus (757) SPVHFKTSFLSSASKSSGKMSEPSLPVEDSQDLYNASPEPKASYLCAGRN
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Cow (807) SPGLFRASLVSSASKSSGKLSEPSLPIEDSQDLYNASPEPKTLFLGAG--
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Human (810) SPGLFRASLVSSASKSSGKLSEPSLPIEDSQDLYNASPEPKTLFLGAG--
951 1000
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Zebrafinch (851) SRFQFSLEDDTQSQLLDADGFLNVGQHRNKYQSSKHQLPLASMDENAMDA
Xenopus (807) SQFQFSLEDDTQSQLLDADGFLNVGRHKSSS--AKHRLALDTMDENAMDA
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Mouse (835) -DFQFCLEDDTQSQLLDADGFLNIRNHRHRYQAVKPQLPLASMDENAMDA
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Chimp (857) -DFQFCLEDDTQSQLLDADGFLNVRNHRNQYQALKPRLPLASMDENAMDA
Human (858) -DFQFCLEDDTQSQLLDADGFLNVRNHRNQYQALKPRLPLASMDENAMDA
1001 1050
Chicken (902) NMDELLDLCSGQFSSQAEHVPS-TSSNKKQN-MEELLNLCSGKFVTQNSP
Zebrafinch (901) NMDELLDLCSGQFSSQAEHVPN-TSSTKKQN-MEELLNLCSGKFVSQSSP
Xenopus (855) NMDELLDLCSGQFKESLSGTSQAAESDAKKQPMDELLELCSGKFVSQADC
Opossum (948) NMDELLDLCTGKFKSQTEKPLR-EKDERKEN-MEELLGLCSGKFTSQGSS
Mouse (884) NMDELLDLCTGQFTSQPEEKCQPRKNDKKEN-MEELLNLCSGKFPTQDAS
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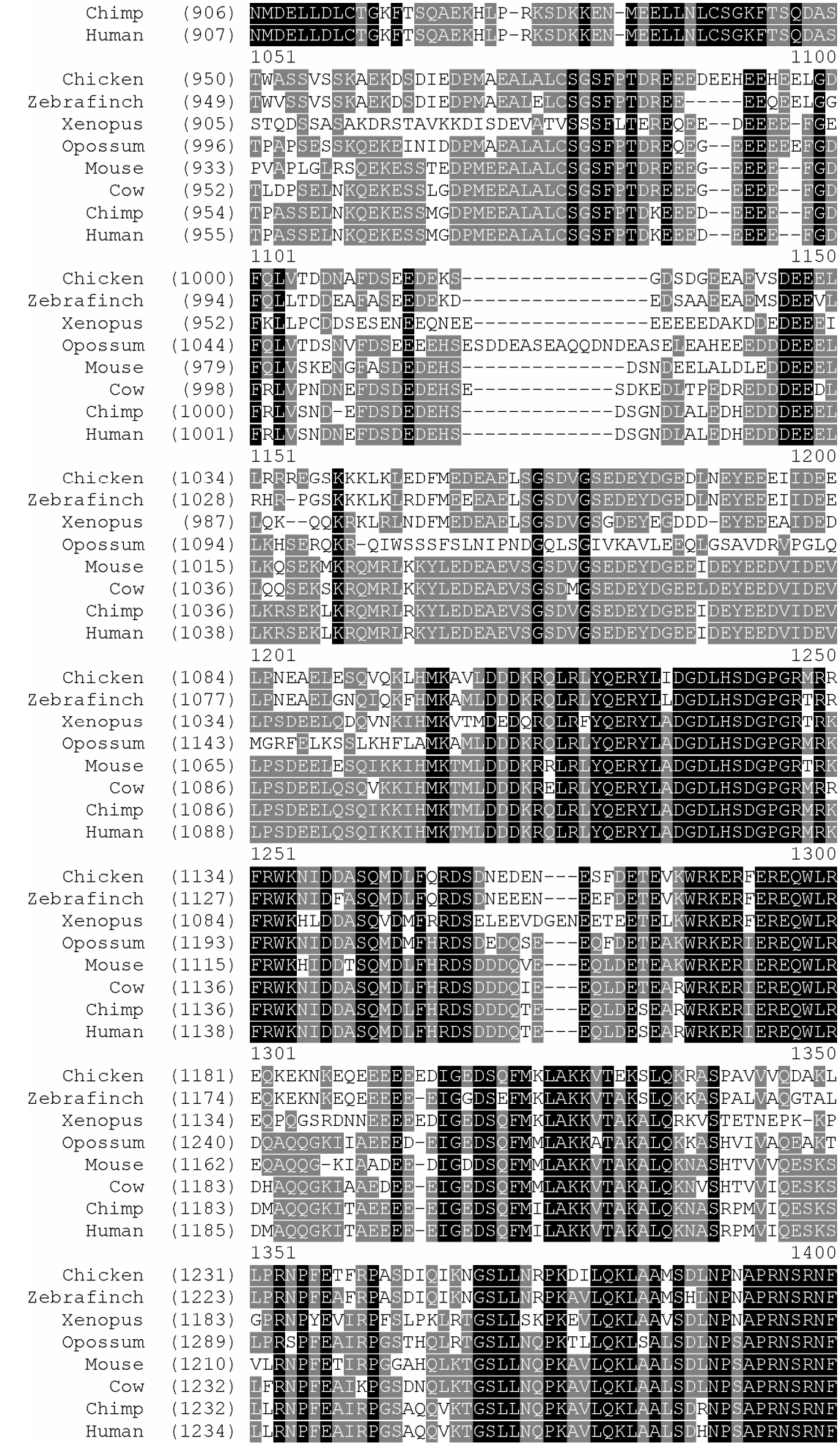
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Human (907) NMDELLDLCTGKFTSQAEKHLP-RKSDKKEN-MEELLNLCSGKFTSQDAS
1051 1100
Chicken (950) TWASSVSSKAEKDSDIEDPMAEALALCSGSFPTDREEEDEEHEEHEELGD
Zebrafinch (949) TWVSSVSSKAEKDSDIEDPMAEALELCSGSFPTDREE-----EEQEELGG
Xenopus (905) STQDSSASAKDRSTAVKKDISDEVATVSSSFLTEREQEE--DEEEE-EGE
Opossum (996) TPAPSESSKQEKEINIDDPMAEALALCSGSFPTDREQEG--EEEEEEFGD
Mouse (933) PVAPLGLRSQEKESSTEDPMEEALALCSGSFPTDREEEG--EEEE--FGD
Cow (952) TLDPSELNKQEKESSLGDPMEEALALCSGSFPTDREEEG--EEEE--FGD
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Human (955) TPASSELNKQEKESSMGDPMEEALALCSGSFPTDKEEED--EEEE--FGD
1101 1150
Chicken (1000) FQLVTDDNAFDSEEDEKS----------GDSDGEEAEVSDEEEL
Zebrafinch (994) FQLLTDDEAFASEEDEKD----------EDSAAEEAEMSDEEVL
Xenopus (952) FKLLPCDDSESENEEQNEE----------EEEEEDAKDDEDEEEI
Opossum (1044) FQLVTDSNVFDSEEEEHSESDDEASEAQQDNDEASELEAHEEEDDDEEEL
Mouse (979) FQLVSKENGFASDEDEHS----------DSNDEELALDLEDDEEEL
Cow (998) FRLVPNDNEFDSDEDEHSE----------SDKEDLTPEDREDDDEEDL
Chimp (1000) FRLVSND-EFDSDEDEHS----------DSGNDLALEDHEDDDEEEL
Human (1001) FRLVSNDNEFDSDEDEHS----------DSGNDLALEDHEDDDEEEL
1151 1200
Chicken (1034) LRRREGSKKKLKLEDFMEDEAELSGSDVGSEDEYDGEDLNEYEEEIIDEE
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Xenopus (987) LQK--QQKRKLRLNDFMEDEAELSGSDVGSGDEYEGDDD-EYEEEAIDED
Opossum (1094) LKHSERQKR-QIWSSSFSLNIPNDGQLSGIVKAVLEEQLGSAVDRVPGLQ
Mouse (1015) LKQSEKMKRQMRLKKYLEDEAEVSGSDVGSEDEYDGEEIDEYEEDVIDEV
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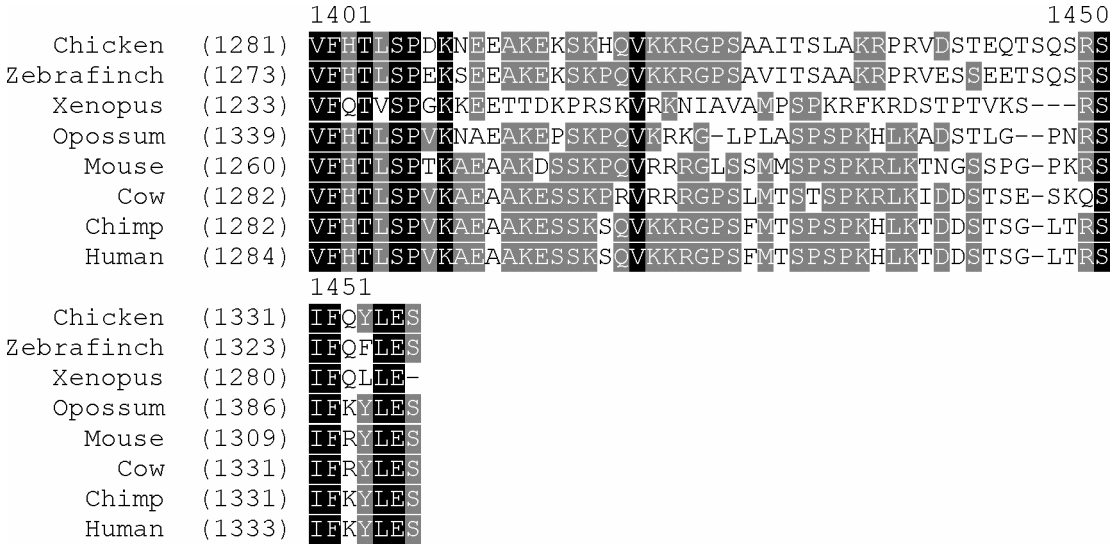
Chimp (1036) LKRSEKLKRQMRLRKYLEDEAEVSGSDVGSEDEYDGEEIDEYEEDVIDEV
Human (1038) LKRSEKLKRQMRLRKYLEDEAEVSGSDVGSEDEYDGEEIDEYEEDVIDEV
1201 1250
Chicken (1084) LPNEAELESQVQKLHMKAVLDDDKRQLRLYQERYLIDGDLHSDGPGRMRR
Zebrafinch (1077) LPNEAELGNQIQKFHMKAMLDDDKRQLRLYQERYLLDGDLHSDGPGRTRR
Xenopus (1034) LPSDEELQDQVNKIHMKVTMDEDQRQLRFYQERYLADGDLHSDGPGRTRK
Opossum (1143) MGRFELKSSLKHFLAMKAMLDDDKRQLRLYQERYLADGDLHSDGPGRMRK
Mouse (1065) LPSDEELESQIKKIHMKTMLDDDKRRLRLYQERYLADGDLHSDGPGRTRK
Cow (1086) LPSDEELQSQVKKIHMKTMLDDDKRELRLYQERYLADGDLHSDGPGRMRR
Chimp (1086) LPSDEELQSQIKKIHMKTMLDDDKRQLRLYQERYLADGDLHSDGPGRMRK
Human (1088) LPSDEELQSQIKKIHMKTMLDDDKRQLRLYQERYLADGDLHSDGPGRMRK
1251 1300
Chicken (1134) FRWKNIDDASQMDLFQRDSDNEDEN---ESFDETEVKWRKERFEREQWLR
Zebrafinch (1127) FRWKNIDFASQMDLFQRDSDNEEEN---EEFDETEVKWRKERFEREQWLR
Xenopus (1084) FRWKHLDDASQVDMFRRDSELEEVDGENEETEETELKWRKERFEREQWLR
Opossum (1193) FRWKNIDDASQMDMFHRDSDEDQSE---EQFDEIEAKWRKERIEREQWLR
Mouse (1115) FRWKHIDDTSQMDLFHRDSDDDQVE---EQLDEIEAKWRKERIEREQWLR
Cow (1136) FRWKNIDDASQMDLFHRDSDDDQIE---EQLDETEARWRKERIEREQWLR
Chimp (1136) FRWKNIDDASQMDLFHRDSDDDQTE---EQLDESEARWRKERIEREQWLR
Human (1138) FRWKNIDDASQMDLFHRDSDDDQTE---EQLDESEARWRKERIEREQWLR
1301 1350
Chicken (1181) EQKEKNKEQEEEEEEDIGEDSQFMKLAKKVTEKSLQKRASPAVVVQDAKL
Zebrafinch (1174) EQKEKNKEQEEEEE-EIGGDSEFMKLAKKVTAKSLQKKASPALVAQGTAL
Xenopus (1134) EQPQGSRDNNEEEEEDIGEDSQFMKLAKKVTAKALQRKVSTETNEPK-KP
Opossum (1240) DQAQQGKIIAEEED-EIGEDSQFMMLAKKATAKALQKKASHVIVAQEAKT
Mouse (1162) EQAQQG-KIAADEE-DIGDDSQFMMLAKKVTAKALQKNASHTVVVQESKS
Cow (1183) DHAQQGKIAAEDEE-EIGEDSQFMMLAKKVTAKALQKNVSHTVVIQESKS
Chimp (1183) DMAQQGKITAEEEE-EIGEDSQFMILAKKVTAKALQKNASRPMVIQESKS
Human (1185) DMAQQGKITAEEEE-EIGEDSQFMILAKKVTAKALQKNASRPMVIQESKS
1351 1400
Chicken (1231) LPRNPFETFRPASDIQIKNGSLLNRPKDILQKLAAMSDLNPNAPRNSRNF
Zebrafinch (1223) LPRNPFEAFRPASDIQIKNGSLLNRPKAVLQKLAAMSHLNPNAPRNSRNF
Xenopus (1183) GPRNPYEVIRPFSLPKLRTGSLLSKPKEVLQKLAAVSDLNPNAPRNSRNF
Opossum (1289) LPRSPFEAIRPGSTHQLRTGSLLNQPKTLLQKLSALSDLNPSAPRNSRNF
Mouse (1210) VLRNPFETIRPGGAHQLKTGSLLNQPKAVLQKLAALSDLNPSAPRNSRNF
Cow (1232) LFRNPFEAIKPGSDNQLKTGSLLNQPKAVLQKLAALSDLNPSAPRNSRNF
Chimp (1232) LLRNPFEAIRPGSAQQVKTGSLLNQPKAVLQKLAALSDRNPSAPRNSRNF
Human (1234) LLRNPFEAIRPGSAQQVKTGSLLNQPKAVLQKLAALSDHNPSAPRNSRNF


1401
1450
Chicken (1281) VFHTLSPDKNEEAKEKSKHQVKKRGPSAAITSLAKRPRVDSTEQTSQSRS
Zebrafinch (1273) VFHTLSPEKSEEAKEKSKPQVKKRGPSAVITSAAKRPRVESSEETSQSRS
Xenopus (1233) VFQTVSPGKKEETTDKPRSKVRKNIAVAMPSPKRFKRDSTPTVKS---RS
Opossum (1339) VFHTLSPVKNAEAKEPSKPQVKRKG-LPLASPSPKHLKADSTLG--PNRS
Mouse (1260) VFHTLSPTKAEAAKDSSKPQVRRRGLSSMMSPSPKRLKTNGSSPG-PKRS
Cow (1282) VFHTLSPVKAEAAKESSKPRVRRRGPSLMTSTSPKRLKIDDSTSE-SKQS
Chimp (1282) VFHTLSPVKAEAAKESSKSQVKKRGPSFMTSPSPKHLKTDDSTSG-LTRS
Human (1284) VFHTLSPVKAEAAKESSKSQVKKRGPSFMTSPSPKHLKTDDSTSG-LTRS
1451
Chicken (1331) IFQYLES
Zebrafinch (1323) IFQFLES
Xenopus (1280) IFQLLE-
Opossum (1386) IFKYLES
Mouse (1309) IFRYLES
Cow (1331) IFRYLES
Chimp (1331) IFKYLES
Human (1333) IFKYLES

Appendix E Identity Table from Multiple Sequence Alignment

	Zebrafinch	Drosophila	Xenopus	Opossum	Mouse	Cow	Chimp	Human
Chicken	78	17	49	54	53	55	56	56
Zebrafinch		17	48	53	54	55	56	56
Drosophila			18	16	17	17	17	17
Xenopus				44	46	46	46	46
Opossum					61	65	66	66
Mouse						75	76	76
Cow							84	84
Chimp								99

Appendix F Conservation Table from Multiple Sequence Alignment

	Zebrafinch	Drosophila	Xenopus	Opossum	Mouse	Cow	Chimp	Human
Chicken	86	36	67	68	69	71	71	71
Zebrafinch		36	66	67	69	72	71	71
Drosophila			35	34	35	35	35	35
Xenopus				60	65	65	64	64
Opossum					74	78	77	77
Mouse						84	86	86
Cow							90	90
Chimp								100

Appendix G Potential cClaspin Interacting Partners

The following data lists the proteins identified by Mass spec that co-purified with SF.cClaspin following the tandem affinity purification method described in section 2.2.2.9. Data has been curated to remove proteins that were identified in the negative control samples – protein purified form DT40 cells not expressing the SF.cClaspin protein. The background (+/- Chk1) and treatment is indicated.

Protein	International Protein Index	Molecular Mass	No. of Unique Peptides
CLSPN similar to claspin	IPI00599884	154kDa	60
LRPPRC leucine-rich PPR-motif containing	IPI00819696	156kDa	20
HSPA5 78 kDa glucose-regulated protein precursor	IPI00590375	72kDa	17
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00589568	73kDa	9
HSPA4L Putative uncharacterized protein	IPI00573597	95kDa	6
TUBB2C Tubulin beta-3 chain	IPI00580626	50kDa	6
HSP70 Heat shock 70 kDa protein	IPI00582091	70kDa	6
MCM2 Putative uncharacterized protein	IPI00577675	100kDa	5
EIF3A similar to p167	IPI00594883	178kDa	4
CSE1L similar to cellular apoptosis susceptibility protein	IPI00582808	110kDa	4
EEF2 Elongation factor 2	IPI00585747	95kDa	4
ATP5B ATP synthase subunit beta, mitochondrial precursor	IPI00584049	57kDa	3
MVP Major vault protein	IPI00597028	95kDa	3
MCM6 Putative uncharacterized protein	IPI00600947	93kDa	2
USP7 126 kDa protein	IPI00580665	128kDa	2
MCM4 similar to MCM4	IPI00820198	97kDa	2
TUBA1C Tubulin alpha-1 chain (Fragment)	IPI00575989	50kDa	2
HSP90B1 Endoplasmin precursor	IPI00570770	92kDa	2
PSMD2 Putative uncharacterized protein (26S proteasome)	IPI00592623	100kDa	2
Eukaryotic translation elongation factor 1	IPI00600541	50kDa	2
TBCD 135 kDa protein [tubulin folding co-factor D]	IPI00574641	113kDa	2
TCP1 T-complex protein 1, alpha subunit	IPI00584300	61kDa	2
NPEPPS aminopeptidase puromycin sensitive	IPI00821924	96kDa	2
TUBB Tubulin beta-7 chain	IPI00591483	50kDa	1
GXPNPEP1	IPI00582583	70kDa	1
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	IPI00594653	44kDa	1
HSPH1 similar to Heat-shock protein 105 kDa	IPI00590633	106kDa	1
LOC422622 hypothetical protein [GTP Binding Protein]	IPI00602336	62kDa	1
MCCC1 Putative uncharacterized protein	IPI00583497	78kDa	1
NCL Nucleolin	IPI00577202	75kDa	1
LOC430499 similar to eEF1, partial	IPI00597210	6kDa	1
RBBP4 Histone-binding protein RBBP4	IPI00592914	48kDa	1
CCT2 Putative uncharacterized protein	IPI00579548	57kDa	1
KRT5 Type II alpha-keratin IIA	IPI00581314	62kDa	1
MLL MLL protein	IPI00820475	430kDa	1
IARS similar to isoleucyl-tRNA synthetase	IPI00581192	145kDa	1
IGSF10 similar to bone specific CMF608	IPI00597899	199kDa	1
Acetyl CoEnzyme Acarboxylase alpha 257 kDa protein	IPI00582369	261kDa	1
PSMD1 106 kDa protein (19S Proteasome)	IPI00579067	106kDa	1
VPS35 [vacuolar protein sorting 35]	IPI00582536	92kDa	1

Table G-1 Chk1-/- Flp-In:SF.cClaspin Cells - Untreated

Protein	International Protein Index	Molecular Mass	No. of Unique Peptides
CLSPN similar to claspin	IPI00599884	154 kDa	35
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00589568	73 kDa	18
HSPA5 78 kDa glucose-regulated protein precursor	IPI00590375	72 kDa	15
HSP70 Heat shock 70 kDa protein	IPI00582091	70 kDa	8
ACOT8 HIV-Nef associated acyl CoA thioesterase isoform 2	IPI00585331	36 kDa	4
eEF2 Elongation factor 2	IPI00585747	95 kDa	4
HSPH1 similar to Heat-shock protein 105 kDa	IPI00590633	106 kDa	4
EEF1A1 Elongation factor 1-alpha 1	IPI00589985	50 kDa	3
HSPA4L Putative uncharacterized protein	IPI00573597	95 kDa	3
Protein	IPI00604233	186 kDa	3
USP7 126 kDa protein	IPI00580665	126 kDa	3
TP53 Cellular tumor antigen p53	IPI00585109	40 kDa	2
MVP Major vault protein	IPI00597028	94 kDa	2
RCJMB04_39i8 Putative uncharacterized protein	IPI00592325	49 kDa	2
MCM2 Putative uncharacterized protein	IPI00577675	100 kDa	2
ATP5B ATP synthase subunit beta, mitochondrial precursor	IPI00584049	57 kDa	2
TUBB Tubulin beta-7 chain	IPI00591483	50 kDa	2
MCM3 DNA replication licensing factor MCM3	IPI00604115	91 kDa	2
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	IPI00594653	36 kDa	2
93 kDa protein	IPI00595984	93 kDa	1
PTPRF similar to protein tyr phosphatase, receptor type, F	IPI00603030	214 kDa	1
DSP similar to desmoplakin isoform II isoform 2	IPI00602986	331 kDa	1

Table G-2 Chk1-/- Flp-In:SF.cClaspin Cells - CHX treated

Protein	International Protein Index	Molecular Mass	No. of Unique Peptides
CLSPN similar to claspin	IPI00599884	154 kDa	26
93 kDa protein	IPI00595984	93 kDa	2
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00589568	73 kDa	1
HSPA5 78 kDa glucose-regulated protein precursor	IPI00590375	72 kDa	1
ACOT8 - HIV-Nef associated acyl CoA thioesterase isoform 2	IPI00585331	36 kDa	1
EEF1A1 Elongation factor 1-alpha 1	IPI00589985	50 kDa	1
PTPRF similar to protein tyr phosphatase, receptor type, F	IPI00603030	214 kDa	1
Upstream stimulatory factor 2	IPI00602660	33 kDa	1

Table G-3 Chk1-/- Flp-In:SF.cClaspin Cells - APH treated

Protein	International Protein Index	Molecular Mass	No. of Unique Peptides
CLSPN similar to claspin	IPI00599884	154 kDa	6
PTPRF similar to protein tyr phosphatase, receptor type, F	IPI00603030	214 kDa	1
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00589568	73 kDa	1
Upstream stimulatory factor 2	IPI00602660	33 kDa	1
93 kDa protein	IPI00595984	93 kDa	1
HSP70 Heat shock 70 kDa protein	IPI00582091	70 kDa	1
FASN Isoform 1 of Fatty acid synthase	IPI00572922	276 kDa	1
TEX10 Testis-expressed sequence 10 protein homolog	IPI00589662	105 kDa	1
TMEM24 similar to DLNB23	IPI00587331	73 kDa	1
ZNF276 similar to zinc finger protein 276 homolog	IPI00573409	64 kDa	1
DSP similar to desmoplakin isoform II isoform 2	IPI00602986	331 kDa	1
STK40 50 kDa protein	IPI00595409	50 kDa	1
PSMD1 106 kDa protein	IPI00579067	106 kDa	1

Table G-4 Chk1 Revertant Flp-In:SF.cClaspin Cells - CHX treated

Protein	International Protein Index	Molecular Mass	No. of Unique Peptides
CLSPN similar to claspin	IPI00599884	154 kDa	23
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00589568	73 kDa	3
HSPA5 78 kDa glucose-regulated protein precursor	IPI00590375	72 kDa	3
HSP70 Heat shock 70 kDa protein	IPI00582091	70 kDa	3
MYH9 Myosin-9	IPI00572165	227 kDa	3
MYH10 Nonmuscle myosin heavy chain	IPI00576130	231 kDa	2
PTPRF similar to protein tyr phosphatase, receptor type, F	IPI00603030	214 kDa	1
Upstream stimulatory factor 2	IPI00602660	33 kDa	1
FASN Isoform 1 of Fatty acid synthase	IPI00572922	276 kDa	1
ACOT8 - HIV-Nef associated acyl CoA thioesterase isoform 2	IPI00585331	36 kDa	1
TEX10 Testis-expressed sequence 10 protein homolog	IPI00589662	105 kDa	1
TMEM24 similar to DLNB23	IPI00587331	73 kDa	1
EEF1A1 Elongation factor 1-alpha 1	IPI00589985	50 kDa	1
HSPA4L Putative uncharacterized protein	IPI00573597	95 kDa	1
DSP similar to desmoplakin isoform II isoform 2	IPI00602986	331 kDa	1

Table G-5 Chk1 Revertant Flp-In:SF.cClaspin Cells - APH treated

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