Functional Analysis of *S. pombe* Cdc37

by

Emma Turnbull BSc Hons

Ph.D Thesis
University of Edinburgh
April 2005

Table of contents

4

Declaration of Originality

I declare that the research work presented in this thesis is my own with acknowledgement for works carried out by others made where appropriate.

Acknowledgements

I would like to give a huge thank you to my supervisor Peter Fantes, who gave me the opportunity to do my PhD in his lab under his expert guidance and for all the valuable help he has given me throughout. I have learnt a great deal and am forever grateful for this opportunity. Thank you to Ina Martin for all her help when I joined the lab and the Bird lab for use of their equipment.

Thank you to Rob Klose for all his advice and for always being great. Thank you to my family, Mum and Dad, little sister Jessie-Kate, Sarah and Andrew for being so supportive.

Abbreviations

Ade	Adenine
AR	Androgen receptor
bp	base pair(s)
BSA	Bovine serum albumin
Ca	Calcium ion
cdc	cell division cycle
Cdk	cyclin dependent kinase
cDNA	complementary DNA
CIP	Calf intestinal alkaline phosphatase
Cl	chloride ion
cm	centimetres
^{0}C	degrees Celsius
DAPI	4,6,diamidino-2-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal media
FACS	fluorescence activated cell sorting
g	grams(s)
H	hydrogen ion
hr(s)	hour(s)
His	Histidine
IPTG	isopropyl-β-thiogalactopyranoside
K	potassium ion
kb	kilobase pair(s)
l	litre(s)
LB	Luria-Bertani medium
Leu	Leucine
M	molar
ME	malt extract medium
Mg	magnesium ion
MOPS	4-morpholinepropane sulphonic acid
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
μg	microgram(s)
μl	microlitre(s)
μM	micromolar
Na	sodium ion
ng	nanogram(s)
nm	nanometre(s)
nmol	nanmole(s)
OAc	acetate

OD	optical density
%	percent
PCR	polymerase chain reaction
PIPES	Piperzine-N,N'-bis[2-ethanesulfonic acid]
pmol	picomole(s)
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
sec	second(s)
SD	synthetic dropout medium
SDS	sodium dodecyl sulphate
SO_4	sulphate ion
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
Thi	Thiamine
T_m	melting temperature
TRITC	Tetramethylrhodamine B isothiocyanate
Tris	tris[hygroxymethyl]aminomethane
ts	temperature-sensitive
Ura	Uracil
v/v	volume per volume
vol	volume
w/v	weight per volume
x	times
X-Gal	5-bromo-4-chloro-3-inodyl-β-galactopyranoside
YE	yeast extract medium

Abstract

Cdc37 is a molecular chaperone that consists of three domains. The N-terminal domain is involved in client protein binding and contains conserved serine residues that are important for function and are phosphorylated by casein kinase II. The middle domain contains the Hsp90-binding and homodimerisation regions. The C-terminal domain is at present of unknown function. The *Schizosaccharomyces pombe cdc37* gene has been identified and was found to be essential for cell viability. To further study *S. pombe* Cdc37, a range of mutants were generated using both random and directed mutagenesis. Temperature-sensitive, truncation, 5 amino acid insertion and point mutation mutants of *cdc37* were created and characterised to gain a better understanding of this chaperone in *S. pombe*.

Expression of only the N-terminal domain of *S. pombe* Cdc37, lacking the postulated Hsp90-binding and homodimerisation regions, can sustain cell viability in a *cdc37Δ* strain, indicating that the middle and C-terminal domains are dispensable for the essential function of Cdc37. The N-terminal domain is the most conserved region of Cdc37 across species and contains highly conserved serine residues known to be phosphorylated in other organisms. Mutational analysis of these conserved serine residues in *S. pombe* Cdc37 identified serine 14 as important for function.

To study the effect of rapid loss of Cdc37 function three temperature-sensitive mutants were generated by random and directed mutagenesis and a fourth was a kind gift from the Shiozaki lab and was analysed in parallel. Cdc37 temperature-sensitive mutant cells at the restrictive temperature stop dividing within a single cell cycle. Cdc37 protein levels were not changed at the non-permissive temperature in mutants, indicating that cell inviability arises from defective function of the mutant Cdc37 protein. Morphologically, temperature-sensitive mutant cells arrest with a uniform phenotype, being elongated, characteristic of the *cdc* phenotype. About 80% of these elongated cells contained a single nucleus with a 2C DNA content, indicating that *cdc37* temperature-sensitive mutants arrest in G2. The only exception was *cdc37-J*, where half the cells leaked through to mitosis after 8 hours at the restrictive temperature and displayed divided nuclei and septa, arresting with defects in

cytokinesis. Characterisation of *cdc37* temperature-sensitive mutants led to the identification of Cdc2 as a client protein and a principal candidate for the cause of the G2 cell cycle arrest. Cdc2 activity was dramatically reduced within one hour at the non-permissive temperature in *cdc37* temperature-sensitive mutants, but Cdc2 protein levels remained constant. Biochemical and genetic interactions between Cdc37 and Cdc2 were observed, supporting the idea of Cdc2 as a client of Cdc37.

Cdc37 is believed to deliver client protein kinases to the co-chaperone Hsp90, forming a heterocomplex. A small fraction of total cellular Cdc37 in *S. pombe* forms a high molecular weight complex which also contains Hsp90. Cdc37 was found to biochemically and genetically interact with Hsp90, indicating that these co-chaperones co-operate in *S. pombe*.

Chapter 1:

Introduction

1.0 Molecular chaperones

1.1 Introduction

Cells are the basic units of life, containing a wide variety of different types of proteins, each carrying out specific functions. Proteins are elegantly regulated by a variety of pathways essential for cell viability and division. The creation of a protein begins with gene expression, transcribing the encoding gene from chromosomal DNA. This ultimately produces an RNA template of the chromosomal DNA sequence, called an mRNA. The mRNA template is then translated by ribosomal complexes, generating a linear nascent polypeptide chain composed of a specific sequence of amino acids, called the primary structure. The amino acid sequence of the linear polypeptide determines its final three-dimensional structure, the native state, which is imperative for achieving proper function of the protein (Dobson, 2004).

Upon translation, the nascent polypeptide chains in their primary structure enter a new regulatory pathway run by molecular chaperone proteins in both prokaryotic and eukaryotic systems. These molecular chaperone proteins are defined as 'proteins that help in the folding of other proteins, typically through cycles of binding and release, but do not form part of the final native structure' (Young et al., 2004). The proteins on which molecular chaperones act are referred to as 'client proteins'. Molecular chaperones represent a highly specialised network of proteins, each with specific roles and interactions with client proteins and co-chaperones to ensure efficient protein quality control within the cell. A list of the different types of molecular chaperone proteins described in this Chapter is given in Table 1.1. The "quality control" mechanisms carried out by molecular chaperone proteins (summarised schematically in Figure 1.1), include client folding, maintaining client proteins in an activation competent state, targeting clients for degradation, client translocation, client complex assembly with partner proteins and refolding misfolded client polypeptides. These chaperone activities towards client proteins are crucial to the cell under normal growth conditions for regulation of biological activity both spatially and temporally.

Table 1.1: A table of molecular chaperone proteins and the names given to homologues from different organisms.

Function	Group		Mammalian	*S. cerevisiae*	*S. pombe*	*E. coli*
Folding chaperones	**Heat shock proteins**	Hsp100	-	-	-	ClpA
			SKD3	Hsp104	Hsp104 (AN: NP_596503)	ClpB
			-	-	-	ClpC
		Hsp90	Hsp90α	Hsc82	Hsp90	HtpG
			Hsp90β	Hsp82		
			Grp94 (ER)	-	-	-
			Trap75 (mitochondrial)	-	-	-
		Hsp70	Hsp70 protein 8 isoform 1 variant (Hsp70 (P8I1V))	Ssa1	AN: NP593704	-
			Hsp70 (P8I1V)	Ssa2	AN: NP593704	-
			Hsp70 (P8I1V)	Ssa3	AN: NP593704	-
			Hsp70 (P8I1V)	Ssa4	AN: NP593704	-
			Hsp70 (P8I1V)	Ssb1	AN: BAA12279	-
			Hsp70 (P8I1V)	Ssb2	AN: BAA12279	-
			Hsp70 9B	Ssc1	AN: NP593459	-
			Hsp70 9B	Ssc3	AN: NP593459	-
			AN: CAI12428	Sse1	AN: NP001018245	-
			Hsp70 protein 4	Sse2	AN: NP001018245	-
			Hsp70 9B	Ssq1		-
			-	Ssz1	AN: BAA13923	
			Bip/GRP78 (ER)	Kar2	AN: NB593245	-
			Grp75 (mitochondrial)	-	AN: NP593459	-
			-	-	-	DnaK
			-	-	-	GrpE
		Hsp 60	Chaperonin (mitochondrial)	-	-	GroEL
			-	-	-	SecB
		Hsp40	DnajB1	Sis1	-	DnaJ
			Hdj1	Ydj1	AN: NP596309	-
			Dnaj A2	Apj1	AN: NP595426	-
		Hsp10	Hsp10	Hsp10 (mitochondrial)	-	GroES
Other chaperones	**Cdc37**		P50	Cdc37	Cdc37	-
	Immunophillin		Cyp40 Cyclophilin D	Cpr6	-	-
			Cyclophilin D	Cpr7	-	-
			FKBP52	-	-	-

Table 1.1 continued:

Function	Group		Mammalian	*S. cerevisiae*	*S. pombe*	*E. coli*
Regulate co-chaperones	p23		p23	Sba1	AN: NP594586	-
Chaperones that bind translating polypeptides	**Trigger Factor**		Mp11	Ssb-Ssz-Zuo	ribosome assembly chaperone (RAC)	Trigger factor
Scaffold chaperones	**Hop**		Hop	Sti1	AN: NP588123	-
	Hip		Hip	-	-	-
Chaperones involved in degradation	**Bag-1**		Bag-1	-	-	-
	CHIP		CHIP	-	-	-

ER   endoplasmic reticulum

-       no orthologue

AN   accession number

Figure 1.1: An overview of some of the roles of molecular chaperone proteins within the cell. **A** Chaperones can themselves be active in folding/activating the client, **B** or deliver the client to a co-chaperone that carries out these processes. **C** Chaperones can promote the association of the client and a partner protein or **D** translocate the client to a different cellular region or organelle so it can be folded/activated by other proteins. **E** Chaperones can also hold the client in an activation competent state so that it can be activated and/or folded by a co-chaperone, preventing the client nascent polypeptides from forming insoluble aggregates.
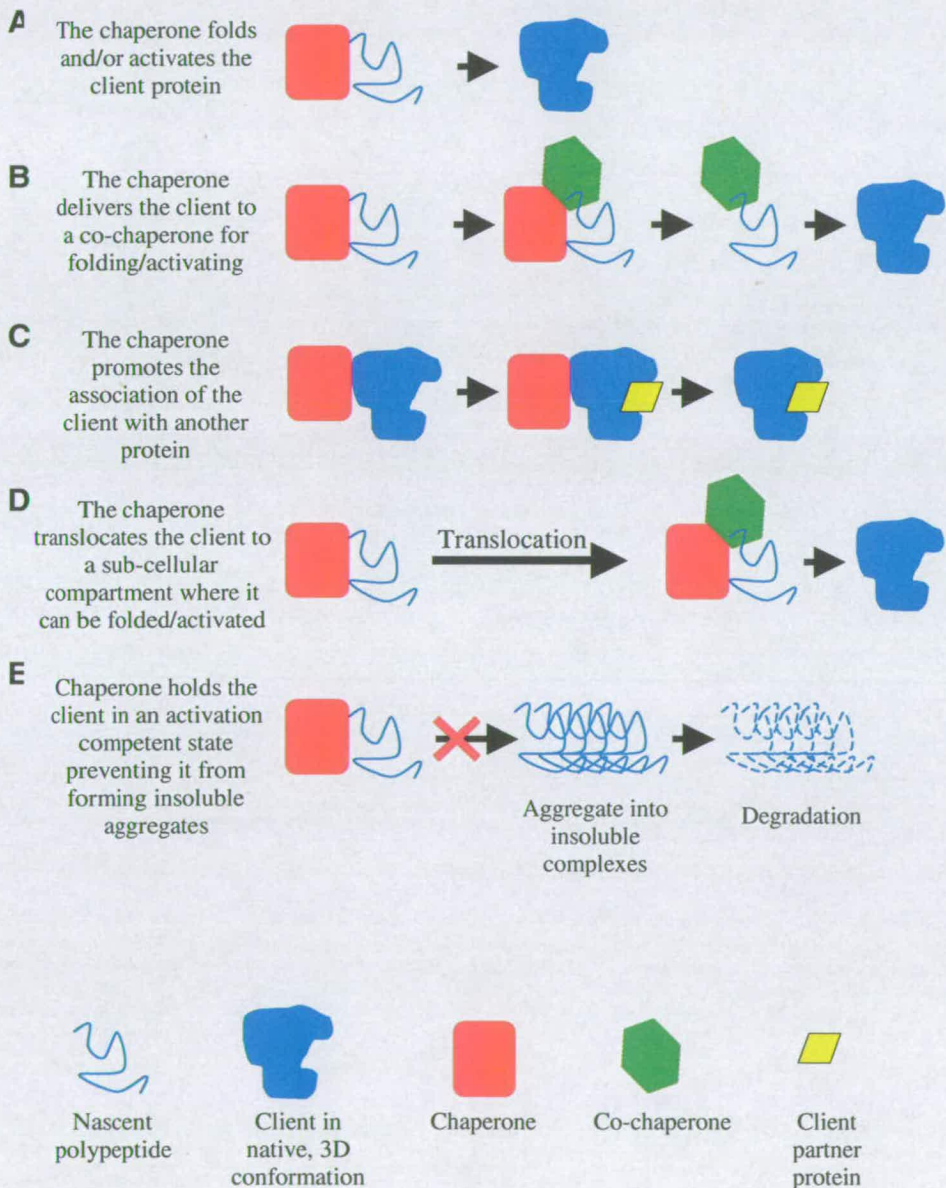
Molecular chaperones also play an important role in cell stress responses stimulated by a number of factors including heat and osmotic stress. In response to heat stress in Drosophila, the production of a group of molecular chaperone proteins called the heat shock proteins (Hsps) dramatically increased (Ish-Horowicz et al., 1979). The elevated expression of molecular chaperones such as Hsps protects the cell under stress conditions by maintaining cellular protein levels and preventing client protein inactivation and aggregation into insoluble complexes. GroEL is an Hsp protein in *E. coli* that protects the client RNA polymerase holoenzyme from heat inactivation (Ziemienowicz et al., 1993). In addition, if this client is already inactivated by heat stress, then in the presence of ATP, GroEL can reactivate the client returning it to its native, biologically functional state (Ziemienowicz et al., 1993). Molecular chaperones also protect clients from being unfolded, but those proteins that are denatured are bound by other chaperones such as small heat shock proteins (e.g. Hsp30), inhibiting their accumulation into insoluble aggregates and maintaining them in a folding-competent state for other chaperones to fold and/or activate (Heikkila, 2004).

1.1.1 Chaperone-chaperone interactions

Molecular chaperone proteins interact with each other in a highly organised network to maintain effective protein quality control within cells. Associations between molecular chaperones are highly specific; for example in *S. cerevisiae* Hsp40 chaperones Sis1 and Ydj1 interact with the Hsp70 co-chaperone Ssa1 but not Ssb1/2, also an Hsp70 chaperone (Lu and Cyr, 1998). Chaperones such as Hop have been found to act as scaffolds, linking specific combinations of co-chaperones such as Hsp90 to Hsp70 (Chen et al., 1996). The interactions between co-chaperones to form these various complexes can be highly competitive. Hop and the Immunophillin Cyp40 compete for binding of Hsc70 (Heat shock cognate 70) of the Hsp70 molecular chaperone family (Carrello et al., 2004). The various interactions between molecular co-chaperone proteins and clients form a range of heterocomplexes *in vivo*. The chaperone function towards clients of individual molecular chaperones can be modulated by associating with different co-chaperones in these heterocomplexes. The

molecular chaperone Hsp70 has a role in protein folding in association with Hsp40 co-chaperones (Lu and Cyr, 1998), but interactions with the co-chaperones CHIP (C-terminus of Hsp70 interacting protein) (Meacham et al., 2001) and Bag-1 (Demand et al., 2001) mediate client protein degradation. Specific combinations of molecular chaperones are required in these heterocomplexes to carry out different types of chaperone function. The Hsp40 chaperone, Ydj1, in association with the Hsp70 chaperone Ssa1, can prevent certain client proteins from forming insoluble aggregates in *S. cerevisiae*, but the combination of Ydj1 and another Hsp70 chaperone, Ssb1, cannot (Cyr, 1995).

1.1.2 Recognition of client proteins by molecular chaperones

Each molecular chaperone recognises and binds a specific set of client proteins in particular conformational states, creating a network of precise chaperone-client interactions that sustain effective cellular quality control. Chaperones display a high degree of substrate specificity, recognising certain types of clients based on their unique polypeptide chain sequence. For example, Ydj1 and Sis1 are both Hsp40 chaperones in *S. cerevisiae* that have very different sequence recognition requirements of client polypeptides (Fan et al., 2004). Sis1 preferentially associates with polypeptides enriched in hydrophobic residues which are mainly aromatic or lysine and generally only occur in pairs, but Ydj1 favours polypeptides with a hydrophobic core of at least 3 or 4 amino acids adjacent to one another (Fan et al., 2004). Molecular chaperone proteins recognise specific clients to ensure they enter the appropriate path in the quality control network.

Molecular chaperones also exhibit substrate specificity based on the structural state of the client protein. Different chaperones preferentially associate with specific conformations and folding states of clients. The prokaryotic GroEL favours clients in a 'molten globule' conformation (see Figure 1.2) (Martin et al., 1991), whereas the *S. cerevisiae* Hsp40 chaperone, Ydj1, binds non-native, unfolded polypeptides (Cyr, 1995). Interestingly, DnaK in *E. coli* binds both short hydrophobic peptides in an extended conformation and proteins such as RepA and Lambda P in a native

Figure 1.2: An overview of protein folding showing how domains of a newly synthesized polypeptide achieve a "molten globule" state. The subsequent folding steps occur at a slower rate and by multiple pathways, which often involve molecular chaperone proteins. Misfolded proteins are recognised by another set of molecular chaperones which target these for degradation. Obtained from (Alberts et al., 2002).
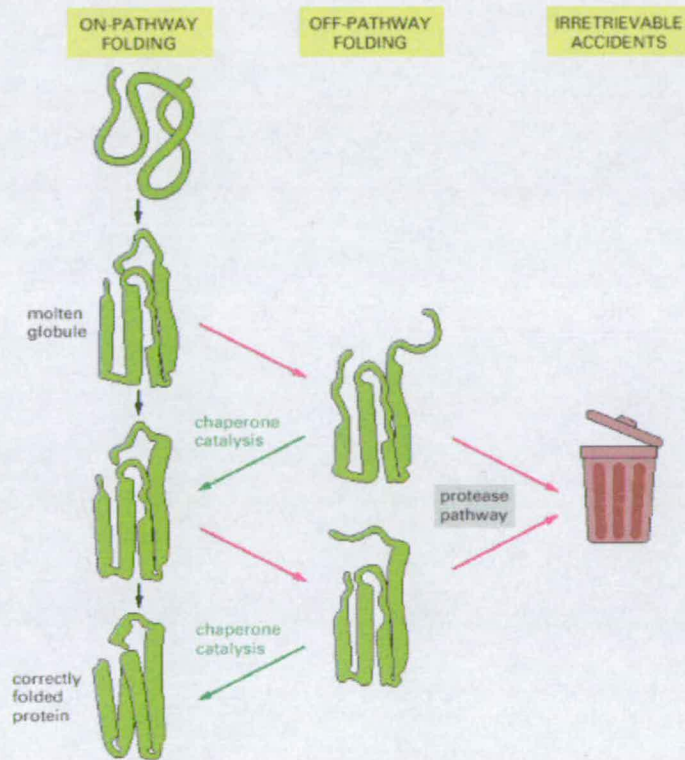
conformation (Wawrzynow and Zylicz, 1995). The ability of molecular chaperones to recognise specific client conformations is most likely to ensure that they carry out their specific type of chaperone activity on clients in the appropriate structural state. The mechanism by which chaperones recognise these different client conformations is still unknown and an intriguing question in the field.

1.1.3 Molecular chaperone function towards client proteins

Molecular chaperones are essential in maintaining quality control of cellular proteins which is fundamental to cell viability. A highly organised network of interacting molecular co-chaperone proteins associates with specific client proteins to promote efficient production, activation, localisation and degradation of the cellular protein pool as required by the cell. In the following section the various roles of molecular chaperone proteins are reviewed, to provide a better understanding of the function of these essential proteins within the cell.

1.1.4 Folding of client proteins by molecular chaperones

The precise folding of a client protein is essential for achieving a biologically functional, native state that corresponds to the structure most stable under physiological conditions (Dobson, 2004). The amino acid sequence of the nascent polypeptide itself contains all the necessary information to reach its functional, native, three-dimensional structure (Dobson, 2004), but a polypeptide cannot spontaneously fold itself accurately to achieve its native state. This is partly due to the peptide bond properties of the polypeptide which confer a high degree of conformational flexibility to the backbone of the protein and the amino acid side chains that enable a number of largely non-covalent interactions (Barral et al., 2004). Consequently, the numbers of possible structures for each polypeptide are numerous. The final native structure is believed to be achieved by a stochastic search through the various conformations that are accessible to the polypeptide (Dobson, 2004). Protein folding as a spontaneous process is slow and often generates misfolded structures. To achieve accurate,

efficient folding, molecular chaperone proteins assist in the folding process (Wegele et al., 2004) as shown for Hsp70 in Figure 1.3.

Client folding is typically carried out by ATP-dependent molecular chaperones. Folding of clients by the *E. coli* chaperone GroEL in association with the co-chaperone GroES requires around 100 ATP molecules per protein client monomer (Martin et al., 1991). The ATP-dependent folding of client proteins is a two step process. Firstly, folding of the denatured client by the molecular chaperone occurs as an ATP-independent formation of a binary complex between the client and chaperone, then secondly, ATP-dependent release of the client in its native state (Gao et al., 1992). Folding of client proteins is often a co-operative process between co-chaperones. *In vitro*, the *E. coli* molecular chaperone DnaK recognises nascent polypeptides and in association with the co-chaperone DnaJ stabilises the client, preventing aggregation (Langer et al., 1992). The client is then transferred by GrpE to the ATPase chaperone GroEL, which in the presence of ATP catalyses the folding and maturation of the client (Langer et al., 1992).

1.1.5   Translocation of client polypeptides by molecular chaperones

Many proteins are synthesised as precursor polypeptides that require post-translational modifications and translocation to cellular organelles or a different region of the cell in order to achieve a native, biologically functional state. Molecular chaperone proteins have a crucial role in promoting the translocation of certain clients during their maturation process. It is imperative that nascent precursor polypeptides are maintained in a translocation-competent state by chaperones such as SecB in *E. coli* to enable efficient translocation to organelles such as the endoplasmic reticulum (ER) (Lecker et al., 1989). The translocation of precursor polypeptides such as invertase in *S. cerevisiae* is carried out by the Hsp70 family chaperone Bip (also known as GRP78), transporting the client into the lumen of the ER (Nguyen et al., 1991; Vogel et al., 1990). The ER is a "protein factory" containing an abundance of molecular chaperones that form various quality control pathways for clients to attain a

Figure 1.3: Folding of nascent polypeptides by the molecular chaperone Hsp70. The chaperone binds specifically to the hydrophobic amino acids of the polypeptide in association with an Hsp40 chaperone protein. The Hsp70 chaperone hydrolyses a molecule of ATP which induces conformational changes that stabilise the complex. Hsp40 disassociates and then Hsp70 releases the client which is induced by the rapid re-binding of ATP after ADP release. Obtained from (Alberts et al., 2002).
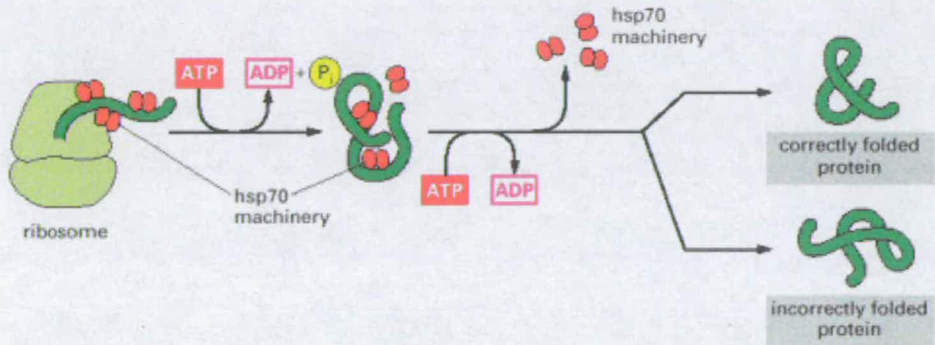
native, biologically functional state. Around one third of all cellular proteins in eukaryotes are translocated to the ER, a highly specialised organelle for protein folding that has a unique oxidising potential that supports disulphide bond formation during protein folding (Kleizen and Braakman, 2004).

1.1.6 Maintenance of clients in a folding or activation competent state by chaperones

Nascent polypeptides must be maintained by molecular chaperones in a folding or activation competent state, ready for folding, translocation or activation by other chaperones. Sustaining client polypeptides in a folding competent state prevents them from binding one another and forming insoluble aggregates, which can be targeted for degradation. Proteins such as proOmpA in *E. coli* are maintained in an export-competent state by the chaperone SecB (a Chaperonin) to enable translocation promoting maturation of the client and to inhibit aggregation into insoluble complexes (Lecker et al., 1990). The ability of molecular chaperones to maintain clients in a folding competent state is also essential during some stress responses to maintain cellular protein levels. In response to heat stress in *Xenopus* oocytes, small heat shock proteins (shsp) bind denatured clients, inhibiting their aggregation and maintaining them in a folding-competent state until other cellular chaperones are able to fold them into their native state (Heikkila, 2004).

1.1.7 Misfolding and refolding of client proteins by molecular chaperones

Client polypeptides can misfold themselves if left unaided. Occasionally client polypeptides are inaccurately folded by molecular chaperones or unfolded from their native state under stress conditions such as heat. An important aspect of the chaperone network is to refold misfolded and unfolded client proteins accurately. A cycle of protein folding in *E. coli* has been identified that ensures that misfolded proteins are refolded and not released by the chaperone GroEL until they have reached their correct native conformation (Martin et al., 1993). GroEL in its ADP-bound state is

stabilised by GroES. Once the nascent polypeptide has bound GroEL, ADP and GroES are released from the complex. Exchanging ADP for ATP promotes the re-association of GroES to the complex; GroEL then carries out ATP hydrolysis and discharges the client. If the client is partially or incorrectly folded, it rebinds GroEL and the cycle continues until the client has achieved its correct native state and is then released.

1.1.8 Molecular chaperones prevent nascent polypeptides forming insoluble aggregates

Chaperones bind nascent polypeptides as early as possible, associating with them at the point of translation from ribosomes, to prevent them from forming insoluble aggregates and to promote their accurate and efficient folding to the native state. Nascent polypeptide chains can form insoluble aggregate complexes, due to their hydrophobic nature and interchain hydrogen bonding (Barral et al., 2004). The rate of translation from ribosomes producing nascent polypeptide chains is much slower than the rate of folding of protein domains, and these domains cannot be folded until the entire sequence required for folding is free from the ribosomal complex (Barral et al., 2004). The problem of nascent polypeptides forming aggregates is accentuated by the fact that numerous ribosomes carrying out translation are usually located within close proximity (Young et al., 2004). Therefore, hydrophobic nascent polypeptide chains must be stabilised to prevent aggregation. The molecular chaperone Trigger Factor in *E. coli* associates with nascent polypeptides as they are being translated by the ribosome to overcome the problem of nascent polypeptides collating into insoluble aggregates (Figure 1.4) (Hesterkamp et al., 1996). Trigger factor is thought to scan the nascent polypeptide as it is being translated by the ribosome, to identify and shield hydrophobic patches of sequence from aggregating (Young et al., 2004). The interaction between Trigger Factor and the client polypeptide is rapid and does not involve any folding by this chaperone (Maier et al., 2001). The Trigger Factor chaperone disassociates from the polypeptide once the ribosome has left the complex (Hesterkamp et al., 1996).

Figure 1.4: Chaperones associate with polypeptides as they are being translated from the ribosome. Molecular chaperones bind the amino terminus of the extending polypeptide chain as it leaves the ribosome, stabilising the client and preventing it from forming insoluble aggregates. Once the polypeptide has been fully translated, chaperones are able to fold the client into is 3-D native state. Obtained from (Cooper, 2000).
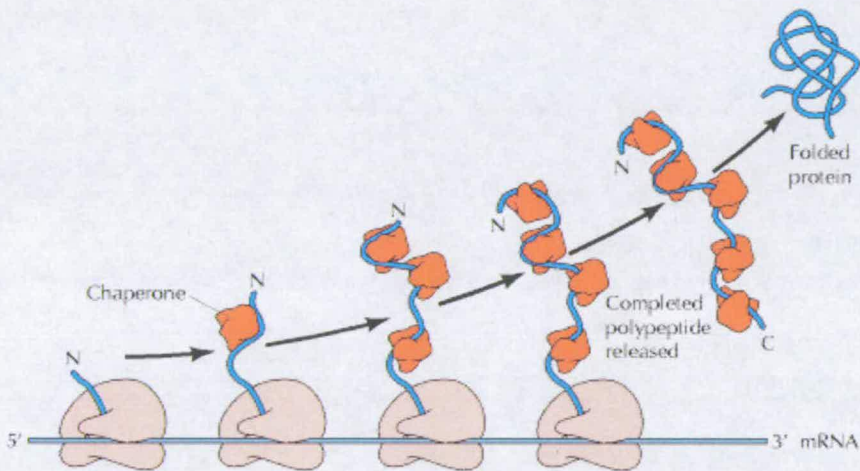
Once non-native polypeptides have been fully translated, another set of chaperones protect them from forming insoluble aggregates, maintaining them in a folding competent state. The Hsp40 chaperone Ydj1 from *S. cerevisiae* forms complexes with non-native polypeptides, preventing formation of insoluble aggregates (Cyr, 1995). Specific sets of co-chaperones interact to stabilise nascent polypeptides, preventing aggregation. Ydj1 associates with the Hsp70 co-chaperone Ssa1 in preventing aggregation of specific client polypeptides, although it is unable to carry out this function with the Hsp70 chaperone Ssb1/2 (Cyr, 1995). If nascent polypeptides do form aggregates, for example after exposure to stresses such as heat, then molecular chaperones are in place in the network to resolve this. Chaperones such as ClpA, a Hsp100 family chaperone, is unable to protect client proteins from thermal denaturation, but can actively resolubilise heat inactivated and denatured clients from insoluble aggregates (Parsell et al., 1994).

Molecular chaperone proteins also interact with aggregates of prion proteins. These insoluble aggregates can have a detrimental affect on individual cells and tissue, causing human diseases such as variant CJD and Huntington's. Recent data has shown that chaperone proteins display a range of activities towards prion proteins and their propagation. Prion aggregates are believed to be formed by nucleated conformational conversion, whereby a prion seed associates with non-prion protein forms which then undergo conversion to prions building the aggregate (Scheibel et al., 2004). Sis1, an Hsp40 molecular chaperone, promotes the propagation of RNQ+ prion proteins in *S. cerevisiae*, whereas Ydj1 does not (Lopez et al., 2003). PSI+ is also a prion protein whose propagation is dependent on an intermediate protein level of the chaperone Hsp104 in *S. cerevisiae*, as excess or limiting levels of this molecular chaperone were found to reduce PSI+ prion protein levels (Chernoff et al., 1995). The reasons for the complexity of the relationships between molecular chaperone proteins and prion protein propagation are currently not fully understood, although it is clear that molecular chaperones have a direct influence.

1.1.9 Targeted degradation of client proteins by molecular chaperones

Quality control of client proteins by molecular chaperones also involves their degradation. This process is necessary to remove misfolded or mutant client proteins, and or reduce protein levels according to cell requirements. CHIP is a chaperone dependent E3 ubiquitin–protein ligase that represents the link between protein folding and protein degradation in quality control. Overexpression of CHIP increases the level of ubiquitination and rate of degradation of client proteins (Jana et al., 2005). CHIP preferentially associates with the misfolded client protein ER-α, which it ubiquitinates and targets for degradation (Tateishi et al., 2004). CHIP mediated degradation of client proteins occurs in association with co-chaperones better known for their folding ability. *In vitro*, the addition of Hsp70 and Hsp40 was found to greatly enhance the level of ubiquitination on client proteins by CHIP (Peng et al., 2004). Client proteins are degraded by the proteasome, as human cells treated with the proteasome inhibitor ALLN were unable to degrade the misfolded cystic fibrosis transmembrane receptor (CFTR) mutant CFTRΔF508, a client known to be targeted for degradation by CHIP (Meacham et al., 2001). There is a further regulatory step to this pathway, as CHIP is negatively regulated by HspBP1 when it is associated with Hsc70 (Alberti et al., 2004). HspBP1 has been shown to interfere with CHIP-induced degradation of clients such as CFTR, preventing their proteolysis and promoting client maturation (Alberti et al., 2004).

1.1.10 Summary

There are many molecular chaperone proteins, interacting with each other to form a highly organized network for the quality control of cellular proteins. Co-chaperones associate in a number of different combinations to carry out a variety of different roles to regulate cellular protein levels and aid the cellular stress response. One of these molecular chaperones, Cdc37, is the focus of this work and will be reviewed in the following section.

1.2   Cdc37

1.2.1   Introduction

Cdc37 is a molecular chaperone that was identified in two distinct ways. The *cdc37* gene was identified during a screen for *S. cerevisiae* mutants that arrest in START (Reed, 1980; Reed, 1980). The Cdc37 protein was concurrently identified as a 50 kDa protein called p50 that interacts with the v-src kinase in chicken cells (Hunter and Sefton, 1980). Clients of the molecular chaperone Cdc37 are predominantly protein kinases such as Cdk4 (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996) and Raf-1 (Grammatikakis et al., 1999) which bind the N-terminal domain of Cdc37 (Shao et al., 2003). Cdc37 interacts with a wide variety of client proteins and co-chaperones, forming a range of high molecular weight complexes (Boudeau et al., 2003; Mahony et al., 1998; Stepanova et al., 1996; Wang et al., 2002). Cdc37 has been found to interact with the co-chaperone Hsp90 (Abbas-Terki et al., 2002; Millson et al., 2004; Miyata and Nishida, 2004; Roe et al., 2004; Shao et al., 2003; Shao et al., 2003; Siligardi et al., 2002; Zhang et al., 2004), and is thought to deliver client protein kinases to this ATP-dependent chaperone (MacLean and Picard, 2003). In this section Cdc37 and its interactions with both the co-chaperone Hsp90 and client proteins including Cdc28 in *S. cerevisiae* will be reviewed.

1.2.2   The *cdc37* gene

The *cdc37* gene has been identified as essential in numerous organisms and appears to be restricted to animal and fungi. An alignment of Cdc37 homologues from different species shows low overall sequence identity (Figure 1.5). There is greater sequence identity among Cdc37 homologues of the lower and higher eukaryotic groups than between them. For example, overall sequence identity between the mammalian Cdc37 homologues from chick and human is 84% (Huang et al., 1998) but only 25% between the human and *S. cerevisiae* proteins. Despite low overall sequence identity, specific regions of Cdc37 are more highly conserved among homologues. The N-

Figure 1.5: An alignment of Cdc37 from different species. There is low overall sequence identity between Cdc37 homologues. Black indicates amino acid residues identical between species and grey denotes amino acids of similar type.

KEY:
1. *Homo sapiens* (human)
2. *Mus musculus* (mouse)
3. *Caenorhabditis elegans* (worm)
4. *Drosophila melanogaster* (fly)
5. *Drosophila virilis* (fly)
6. *Gallus gallus* (chicken)
7. *Rattus norvegicus* (rat)
8. *Saccharomyces cerevisiae* (budding yeast)
9. *Schizosaccharomyces pombe* (fission yeast)
10. *Tetraodon fluviatilis* (pufferfish)
11. *Xenopus laevis* (toad)
12. *Cabdida albincan* (yeast)

terminal domain of Cdc37 is the most highly conserved region and is involved in client protein interactions (Shao et al., 2003). The N-terminal domain protein sequence displays 80% identity between the yeasts *S. pombe* and *S. cerevisiae* and 50% identity between *S. pombe* and human. Cdc37 homology across species may be more highly conserved at the structural or functional level of the protein, than in the sequence of amino acids, as Cdc37 proteins from different organisms are functional homologues. Cdc37 from *C. albicans* can rescue the *S. cerevisiae* temperature-sensitive mutant *cdc37-34* at the restrictive temperature (Ni et al., 2004).

Interestingly, a protein called Harc (Hsp90-associating relative of Cdc37) with similarity to human Cdc37 has been identified in human cells and is thought to be a duplication of Cdc37 that occurred during vertebrate evolution (Scholz et al., 2001). The protein sequence of Harc shares only 31% homology with human Cdc37 across the whole protein (Scholz et al., 2001). Harc is a 35 kDa protein comprised of three domains that interacts with a range of co-chaperones including Hsp70, Hop, the Immunophillin Cyp40, and like its Cdc37 homologue binds the co-chaperone Hsp90 by its middle domain (Scholz et al., 2001). In contrast to Cdc37, Harc does not bind protein kinases such as Raf-1 and v-src (Scholz et al., 2001).

1.2.3   The Cdc37 protein

Cdc37 comprises around 0.1% of total cellular soluble protein in Drosophila cells (Kimura et al., 1997). The mRNA in *S. pombe* (Rustici et al., 2004) and protein levels in *S. cerevisiae* (Gerber et al., 1995) of Cdc37 do not fluctuate during the cell cycle. Furthermore, cellular Cdc37 protein levels in *S. cerevisiae* remain at a constant level in both stationary and exponentially growing cells (Gerber et al., 1995). It has been suggested that Cdc37 protein levels may be affected by post-translational mechanisms, as human cells expressing a Cdc37ΔC-terminus mutant contained elevated Cdc37 protein levels (Schwarze et al., 2003). The localisation of the Cdc37 protein in *S. pombe* (Tatebe and Shiozaki, 2003) and human (Lange et al., 2002; Perdew et al., 1997) cells is largely cytoplasmic, being diffuse throughout this region. However, in *S. pombe* cells, distinct localisation of Cdc37 in the chromatin region of

the nucleus was also observed (Tatebe and Shiozaki, 2003). In contrast, the Cdc37 protein in human cells displayed specific perinuclear localization (Lange et al., 2002; Perdew et al., 1997) and was also seen to localize specifically to the spindle microtubules and midbody co-localising with the client protein kinase Aurora B (Lange et al., 2002).

1.2.4   Cdc37 protein structure

Structurally, three domains of human Cdc37 have been defined by limited proteolysis and peptide analysis and are referred to as the N-terminal, middle and C-terminal domains (Shao et al., 2003) The N-terminal domain of Cdc37 was found to consist of amino acids 1-126, the middle region is composed of residues 128-282 and the C-terminal domain of amino acids 283-378 (Shao et al., 2003). Intriguingly, the C-terminal domain of Cdc37 in *S. cerevisiae* is completely dispensable, as expression of the mutant Cdc37ΔC-terminus does not compromise chaperone function, maintaining the activity of clients such as Hck (Scholz et al., 2000) and v-src (Lee et al., 2002). Removal of both the middle and C-terminal domains of *S. cerevisiae* Cdc37 reduces chaperone activity by 70% (Lee et al., 2002). Crystallisation studies of portions of the middle and C-terminal domains of human Cdc37 (amino acids 148 to 347) identified a 6-helix bundle (amino acids 148-245), connected by a long single helix (residues 246 to 286) and a disordered polypeptide chain (amino acids 309-315) to a small 3-helix bundle (residues 316-347) (Figure 1.6) (Roe et al., 2004).

The Cdc37 protein has a molecular weight of 68 kDa in *S. cerevisiae*, but migrated at 130 kDa by gel filtration chromatography, indicating that Cdc37 occurs as a dimer (Kimura et al., 1997). Crystallisation studies of a truncated human Cdc37 protein comprising codons 148 to 347 showed that it forms a dimer with amino acids 218 to 255 forming the main hydrophobic interface between the two monomers (Figure 1.7) (Roe et al., 2004). The core of the dimerisation interaction involves the close packing of the main and side chains of Gln247 an Tyr248 in the first turn of the long

Figure 1.6: The crystal structure determined for human Cdc37 amino acids 148 to 347 (Roe et al., 2004). A 6-helix bundle (amino acids 148-245) was identified, connected by a long single helix (residues 246 to 286) and a disordered polypeptide chain (amino acids 309-315) to a small 3-helix bundle (residues 316-347)
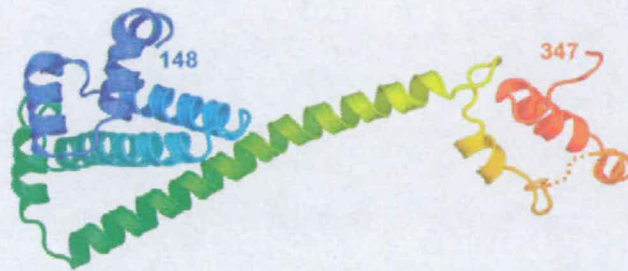
Figure 1.7: Crystal structure of amino acids 148 to 347 of human Cdc37 forming a dimer, and a 90° rotation of the diagram (Roe et al., 2004).
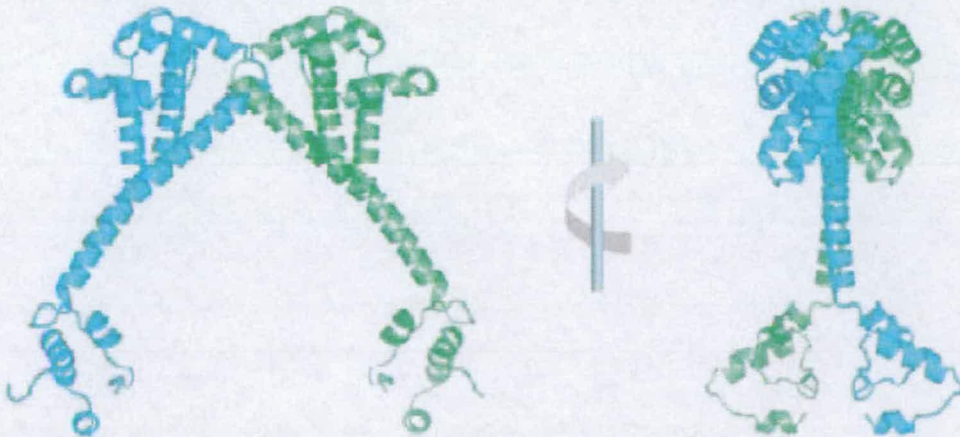
connecting helix with their equivalent partners in the other monomer (Roe et al., 2004). The interaction between the hydrophobic patches of either Cdc37 monomer is reinforced by carboxyl-carboxylate interactions between the side chains of Asp294 from each of the monomers, a network of hydrogen bonds involving side chains of Arg246 and Glu250 on one monomer and Glu221 and Glu225 on the other and ion-pairs between Lys240 and Asp245 from separate monomers (Roe et al., 2004). However, by analytical centrifugation the C-terminal half (amino acids 128-379) and the N-terminal half (30-127aa) of human Cdc37 were both seen to homodimerise as N- and N-terminal and C- and C-terminal domain interactions, although the C- and C-terminal homodimerisation appears stronger (Siligardi et al., 2002).

1.2.5   Phosphorylation of Cdc37

The extreme N-terminal region (first 40 amino acids) of Cdc37 displays the highest level of homology among species and contains a number of conserved serine residues. Serines 14 and 17 in *S. cerevisiae* (Bandhakavi et al., 2003) and serine 13 (equivalent to *S. cerevisiae* serine 14) in rat (Miyata and Nishida, 2004) and human (Shao et al., 2003), have been identified as important sites of phosphorylation. Treatment of cells with TBB, a specific inhibitor of the kinase Casein kinase II (CKII), significantly reduced phosphorylation of Cdc37 *in vivo* (Miyata and Nishida, 2004). CKII phosphorylates recombinant Cdc37 *in vitro* (Shao et al., 2003) and mutation of the conserved serine 13 residue to alanine in the rat Cdc37 protein inhibited phosphorylation by CKII (Miyata and Nishida, 2004). Phosphorylation by CKII of the conserved N-terminal serine residues, serine 13 in rat and serines 14 and 17 in *S. cerevisiae*, are required for Cdc37 activity (Bandhakavi et al., 2003; Miyata and Nishida, 2004). There is evidence that Cdc37 and CKII maintain each other's activity in a feedback loop of activation (Bandhakavi et al., 2003). Zds1 and Zds2, multi-copy suppressors of the *S. cerevisiae* CKII temperature-sensitive mutant *cka2-13*, may play a crucial role in Cdc37-CKII interactions because their overexpression enhances Cdc37 protein levels (Bandhakavi et al., 2003).

Phosphorylation of conserved Cdc37 serine residues by CKII is important for client protein binding and molecular chaperone activity. Inhibition of Cdc37 phosphorylation by mutating conserved N-terminal serine 13 to alanine results in significantly reduced binding affinity *in vivo* towards several client kinases such as Akt, Aurora-B and Cdk4 and reduction in protein levels of client proteins MOK and Raf-1 (Miyata and Nishida, 2004). Phosphorylation of serine 13 in human and rat Cdc37 is crucial for the ability of Cdc37 to form heterocomplexes with the co-chaperone Hsp90 and client proteins (Miyata and Nishida, 2004; Shao et al., 2003). Mutation of rat Cdc37 serine 13 to alanine inhibited interactions between Hsp90 and protein clients, but binding between Hsp90 and the mutant Cdc37 was not compromised (Miyata and Nishida, 2004). Mutation of human Cdc37 serine 13 to alanine or glutamic acid only reduced Cdc37 binding affinity towards Hsp90 by 40 and 10% respectively (Shao et al., 2003). However, phosphorylation site mutants of *S. cerevisiae* Cdc37 reduced binding affinity towards Hsp90 in the yeast-two-hybrid assay (Millson et al., 2004). Mutations of serine 13 to alanine, inhibiting CKII phosphorylation, appear to affect human Cdc37 function towards Hsp90, as these Cdc37 mutants were unable to stimulate nucleotide modulated conformational switching in Hsp90 (Shao et al., 2003).

1.2.6 Cdc37 chaperone function towards client proteins

Cdc37 has been found to interact with a variety of clients which are predominantly protein kinases but also include steroid hormone receptors in mammalian systems (MacLean and Picard, 2003). Cdc37 is essential for maintaining protein levels and activity of specific protein clients. Cdc37 is also involved in the translocation of clients, promoting the assembly of complexes between clients and their partner proteins and delivering clients to other co-chaperones. In this section the role of Cdc37 as a molecular chaperone towards client proteins will be reviewed.

1.2.6.1 Client proteins of the Cdc37 molecular chaperone

Cdc37 was first isolated as a 50 kDa protein by co-immunoprecipitating with the client protein kinase v-src from transfected chicken cells (Hunter and Sefton, 1980). An ever expanding list of Cdc37 client proteins now exists, the majority of which are protein kinases such as Cdk4 (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996) and Raf-1 (Grammatikakis et al., 1999). However, Cdc37 also associates with non-kinase clients such as the steroid hormone receptor, the androgen receptor (AR) (Rao et al., 2001), the polysaccharide Hyalurinan and related glycosaminoglycans (Grammatikakis et al., 1995). Cdc37 exhibits a high degree of specificity towards the type of client proteins it associates with, for example, Cdc37 interacts with the androgen hormone receptor (Rao et al., 2001), but not the glucocorticoid receptor (Kimura et al., 1997).

Cdc37 binds client proteins in a dynamic manner, and the addition of molybdate can stabilise the interaction to enable detection of associating proteins (Hartson et al., 2000; Hutchison et al., 1992). The N-terminal domain of human Cdc37 binds client proteins (Shao et al., 2003). Cdc37 binds the kinase catalytic domain of client proteins such as MOK (Miyata and Nishida, 2004), Lck (Prince and Matts, 2004), Raf (Silverstein et al., 1998) and LKB1 (Boudeau et al., 2003). More specifically, Cdc37 interacts with the N-terminal lobe of the kinase catalytic domain of the client protein kinases Lck and eIF2α (Scroggins et al., 2003). The residues Gly15 and Lys18 of the client kinases' ATP binding motif, that are important for kinase activity of the client protein Cdk4, are crucial for Cdc37 binding (Zhao et al., 2004). Cdc37 binds non-kinase hormone receptor clients, such as AR, by their C-terminal domain (Rao et al., 2001).

The molecular chaperone Cdc37 is associated with protein clients at a very early stage in their maturation. Cdc37 was identified in purified ribosomal complexes, indicating that Cdc37 associates with clients prior to their full translation (Shao et al., 2001). Cdc37 preferentially associates with newly synthesised immature and inactive polypeptides instead of mature forms of the protein kinase client Lck (Hartson et al.,

2000) and eIF2α (Shao et al., 2001). Cdc37 associates with client proteins like eIF2α until they have attained a mature and activated state (Shao et al., 2001).

1.2.6.2 Cdc37 chaperone activity – maintaining client protein levels

Removal of Cdc37 from Drosophila cells by RNAi treatment resulted in considerably reduced Aurora B protein levels (Lange et al., 2002). The chaperone function of Cdc37 is required for the stability of a number of client proteins including Ste11 in *S. cerevisiae* (Abbas-Terki et al., 2000) and LKB1 in HeLa cells (Boudeau et al., 2003). Human Cdc37 can sustain the protein levels of clients such as the kinase Zap70 in a concentration dependent manner, but was unable to prolong the half life of a Zap70 kinase domain mutant protein, indicating that Cdc37 is not involved in targeting clients for degradation (Matsuda et al., 1999). The ability of Cdc37 to stabilise protein levels of clients such as Raf-1, can occur independently of maintaining the activity of the client (Grammatikakis et al., 1999). However, the ability of Cdc37 to maintain protein levels and client activity often goes hand-in-hand, as Cdc37 can hold client proteins in an activation competent state ready for folding and activation by other chaperones such as Hdj-1 and Hsp70 (Kimura et al., 1997).

1.2.6.3 Cdc37 chaperone activity – sustaining client activity

Cdc37 is essential for preserving the activity of a number of client proteins including Aurora B (Lange et al., 2002), IKK (Chen et al., 2002), reverse transcriptase (Wang et al., 2002) and Raf-1 (Grammatikakis et al., 1999). Cdc37 promotes the activation and maturation of client protein kinases like eIF2α kinase and only disassociates when they have achieved a mature and active state (Shao et al., 2001). However, Cdc37 does not appear to activate client proteins itself, but promotes client activation by associating with ATPase chaperones. Cdc37 can stimulate activation of client proteins such as the eIF2α kinase in the presence of the ATPase chaperone Hsp90 (Shao et al., 2001). The ability to maintain the activity of a protein client often occurs in parallel with maintaining its protein levels. However, in the *S. cerevisiae* temperature-

sensitive mutant *cdc37-34*, AR activation was dramatically reduced at the restrictive temperature, but its protein levels remained constant (Fliss et al., 1997).

1.2.6.4   Cdc37 chaperone activity – Translocation of client proteins to promote their activation

Cdc37 is also involved in the activation of client proteins by playing a role in their translocation from one cellular region to another in association with ATPase chaperones. Certain clients require translocation in order to be properly folded and activated by other protein partners. One such client is Raf that requires translocation from the cytosol to the plasma membrane, where it is activated by Ras. Cdc37 and Hsp90 bind the client protein kinase Raf in the cytosol and at the plasma membrane, indicating that these chaperones may be involved in its translocation promoting the clients activation (Wartmann and Davis, 1994).

1.2.6.5   Cdc37 chaperone activity – promoting complex assembly of client proteins with their partner proteins

In the presence of Cdc37 a 3-fold increase in the kinase activity of the Cdk4-cyclin D complex was observed (Lamphere et al., 1997). Interestingly, Cdc37 itself does not increase the activity of the complex (Lamphere et al., 1997) as the addition of Cdc37 to bound Cdk4-cyclin complexes does not enhance their activity (Stepanova et al., 1996). Cdc37 only binds Cdk4 (Lamphere et al., 1997; Stepanova et al., 1996) and not its cyclin partners (Stepanova et al., 1996). Cdc37 binds Cdk4 and facilitates assembly with the cyclin partners in a concentration dependent manner (Lamphere et al., 1997; Stepanova et al., 1996). This has also been observed for Cdk9 and its partner cyclin T in human cell lines (Lamphere et al., 1997; O'Keeffe et al., 2000).

1.2.6.6 Cdc37 chaperone activity – in association with other chaperones

A number of interactions between Cdc37 and co-chaperones have been identified both biochemically and genetically. Cdc37 genetically interacts with Hsp40 chaperones in *S. cerevisiae*, as a double mutant of *CDC37* and *YDJ1* temperature-sensitive mutants was lethal at temperatures permissive for each individual mutant (Mort-Bontemps-Soret et al., 2002). Biochemically interactions have been detected between Cdc37 and the co-chaperones p23, Hop, FKBP52, Cyp40 (immunophillin) (Hartson et al., 2000) and Cpr7 (immunophillin) (Abbas-Terki et al., 2002). Cdc37 and Sti1, the *S. cerevisiae* homologue of mammalian Hop, physically interact (Abbas-Terki et al., 2002) and have similar chaperone roles towards clients, as overexpression of Cdc37 can restore v-src activity to near wildtype levels in a *S. cerevisiae Sti1*Δ strain (Lee et al., 2002).

Cdc37 interacts with a variety of clients and co-chaperones, forming a range of heterocomplexes *in vivo* (Boudeau et al., 2003; Mahony et al., 1998; Stepanova et al., 1996; Wang et al., 2002). For example, human Cdc37 and FKBP52 can simultaneously occur in association with Hsp90 in complexes with immature clients HRI or Lck (Hartson et al., 2000). Research to date has concentrated on the interaction between the chaperone proteins Hsp90 and Cdc37 which suggests that Cdc37 directs client protein kinases to Hsp90 forming a heterocomplex (MacLean and Picard, 2003). Client protein kinases such as Cdk4 bind both Cdc37 and the chaperone Hsp90 as determined by co-immunoprecipitation (Dai et al., 1996; Stepanova et al., 1996) and interactions between Cdc37 and Hsp90 have also been identified in both mammalian and yeast systems (Abbas-Terki et al., 2002; Lamphere et al., 1997; Millson et al., 2004; Miyata and Nishida, 2004; Perdew et al., 1997; Roe et al., 2004; Shao et al., 2003; Shao et al., 2003; Siligardi et al., 2002; Zhang et al., 2004). Cdc37 appears to compete for Hsp90 binding with the TPR binding domain co-chaperones Cpr6/Cyp40 (Siligardi et al., 2002) and Hop (Silverstein et al., 1998) which form an alternative range of heterocomplexes with Hsp90.

Loss of function of one chaperone heterocomplex can result in the client being taken up in another with a different set of co-chaperones. Geldanamycin (GA) inhibits Hsp90 ATPase activity and the ability of the Hsp90-Cdc37 complex to bind client proteins such as guanylate cyclase (Kumar et al., 2001) and Cdk9 (O'Keeffe et al., 2000), which are instead found complexed with the ATPase chaperone Hsp70. Cdc37 can also interact with Hsp70 in certain instances, maintaining client proteins in an activation competent state *in vitro*, ready for reactivation by Hsp70 (Kimura et al., 1997). The molecular chaperone Hsp90 and its association with Cdc37 will be reviewed in the following section.

1.2.7   Hsp90

1.2.7.1   The Hsp90 gene and protein

The Hsp90 gene(s) is essential as *HSP90* temperature-sensitive mutants in *S. cerevisiae* arrest cell division at the restrictive temperature (Kimura et al., 1994). The *HSP90* gene was originally identified due to its radically increased RNA levels in response to heat shock in yeast (Finkelstein et al., 1982) that produced a 90 kDa protein (Finkelstein and Strausberg, 1983). Under normal growth conditions, the Hsp90 molecular chaperone represents ~0.5-1% of total cellular soluble protein in *S. cerevisiae* (Kimura et al., 1997). Homology of the Hsp90 protein sequence among species is much greater than observed for Cdc37, with 60% sequence conservation between human and *S. cerevisiae* homologues. In mammalian and yeast systems two isoforms of Hsp90 have been identified. In *S. cerevisiae* the Hsp90 isoforms, referred to as Hsc82 and Hsp82, exhibit 97% homology in protein sequence and are functional equivalents, but have very different expression patterns, with Hsc82 being much more abundant (Borkovich et al., 1989). In multicellular organisms additional versions of Hsp90 have been identified in organelles such as Grp94 in the endoplasmic reticulum of human cells (Sorger and Pelham, 1987).

1.2.7.2 The Hsp90 molecular chaperone

Hsp90 associates with a wide range of client proteins that are predominantly involved in signal transduction and cell cycle control, such as protein kinases (Picard, 2002) which it binds at both the N- and C- terminal lobes of the catalytic domains of the client kinases Lck and eIF2α (Scroggins et al., 2003). Like Cdc37, Hsp90 binds protein clients at a very early stage in their maturation process, associating with them as polypeptides in ribosomal complexes, interacting with clients prior to their full translation (Shao et al., 2001). Hsp90 is active in promoting the maturation of newly synthesised polypeptides to native state proteins of clients such as c-src (Xu et al., 1999). Hsp90 is also involved in maintaining the protein levels of clients. Under stress conditions such as heat shock, Hsp90 stabilises client proteins such as citrate synthase by preventing their ability to form thermal insoluble aggregates and sustaining their activity (Jakob et al., 1995).

Hsp90 is an molecular chaperone whose function requires the hydrolysis of ATP (Obermann et al., 1998; Panaretou et al., 1998). The addition of the Hsp90 ATP-hydrolysis inhibitor Geldanamycin (GA) to NIH-3T3 cells reduces the stability of the client protein kinase Cdk4, decreasing protein levels 5-fold (Stepanova et al., 1996). GA binds Hsp90 in the ATP binding domain, preventing ATP hydrolysis from being carried out (Roe et al., 1999). Several co-chaperones of Hsp90 can stimulate its ATP activity promoting chaperone function towards client proteins. Aha1 interacts with Hsp90 and can stimulate its ATPase activity up to 12 times its basal level (Panaretou et al., 2002). The ATPase activity of Hsp90 in association with Aha1 can be inhibited by the presence of other co-chaperones such as Sti1/Hop (Panaretou et al., 2002), forming a regulatory network for Hsp90 chaperone function.

1.2.7.3 Hsp90 associations with co-chaperones

Hsp90 interacts with a range of co-chaperones forming different heterocomplexes. The best characterised model of Hsp90 chaperone activity in association with co-chaperones involves the maturation of the steroid hormone receptor (Pain, 2000). The

early chaperone complex associated with the steroid hormone receptor involves delivery of the client by Hsp40 to Hsp70, forming a heterocomplex that also includes Hip. The chaperone Hop acts as a scaffold linking Hsp70 of the early complex and Hsp90 of the intermediate complex. The hormone receptor is then passed to the intermediate complex which contains Hsp90, Hip and Hop. The final stages of maturation of the client protein before release involve the mature complex comprising Hsp90 with p23 and different Immunophillin. This model represents just one of the pathways in which Hsp90 and co-chaperones interact to carry out appropriate chaperone activity towards client proteins.

1.2.7.4   Tertiary complex of Hsp90-Cdc37-client protein

Hsp90 also interacts with the co-chaperone Cdc37 and is believed to form a heterocomplex that includes client proteins. Reconstitution in rabbit reticulate lysates of the complex between the co-chaperone Hsp90 and the protein kinase client pp60^{v-src} identified the presence of Cdc37, indicating that these proteins form a heterocomplex (Hutchison et al., 1992). In addition, Cdc37 and Hsp90 have been found in the same 450 kDa high molecular weight complex associated with the client Cdk4 in NIH-3T3 cells (Stepanova et al., 1996). Cdc37 and Hsp90 associate at stoichiometric levels in the heterocomplexes, but the protein levels of specific clients are greatly reduced by comparison (Grammatikakis et al., 1999). It is believed that Cdc37 delivers client kinases to the co-chaperone Hsp90, forming a tertiary complex (MacLean and Picard, 2003). This concept is supported by the observation that associations between the client Raf-1 and Hsp90 are greatest in the presence of Cdc37 (Grammatikakis et al., 1999). Furthermore, Cdc37 can compensate for loss of another Hsp90 co-chaperone, Sti1, by restoring Hsp90 binding to the client protein kinase Ste11 when Cdc37 is overexpressed in a *S. cerevisiae Sti1*Δ strain (Lee et al., 2004). The addition of Geldanamycin (GA) blocks the interaction between Cdc37 and certain client proteins, indicating that interactions with Hsp90 are essential for these associations (Hartson et al., 2000; Lange et al., 2002; Rao et al., 2001; Shao et al., 2003). Formation of the heterocomplex involving both Hsp90 and Cdc37 appears to

be crucial for promoting chaperone activity towards certain client proteins such as sustaining the activity of Raf-1 (Grammatikakis et al., 1999).

1.2.7.5 The interaction between the chaperones Cdc37 and Hsp90

Interactions between Hsp90 and Cdc37 have been identified in both lower and higher eukaryotic systems, although the interaction has been studied in greater detail in higher organisms. The middle domain of human Cdc37 interacts with the co-chaperone Hsp90 (Scholz et al., 2001). The complex between Hsp90 and Cdc37 was purified and subjected to limited proteolysis which identified an extremely stable 16 kDa unit comprising amino acids 147 to 276 of Cdc37 and amino acids 4 to 261 of Hsp90 (Zhang et al., 2004). A complex between these interacting domains of yeast Hsp90 and human Cdc37 has been crystallised and its structure determined identifying amino acids 164-170 and 204-208 of human Cdc37 that form a hydrophobic patch which interacts with the N-terminal region of yeast Hsp90 (Figure 1.8) (Roe et al., 2004). Human Cdc37 binds both the N-terminal domains and the linker regions of Hsp90 (Zhang et al., 2004). Cdc37 binds to Hsp90 as a dimer (Siligardi et al., 2002; Zhang et al., 2004) at a 1:1 molar ratio (Zhang et al., 2004).

Cdc37 preferentially binds a non-ATP bound form of Hsp90, suppressing ATP turnover (Siligardi et al., 2002). Consistent with this, Cdc37 forms a stable complex with Hsp90 bound to the ATP hydrolysis inhibitor GA (Grammatikakis et al., 1999; Siligardi et al., 2002). The Hsp90-binding domain of Cdc37 is better at inhibiting ATP turnover by Hsp90 than the full length chaperone protein (Zhang et al., 2004). Displacement of Cdc37 from Hsp90 by immunophillin Cpr6/Cyp40 removes the suppression of Hsp90 ATP turnover (Siligardi et al., 2002). After Cdc37 has been released from the tertiary complex with Hsp90 and the client, ATP turnover by Hsp90 is carried out as a two step process, promoting conformational changes of the Hsp90-client complex (McLaughlin et al., 2004).

Figure 1.8: A diagram to show the interacting domains of yeast Hsp90 and human Cdc37 determined by crystallisation studies. Amino acids 164-170 and 204-208 of human Cdc37 were seen to form a hydrophobic patch that interacts with the N-terminal region of yeast Hsp90 (Roe et al., 2004).
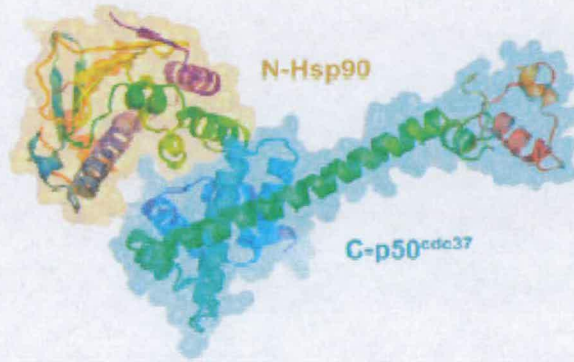
At present there is less biochemical evidence of Cdc37-Hsp90 interactions in yeast systems than mammalian. A genetic interaction between Cdc37 and Hsp90 was observed in *S. cerevisiae*; strains mutationally compromised for Cdc37 and Hsp90 were synthetically lethal (Kimura et al., 1997). In yeast systems, Cdc37 interactions with clients and co-chaperones are more unstable and harder to detect biochemically (Farrell and Morgan, 2000) and human Cdc37 binds Hsp90 tighter than *S. cerevisiae* Cdc37 (Siligardi et al., 2002). The non-specific binding affinity of these co-chaperones and the stability of the Cdc37 complexes have made identifying interactions problematic, and *in vitro* and mutational analysis has had to be employed to identify interactions between Hsp90 and Cdc37 in yeast. In *S. cerevisiae*, a physical interaction between Hsp90 and Cdc37 has been shown using recombinant GST-Cdc37 in pull-down experiments (Abbas-Terki et al., 2000). The yeast-two hybrid assay also indicates an interaction between these co-chaperones, but only when a mutant form of Hsp90 was used in which ATP hydrolysis was inhibited (Millson et al., 2004). Interestingly, in *S. cerevisiae* Cdc37 binds both of the Hsp90 isoforms, Hsc82 and Hsp82 (Abbas-Terki et al., 2000).

1.2.8   Cdc37 is important for maintenance of the cell cycle and proliferation

Cdc37 RNAi treatment of Drosophila cells abrogates proliferation and cells display defects in cytokinesis and chromosome segregation (Lange et al., 2002). The chaperone function of Cdc37 is important for maintenance of the cell cycle, promoting cell proliferation. Consistent with this, Cdc37 expression is elevated in proliferative zones of human tissues and embryonic development (Stepanova et al., 2000). In cancerous cells that undergo uncontrolled proliferation in patients with acute myelocytic leukaemia (Casas et al., 2003) and in prostatic tumours, neoplasias and pre-malignant tumours (Stepanova et al., 2000) Cdc37 expression is upregulated. Cdc37 has oncogenic properties, being able to promote irregular and unchecked cell proliferation. Cdc37 overexpression in mammary gland tissues in transgenic mice promoted abnormal growth and occasionally necrosis (Stepanova et al., 2000). Cdc37

overexpression drives cell proliferation whereas loss of Cdc37 arrests the cell and leads to apoptosis (Schwarze et al., 2003).

Cdc37 is essential for cell viability and maintenance of the cell cycle as cells deleted for the *CDC37* gene in *S. cerevisiae* can only produce micro-colonies of 4-60 cells which are heterogeneous in phenotype (Gerber et al., 1995). The heterogeneity suggests that Cdc37 is involved in a variety of cellular processes. Temperature-sensitive mutants of *CDC37* in *S. cerevisiae* have been valuable in understanding the role of this molecular chaperone in the cell cycle. *cdc37* was first isolated in *S. cerevisiae* as two temperature-sensitive mutants, *cdc37-1* and *cdc37-2*, that arrest in START of the cell cycle (Reed, 1980; Reed, 1980). Other *S. cerevisiae CDC37* temperature-sensitive mutants identified to date include *cdc37-184* which also arrests in START (Valay et al., 1995) and *cdc37-34* which arrests with a cell population split between G1 and a G2/mitosis arrest (Dey et al., 1996). It is interesting to note that the *cdc37-1* (Gerber et al., 1995) and *cdc37-184* (Valay et al., 1995) mutations are located around the joining region of the middle and C-terminal domains, whereas the *cdc37-34* mutation is located at the extreme N-terminus (Dey et al., 1996). Therefore, temperature-sensitive mutations in the N-terminal and middle domains of Cdc37 may cause different types of cell cycle arrests as they affect different aspects of Cdc37 function and interactions with different cell cycle clients.

Cdc37 has chaperone activity towards a range of client protein kinases that are crucial for cell cycle progression. These clients include cyclin dependent kinases (Cdk) such as Cdk4 (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996) that is essential for G1/S-phase transition. The kinase *MPS1* is also a client of Cdc37 that is involved in spindle pole body duplication and G1 control in *S. cerevisiae* and the lethality of the temperature-sensitive mutant *mps1-1* is rescued by overexpression of Cdc37 (Schutz et al., 1997). These data indicate that the role of Cdc37 as a chaperone is of great importance to maintenance of components of the cell cycle.

1.2.8.1   The cell cycle

Cell proliferation occurs by the ordered and regulated progression of the cell cycle, enabling a parent cell to duplicate and divide, producing two identical daughter cells. The progression of the cell cycle is highly regulated and various checkpoints exist that are activated by malfunction of a step in the cell cycle to ensure that cell growth and division are accurately executed generating viable progeny. The cell cycle consists of four distinct stages; G1, S phase, G2 and mitosis (Figure 1.9). In G1 and G2 the cells undergo a period of growth. During S phase the genetic material is replicated and mitosis involves the segregation of genetic material, followed by cleavage to generate two daughter cells.

Cells can exit from the cell cycle in either G1 or G2, and cells in multicellular organisms that exit the cell cycle in G1 at the restriction point, arrest in a stationary phase called G0 (Pardee, 1974). If cells do not exit the mitotic cell cycle at this point, then they are committed to complete the remaining cell cycle stages. In *S. cerevisiae* the restriction point in called Start (Hartwell, 1974), and occurs in G1 which is the longest phase in the cell cycle for this organism. In contrast, G2 is the longest phase in the *S. pombe* and higher eukaryotic cell cycle. *S. pombe* is an ascomycetous fungus whose cell division occurs by medial fission, as observed in higher eukaryotes to produce two daughter cells of equal size (Figure 1.10). *S. pombe* cells are rod shaped and after division grow by apical extension, initially at the old end and then also from the new. The length of the rod shaped *S. pombe* cells is indicative of the cell cycle (Mitchison, 1957; Mitchison, 1990).

Entry into each phase of the cell cycle is regulated by a family of protein kinases known as cyclin dependent kinases (Cdks). To initiate entry into the next cell cycle phase, the Cdk must be active which requires its association with an appropriate cyclin partner and both positive and negative phosphorylation at specific residues. Activation of some cell cycle checkpoints is due to a malfunction that can induce inactivation of the Cdk, preventing cell cycle progression until the discrepancy has been resolved.

Figure 1.9: A schematic of the cell cycle. This schematic is representative of the *S. pombe* cell cycle during which G2 is the longest phase. This is in contrast to *S. cerevisiae* cells which spend the longest period of time in G1 phase.
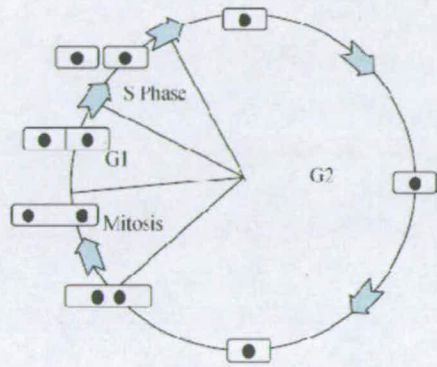
Figure 1.10: A schematic diagram of the *S. pombe* life cycle. Cells usually divide by means of the haploid mitotic cell cycle. Under nutrient deprivation, cells exit the mitotic cell cycle, arresting in stationary phase or undergo conjugation to form diploid zygotes. The continuation of nutrient starvation of cells results in entry into meiosis, resulting in sporulation producing an ascus that contains four spores. If nutrients are provided, the spores undergo outgrowth and resume the mitotic cell cycle (MacNeill and Fantes, 1993).
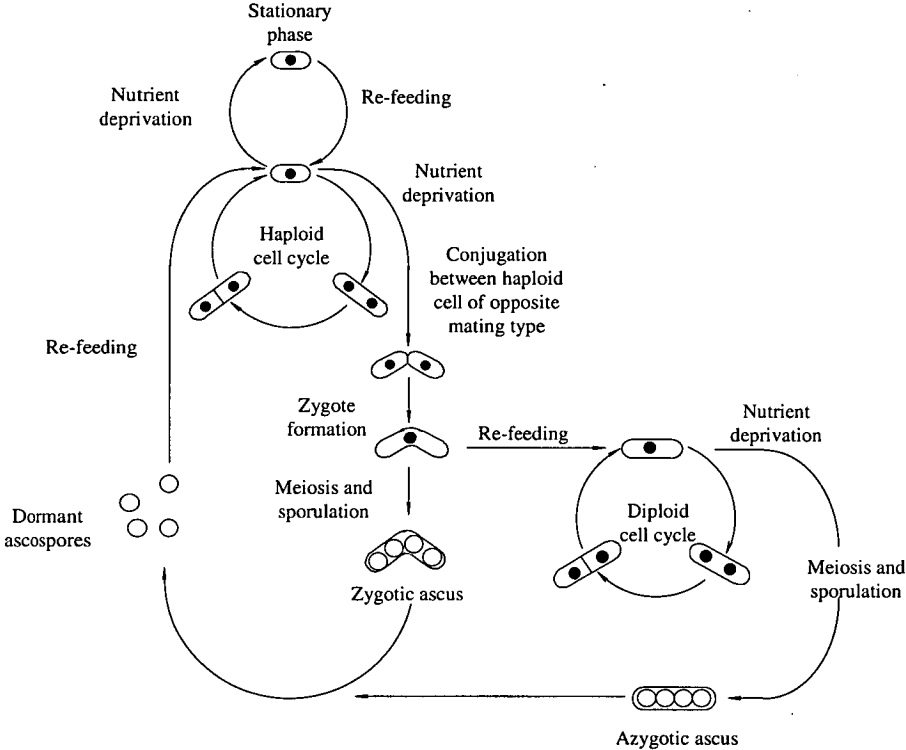
In *S. cerevisiae* and *S. pombe*, Cdc28 and Cdc2 respectively, are the main Cdks that promote the passages between G1/S phase and G2/mitosis. The cyclin partner proteins are periodically present and rapidly degraded, which modulates their association with the Cdk and acts in part to regulate progression of the cell cycle. In *S. cerevisiae*

Cdc28 associates with two different types of cyclin. To initiate the transition between G1 and S phase, Cdc28 forms an active complex with one of three Cln cyclins and one of six different Clb cyclins to modulate the progression between S, G2 and M phase (Mendenhall and Hodge, 1998). In *S. pombe*, two principal cyclin partners have been identified, Cdc13 and Cig2, that associate with Cdc2 and regulate the G2/mitosis transition and entry into G1 during the cell cycle (Moser and Russell, 2000). In contrast to these yeasts, higher eukaryotic cells contain a number of different Cdks to regulate the various stages of the cell cycle, including the Cdc2 homologue Cdk1 (Lee and Nurse, 1987). Other Cdks include Cdk2 that regulates G1/S and G2/M transitions, Cdk4 and Cdk6 are involved in passage of the restriction point and Cdk7 is important for the activation of other Cdks (Pollard and Earnshaw, 2002)

1.2.8.2 The cell cycle role of *S. pombe* cyclin-dependent kinase Cdc2

In *S. pombe*, Cdc2 is the Cdk that is crucial for the transitions between G2/mitosis and G1/S phase. Cdc2 protein levels remain constant throughout the cell cycle (Alfa et al., 1989). Regulation of cell cycle progression by Cdc2 is controlled by modulating its activity at different stages by associating with specific cyclin partners and both negative and positive phosphorylation. Prior to the G2/mitosis transition, Cdc2 is phosphorylated on threonine 167 (Gould et al., 1991) to promote cyclin association and on tyrosine 15 (Gould and Nurse, 1989) to keep the Cdk inactive during interphase. Phosphorylation on threonine 167 is carried out by the Cdk-activating kinases (CAKs) (Lee et al., 1999) while tyrosine 15 is phosphorylated primarily by Wee1 (Gould and Nurse, 1989). Phosphorylation on threonine 167 allows Cdc2 to stably associate with a 482 amino acid B-type cyclin, Cdc13 (Booher and Beach, 1988; Booher et al., 1989; Hagan et al., 1988). The Cdc13 protein shows periodic changes in abundance (Alfa et al., 1989), accumulating through interphase and then

being actively degraded at the metaphase-anaphase transition. To initiate mitosis, Cdc2 bound to Cdc13 is activated by dephosphorylation on tyrosine 15 by the phosphatase Cdc25 (Nurse, 1997). Active Cdc2 is then localised to the nucleus by cyclin Cdc13 and the active complex promotes mitosis (Alfa et al., 1989).

1.2.8.3   Cdc37 and the client Cdc28

Temperature-sensitive mutants of Cdc37 in *S. cerevisiae*, *cdc37-184* and *cdc37-1*, arrest at Start within G1 phase. As previously stated, the Cdk Cdc28 in *S. cerevisiae* is pivotal at this transitional stage of the cell cycle, and overexpression of Cdc28 rescues the lethality of *cdc37-184* (Mort-Bontemps-Soret et al., 2002) and partially rescues the lethality of *cdc37-1* at the restrictive temperature (Gerber et al., 1995). In contrast, overexpression of Cdc37 in the *S. cerevisiae* temperature-sensitive mutants *cdc28-1* and *cdc28-4* could not suppress the lethality at the non-permissive temperature (Mort-Bontemps-Soret et al., 2002). Cdc28 may be a client of Cdc37, as in the *S. cerevisiae* temperature-sensitive mutant *cdc37-1* at the restrictive temperature, Cdc28 protein levels were dramatically reduced and activity was lowered 5-fold (Gerber et al., 1995). Cdc37 also aids Cdc28 activation by facilitating its association with its cyclin partner, as in the *cdc37-1* temperature-sensitive mutant there was a 6-fold drop in the amount of cyclin Cln2 associated with Cdc28 (Gerber et al., 1995). Furthermore, the N-terminal domain of Cdc28 binds the C-terminal domain of *S. cerevisiae* Cdc37 as determined by yeast-two hybrid (Mort-Bontemps-Soret et al., 2002), showing that these two proteins can biochemically interact.

1.3   This work

1.3.1   The molecular chaperone Cdc37 in *S. pombe*

Cdc37 in *S. pombe* was isolated and found to be essential for cell viability (Westwood et al., 2004). Little is known about the molecular chaperone Cdc37 in *S. pombe* and its interactions with the co-chaperone Hsp90 or client proteins. The only client of Cdc37 identified in *S. pombe* to date is the protein kinase Spc1 with which a biochemical

interaction has been detected (Tatebe and Shiozaki, 2003). To study Cdc37 further a range of *cdc37* temperature-sensitive, 5 amino acid insertion, truncation and point mutation mutants were created using both random and directed mutagenesis. Each of these mutants was characterised to gain a better understanding of the molecular chaperone Cdc37 in *S. pombe* and the results are presented and discussed in this work.

Chapter 2:

Materials and Methods

2.0 Methods & Materials

2.1 General

2.1.1 Chemicals and enzymes

Materials were generally purchased from Sigma-Aldrich, New England Biotech or Roche unless otherwise stated.

2.1.2 Commonly used reagents

DNA loading buffer (6x stock):	0.25% bromophenol blue 40% sucrose
PBS (10x stock):	800 mM Na_2HPO_4 200 mM NaH_2PO_4 1 M NaCl
TBS (10x stock):	125 mM NaCl 25 mM Tris-HCl pH8.0
PBST/TBST:	1 x PBS/TBS 0.1% (v/v) Tween-20
Protein gel running buffer:	25 mM Tris-HCL 200 mM glycine 0.1% (w/v) SDS
2x SDS protein buffer:	100 mM Tris-HCL pH6.8 200 mM DTT 4% (w/v) SDS 0.2% (w/v) bromophenol blue sodium phosphate buffer pH5 7.7 mM Na_2HPO_4 42.3 mM NaH_2PO_4
TAE (50x):	2 M Tris 17.5% glacial acetic acid 50 mM EDTA pH8
TE (10x pH8):	100 mM Tris-HCl

	10 mM EDTA
Transfer buffer:	20 mM glycine
	25 mM Tris-HCL
	20% (v/v) methanol

	39 mM glycine
Semi-dry Transfer Buffer:	48 mM Tris-HCL
	0.037% (w/v) SDS
	20% (v/v) methanol

2.2   DNA techniques

2.2.1   Plasmids

2.2.1.1   General vectors

Table 2.1: DNA vectors that were employed for cloning and expression of various genes.

Name	Description	Reference	Use
PFA6a-kanMX6	*S. pombe* expression vector containing a G418 cassette	(Bahler et al., 1998)	Construction of Random Mutagenic PCR template to generate *cdc37* temperature-sensitive mutants
pBDC	Yeast two-hybrid vector	(Millson et al., 2003)	Yeast two-hybrid assay
pADC	Yeast two-hybrid vector	(Millson et al., 2003)	Yeast two-hybrid assay
pGEX-1	GST fusion vector for p50 and Cdc37	Amersham	Make recombinant proteins with a cleavable N-terminal GST tag

2.2.1.2   pREP vectors

In 1990 Maundrell identified a highly expressed *S. pombe* gene, *nmt1* (no message in thiamine) that in the presence of thiamine was completely repressed at the

transcriptional level within 3 hours. The mechanism of repression of the *nmt1* gene is fully reversible. The *nmt* promoter was sub-cloned, creating an extra-chromosomally replicating plasmid for use in *S. pombe*, pREP3 with nutritional markers for selection (Maundrell, 1993). A general map of the pREP vector is shown schematically in Diagram 2.1. Deletion of bases in the TATA box of the *nmt1* promoter was found to reduce expression levels (Basi et al., 1993). Two plasmids were subsequently generated with reduced expression levels, pREP41 and pREP81, detailed in Table 2.2. The pREP vectors that were used in this work are listed in Table 2.3.

2.2.1.3 Modification of the pREP81 vector for N-terminal HA-tagging of proteins

To N-terminally HA-tag proteins, the promoter region of the pREP81 vector was modified by PCR amplification using a 3' primer incorporating an HA-tag sequence upstream of *NdeI*. The customized promoter was cloned into pREP81 using restriction sites *NdeI* and *PstI*. Cdc37 mutants were cloned into the pREP81-HA vector using restriction sites *NdeI* and *XmaI*.

2.2.1.4 Constructs

All constructs generated and used in this work are listed in Tables 2.4 to 2.12. For expression in *S. pombe* genes were cloned into pREP vectors. The general cloning strategy for genes such as *cdc37* into pREP vectors in shown in Diagram 2.2.

Table 2.2: pREP vectors with modified TATA box (Basi et al., 1993). Mutations within the TATA box of the *nmt1* promoter enable different levels of expression from for pREP vectors. The relative promoter activity was measured as CAT (Cm acetyltransferase activity.)

Vector	TATA box	Relative Promoter Activity	
		- thiamine	+ thiamine
pREP3 or 1	ATATATAAA	80	1
pREP41	ATAAA	12	0.06
pREP81	AT	1	0.004

Table 2.3: pREP vectors used in this work.

Name	Description	Origin
pREP1	A high level expression vector with a leucine marker gene	(Maundrell, 1990)
pREP41	A pombe expression vector with a leucine marker gene	(Maundrell, 1993)
pREP81	A pombe expression vector with a leucine marker gene	(Maundrell, 1993)
pREP82	A pombe expression vector with a uracil marker gene enables expression at a level equal to pREP81	

Diagram 2.1: A general map of a pREP vector denoting the nutritional marker and polyclonal linker of pREPs 1, 41 and 81.
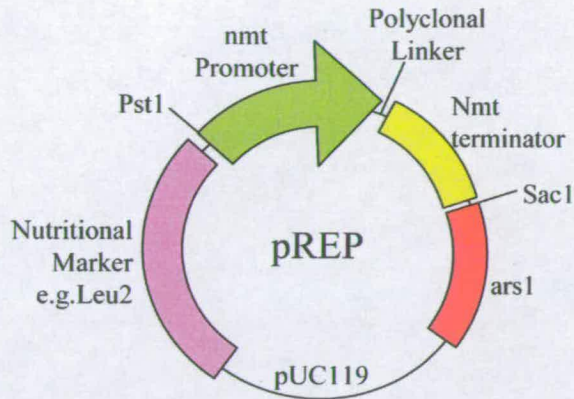
Polyclonal linker sequence of pREP1, 41 and 81:

CAT ATG GGA TCC CCG GG

Nde1   BamH1   Xma1
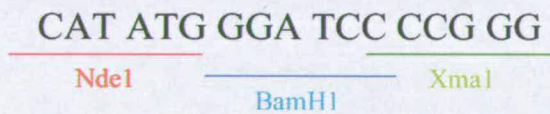
Diagram 2.2: General strategy for cloning genes such as *cdc37* into pREP vectors.
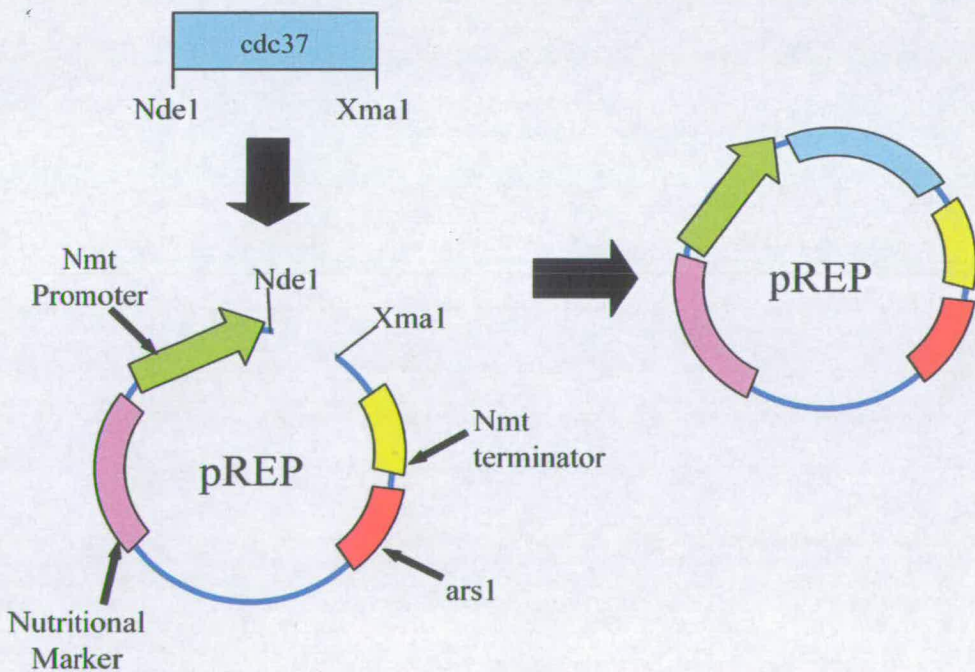
Table 2.4: Constructs generated by directed mutagenesis for mutational analysis of the conserved N-terminal serine residues of *cdc37*.

Name	Origin	Description
pREP1-cdc37-S1417A	ET	Mutate Serine 14 and 17 to alanine
pREP81-cdc37-S1417A	ET	Mutate Serine 14 and 17 to alanine
pREP1-cdc37-S6A	ET	Mutate Serine 6 to alanine
pREP81-cdc37-S6A	ET	Mutate Serine 6 to alanine
pREP1-cdc37-S14A	ET	Mutate Serine 14 to alanine
pREP81-cdc37-S14A	ET	Mutate Serine 14 to alanine
pREP1-cdc37-S14E	ET	Mutate Serine 14 to glutamic acid
pREP81-cdc37-S14E	ET	Mutate Serine 14 to glutamic acid
pREP1-cdc37-S17A	ET	Mutate Serine 17 to alanine
pREP81-cdc37-S17A	ET	Mutate Serine 17 to alanine
pREP1-cdc37-S14L	ET	Recapitulate of *cdc37-34* from *S. cerevisiae*
pREP81-cdc37-S14L	ET	Recapitulate of *cdc37-34* from *S. cerevisiae*
pREP81-HA-cdc37-S14A	ET	N-terminally HA tag mutate S14 A
pREP81-HA-cdc37-S14E	ET	N-terminally HA tag mutate S14 E
pREP81-HA-cdc37-S14L	ET	N-terminally HA tag of *cdc37-34*
pREP81-HA-cdc37	ET	N-terminally tag *cdc37*

Origin key: ET: Emma Turnbull
               KS: Katie Single – Honours Student
               IM: Ina Martin – Post-doc
               CP: Chris Prodromou
               SM: Stuart MacNeill

Table 2.5: Truncation mutants of *cdc37* generated by directed mutagenesis.

Name	Origin	Description
pREP1-cdc37-120	ET	Introduce a STOP codon at amino acid 120
pREP81-cdc37-120	KS	Introduce a STOP codon at amino acid 120
pREP1-cdc37-120T	ET	Introduce a STOP codon at amino acid 120 and remove remaining sequence
pREP81-cdc37-120T	KS	Introduce a STOP codon at amino acid 120 and remove remaining sequence
pREP1-cdc37-360	ET	Introduce a STOP codon at amino acid 360
pREP41-cdc37-360	ET	Introduce a STOP codon at amino acid 360
pREP81-cdc37-360	ET	Introduce a STOP codon at amino acid 360
pREP1-cdc37-360T	ET	Introduce a STOP codon at amino acid 360 and remove remaining sequence
pREP41-cdc37-360T	ET	Introduce a STOP codon at amino acid 360 and remove remaining sequence
pREP81-cdc37-360T	ET	Introduce a STOP codon at amino acid 360 and remove remaining sequence
pREP1-cdc37-155	ET	introduce stop codon at amino acid 155 and remove remaining sequence
pREP81-cdc37-155	ET	introduce stop codon at amino acid 155 and remove remaining sequence
pREP1-cdc37-190	ET	introduce stop codon at amino acid 190 and remove remaining sequence
pREP81-cdc37-190	ET	introduce stop codon at amino acid 190 and remove remaining sequence
pREP1-cdc37-220	ET	introduce stop codon at amino acid 220 and remove remaining sequence
pREP81-cdc37-220	ET	introduce stop codon at amino acid 220 and remove remaining sequence

Table 2.6: Cdc37 mutants created by *in vitro* pentapeptide mutagenesis.

Name	Origin	Description
pREP81-cdc37-PA	ET	Insertion of 5 amino acids at codon 421
pREP1-cdc37-PA	ET	Insertion of 5 amino acids at codon 421
pREP81-cdc37-P1	ET	Truncation, STOP codon at amino acid 61
pREP1-cdc37-P1	ET	Truncation, STOP codon at amino acid 61
pREP81-cdc37-P2	ET	Truncation, STOP codon at amino acid 421
pREP1-cdc37-P2	ET	Truncation, STOP codon at amino acid 421
pREP81-cdc37-P3	ET	Truncation, STOP codon at amino acid 40
pREP1-cdc37-P3	ET	Truncation, STOP codon at amino acid 40
pREP81-cdc37-P5	ET	Truncation, STOP codon at amino acid 354
pREP1-cdc37-P5	ET	Truncation, STOP codon at amino acid 354
pREP81-cdc37-P6	ET	Insertion of 5 amino acids at codon 252
pREP1-cdc37-P6	ET	Insertion of 5 amino acids at codon 252
pREP81-cdc37-P7	ET	Truncation, STOP codon at amino acid 273
pREP1-cdc37-P7	ET	Truncation, STOP codon at amino acid 273
pREP81-cdc37-P8	ET	Truncation, STOP codon at amino acid 396
pREP1-cdc37-P8	ET	Truncation, STOP codon at amino acid 396
pREP81-cdc37-P9	ET	Truncation, STOP codon at amino acid 333
pREP1-cdc37-P9	ET	Truncation, STOP codon at amino acid 333
pREP81-cdc37-P11	ET	Truncation, STOP codon at amino acid 301
pREP1-cdc37-P11	ET	Truncation, STOP codon at amino acid 301
pREP81-cdc37-P15	ET	Truncation, STOP codon at amino acid 261
pREP1-cdc37-P15	ET	Truncation, STOP codon at amino acid 261
pREP81-cdc37-P23	KS	Insertion of 5 amino acids at codon 120
pREP1-cdc37-P23	KS	Insertion of 5 amino acids at codon 120
pREP81-cdc37-P30	KS	Insertion of 5 amino acids at codon 386
pREP1-cdc37-P30	KS	Insertion of 5 amino acids at codon 386
pREP81-cdc37-P35	ET	Truncation, STOP codon at amino acid 428
pREP1-cdc37-P35	ET	Truncation, STOP codon at amino acid 428

Table 2.7: Constructs generated by directed mutagenesis of N-terminal serine residues of *cdc37*.

Name	Origin	Description
pREP1-cdc37-S1417A	ET	Mutate Serine 14 and 17 to alanine
pREP81-cdc37-S1,17A	ET	Mutate Serine 14 and 17 to alanine
pREP1-cdc37-S6A	ET	Mutate Serine 6 to alanine
pREP81-cdc37-S6A	ET	Mutate Serine 6 to alanine
pREP1-cdc37-S14A	ET	Mutate Serine 14 to alanine
pREP81-cdc37-S14A	ET	Mutate Serine 14 to alanine
pREP1-cdc37-S14E	ET	Mutate Serine 14 to glutamic acid
pREP81-cdc37-S14E	ET	Mutate Serine 14 to glutamic acid
pREP1-cdc37-S17A	ET	Mutate Serine 17 to alanine
pREP81-cdc37-S17A	ET	Mutate Serine 17 to alanine
pREP1-cdc37-S14L	ET	Recapitulate temperature-sensitive mutant *cdc37-34* from *S. cerevisiae* mutating Serine 14 to leucine
pREP81-cdc37-S14L	ET	Recapitulate temperature-sensitive mutant *cdc37-34* from *S. cerevisiae* mutating Serine 14 to leucine
pREP81-HA-cdc37-S14A	ET	N-terminally HA tag mutate Serine 14 to alanine
pREP81-HA-cdc37-S14E	ET	N-terminally HA tag mutate Serine 14 to glutamic acid
pREP81-HA-cdc37-S14L	ET	N-terminally HA tag recapitulated temperature-sensitive mutant *cdc37-34* from *S. cerevisiae* mutating Serine 14 to leucine
pREP81-HA-cdc37	ET	N-terminally tag *cdc37* to compare expression levels with N-terminally HA tagged Serine mutants

Table 2.8: Plasmid used to express *S. pombe cdc2*

Name	Origin	Description
pIRT2-cdc2	SM	For expression of *cdc2*

Table 2.9: Plasmids used in generating and analyzing *cdc37* temperature-sensitive mutants.

Name	Origin	Description
PFA6a-kanMX6-cdc37	ET	Template for Random mutagenic PCR incorporating *cdc37*, G418 cassette and *cdc37* flanking sequence for homologous recombination into the chromosome
PFA6a-kanMX6-cdc37-184	ET	Template for introducing *cdc37-184* into the genome by homologous recombination
pREP81-cdc37-RM13-LH	ET	Recapitulate both *cdc37-13* mutations
pREP1-cdc37-RM13-LH	ET	Recapitulate both *cdc37-13* mutations
pREP81-cdc37-RM13-L	ET	Recapitulate *cdc37-13* mutation glutamic acid 237 to lysine
pREP1-cdc37-RM13-L	ET	Recapitulate *cdc37-13* mutation glutamic acid 237 to lysine
pREP81-cdc37-RM13-H	ET	Recapitulate *cdc37-13* mutation tyrosine 261 to histidine
pREP1-cdc37-RM13-H	ET	Recapitulate *cdc37-13* mutation tyrosine 261 to histidine
pREP81-cdc37-RMJ-1	ET	Recapitulate *cdc37-J* mutation leucine 286 to methionine
pREP1-cdc37-RMJ-1	ET	Recapitulate *cdc37-J* mutation leucine 286 to methionine
pREP81-cdc37-RMJ-2	ET	Recapitulate *cdc37-J* mutation histidine 305 to leucine
pREP1-cdc37-RMJ-2	ET	Recapitulate *cdc37-J* mutation histidine 305 to leucine
pREP81-cdc37-RMJ-3	ET	Recapitulate *cdc37-J* mutation arganine 314 to glycine
pREP1-cdc37-RMJ-3	ET	Recapitulate *cdc37-J* mutation arganine 314 to glycine
pREP81-cdc37-RMJ-123	ET	Recapitulate all 3 *cdc37-J* mutations
pREP1-cdc37-RMJ-123	ET	Recapitulate all 3 *cdc37-J* mutations

Table 2.10: The *cdc37* constructs for use in the yeast two-hybrid.

Name	Origin	Description
cdc37-pADC	ET	C-Terminal activating domain
cdc37-pBDC	ET	C-Terminal DNA binding domain
pADC-cdc37	ET	N-Terminal activating domain
pBDC-cdc37	ET	N-Terminal DNA binding domain

Table 2.11: Constructs of human *cdc37* (p50) generated and used in this work.

Name	Origin	Description
pREP1-p50	ET	For overexpression of p50 in *S. pombe*
pREP81-p50	CP	For expression of P50 in *S. pombe*
pGEX-1b-GST-p50	ET	For expression of GST-p50 for GST Pull downs
pREP81-P50-myc	ET	For size exclusion chromatography and IP's
pREP1-P50-myc	ET	For size exclusion chromatography and IP's
p50-pADC	ET	C-Terminal activating domain for use in the Yeast two-hybrid
p50-pBDC	ET	C-Terminal DNA binding domain for use in the Yeast two-hybrid
pADC-p50	ET	N-Terminal activating domain for use in the Yeast two-hybrid
pBDC-p50	ET	N-Terminal DNA binding domain for use in the Yeast two-hybrid

Table 2.12: Constructs of *S. pombe swo1* (the gene encoding *S. pombe* Hsp90).

Name	Origin	Description
pREP81-Swo1	ET	For expression of *swo1*
pREP1-Swo1	ET	For overexpression of *swo1*
pADC-Swo1	ET	N-Terminal activating domain for use in the Yeast two-hybrid
pBDC-Swo1	ET	N-Terminal DNA binding domain for use in the Yeast two-hybrid

2.2.2 Purification of plasmid DNA using QIAfilter midi kit

Plasmid DNA from *E. coli* was purified using the Qiagen midi kit manufacturer's protocol (#12243) for a final yield of up to 100 µg.

2.2.3 Small scale purification of plasmid DNA using QIAprep spin miniprep kit

Up to 20 µg of plasmid DNA was purified from *E. coli* using the QIAprep Spin Miniprep kit's protocol (#27106).

2.2.4 Crude preparation of plasmid DNA from *E. coli*

Crude preparations of plasmid DNA for analytical purposes were made by inoculating 4 ml of LB plus appropriate antibiotics with a single *E. coli* colony. Cultures were grown overnight at 37 °C shaking at 200 rpm. 3 ml of overnight cell culture was harvested by centrifugation at 11,000 xg for 1 minute. Supernatant was removed and the pellet was resuspended in 100 µl of STET by vortexing. 25 µl of Lysozyme buffer was added and samples were vortexed.

STET: 8% sucrose
                               5% Triton x-100
                               50 mM Tris-HCL pH8.0
                               50 mM EDTA

Lysozyme buffer: 10 mM Tris-HCL pH8.0
                               1 mM EDTA
                               10 mg/ml of lysozyme

Samples were boiled for 45 seconds then allowed to cool on ice for 1 minute. Tubes were centrifuged for 15 minutes at 11,000 xg and resulting pellets removed using a sterile toothpick. 125 µl of Isopropanol was added to each tube and vortexed. Samples were centrifuged for 10 minutes at 11,000 xg, the supernatant was discarded and pellets were allowed to air dry for 5 minutes. DNA pellets were resuspended in 30 µl

of Tris-RNAase (RNAase 10 mg/ml in Tris-HCL pH8.0). Samples were incubated at room temperature for 1 hour and then stored at -20 °C.

2.2.5   *E. coli* colony PCR for analysis of plasmid DNA

Colony PCR was used for crude analysis of plasmid DNA from *E. coli*. A sterile toothpick was dipped into an *E. coli* colony, streaked on a LB-agar plate containing appropriate antibiotics and then swirled in an eppendorf tube containing 10 µl of dH$_2$O. Tubes were boiled at 95 °C for 5 minutes, cooled on ice for 2 minutes and then spun down at 5000 xg for 2 minutes. 2 µl of each preparation was used in a Standard PCR reaction (Section 2.3.2.2). Reactions were then run on an agarose gel (Section 2.2.7) for visualisation of the PCR amplified product. Plates containing streaked *E. coli* candidates were incubated overnight at 37 °C and then used as a source to make a clean DNA prep.

2.2.6   Restriction digest of DNA

Restriction digests of DNA were carried out using a 1:10 dilution of appropriate buffer, 1 µl BSA and 0.5-1 µl of restriction enzymes from Promega or NEB. Analytical digests were allowed to run at the optimum temperature for 2 hours in a total volume of 20 µl. Restriction digests to isolate DNA fragments for cloning were carried out overnight in a 50 µl reaction volume.

2.2.7   Visualization of DNA by electrophoresis in agarose gel

DNA samples were run on agarose (Roche #1-388-991) gels for visualization, 0.7% agarose in TAE for fragments larger than 1kb and 1% for smaller. Ethidium bromide (Sigma-Aldrich #160539) was used at a 1:20,000 dilution to permit fluorescence of DNA bands and gels were run using the gel sub-cell (Bio-rad #170-4417).

2.2.8 Purification of plasmid DNA from agarose gels

DNA plasmids smaller than 4kb that required a concentrated output in a small volume were purified using the Minelute^{TM} gel extraction kit (#28604) following the instructions provided by the manufacturer. DNA that was greater than 4 kb was cleaned by means of the QIAquick gel extraction kit (#28704) using the kit protocol.

2.2.9 Purification of DNA by Phenol-chloroform extraction and ethanol precipitation

One volume of 25phenol:24chloroform:1isoamylalcol (phenol chloroform) was added to samples and vortexed for 30 seconds. Samples were centrifuged at 11,000 xg for 5 minutes at room temperature and the upper phase was carefully transferred into a new tube. 2.5 volumes of 100% ethanol and 0.1 volume of 3 M NaOAc were added to samples and vortexed. Tubes were incubated at -70 °C for 20-30 minutes. Samples were spun at 4 °C at 11,000 xg for 10 minutes and ethanol was discarded. One volume of 70% ethanol was added to partially re-hydrate the DNA and centrifuged at 11,000 xg for 5 minutes at 4 °C. The supernatant was removed and DNA pellets allowed to air dry. Finally, pellets were resuspended in 50 µl of appropriate buffer and stored at -20 °C.

2.2.10 Quantification of purified plasmid DNA

The DNA content of samples was quantified using a Hitachi U-2000 Spectrophotometer. A 1:200 dilution of DNA samples was made into dH$_2$0 and placed in a quartz cuvette. Absorbency was determined at OD$_{260}$ and OD$_{280}$. The concentration of nucleic acids can be calculated from the OD$_{260}$ reading where an OD 1 corresponds to approximately 50 µg/µl assuming that the mass of a nucleotide pair is 660 daltons. The purity of nucleic acid can be estimated by the ratio between the readings at OD$_{260}$ and OD$_{280}$. A value of 1.8 for DNA indicates a relatively pure sample, lower figures indicate greater contamination.

2.2.11   Ligation of DNA fragments

Ligation of DNA fragments was carried out using 400 units of T4 DNA ligase (NEB #M0202) in a 1:10 dilution of buffer provided by the manufacturer. Ligation of DNA fragments with overhang ends were performed at room temperature for 30 minutes. Ligation of blunt ended fragments was carried out at room temperature overnight at 4°C.

2.2.12   Phosphatase treatment of DNA fragments

Vectors cut with a single restriction enzyme were treated with CIP (calf intestinal alkaline phosphatase (NEB #M0290)) to reduce plasmid re-ligation during cloning. Reactions were carried out at 37 °C for 60 minutes using a 1:10 dilution of buffer provided by the manufacturer and 10 units of CIP. DNA was then purified using the QIAquick gel extraction kit (Section 2.2.8), substituting Buffer QG for Buffer PBS according to the manufacturer's protocol.

2.2.13   *In vitro* pentapeptide mutagenesis

The GPS-LS linker scanning system (NEB #E7102) was used for *in vitro* pentapeptide transposition as shown in Diagram 2.3.

Reactions were set up according to manufacturers instructions as shown below.

Reaction: 2 μl 10X GPS Buffer
1 μl pGPS5 Donor DNA (0.02 μg)
0.08 μg Target DNA (variable volume)
dH$_2$0 to a final volume of 18 μl

1 μl TnsABC* Transposase was added to each reaction, mixed thoroughly and incubated for 10 minutes at 37 °C. 1 μl of Start Solution was added to each sample and mixed. Reactions were incubated for 1 hour at 37 °C and then heat inactivated at 75 °C for 10 minutes.

Diagram 2.3: A schematic diagram to show how the *cdc37* gene was mutagenised by *in vitro* pentapeptide transposition mutagenesis and cloned into pREP vectors. **A** *cdc37* was cut from its vector, pREP81, using restriction enzymes *Nde1* and *Xma1* and purified. **B** TnsABC* Transposase was used to randomly insert a transposon into target DNA fragments from the GPS5 Transprimer-5 donor plasmid. **C** *cdc37* fragments were run on an agarose gel and those fragments containing the 1.7kb GPS5 insertion were excised and purified using the QIAquick gel extraction kit. **D** Fragments were then ligated into the pREP81 vector using the restriction sites *Nde1* and *Xma1*. **E** The transprimer was removed from resulting plasmids by digestion with the restriction enzyme *Pme1*. Plasmids were recircularised in the presence of T4 DNA ligase, leaving a 15 bp insertions. Plasmids were then sequenced to identify the insertion.
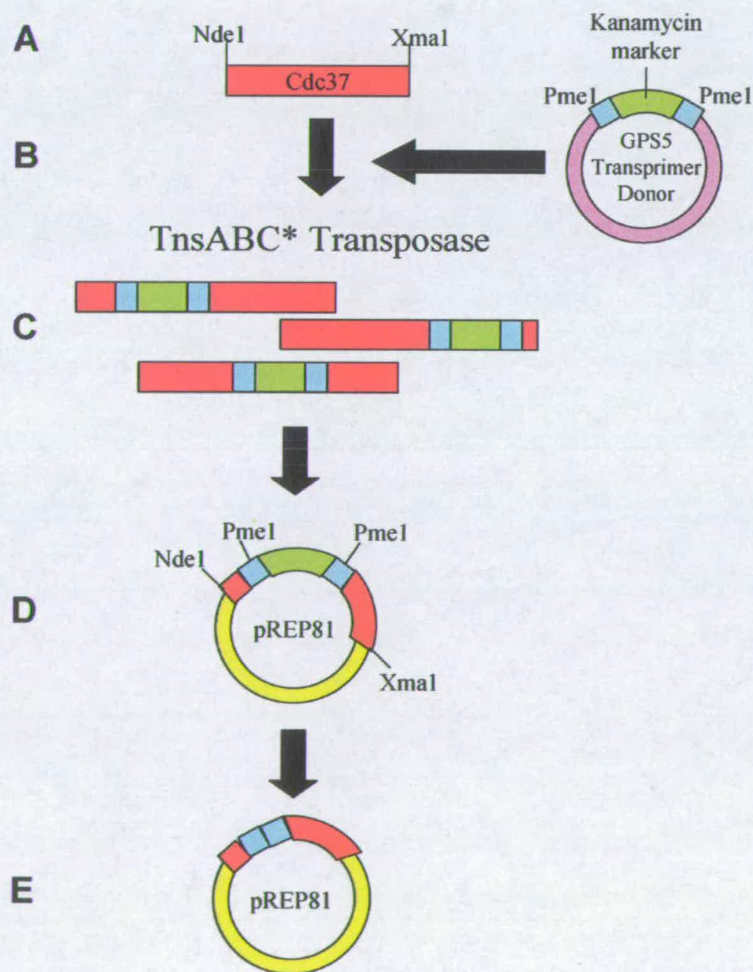
2.3   PCR

2.3.1   Oligonucleotides

All oligonucleotides were purchased from MWG Biotech or Sigma-Aldrich. Oligonucleotide pellets were re-hydrated as a 100 μM stock with dH$_2$0 for 1 hour at 37 °C. PCR amplifications were carried out using 10 μM oligonucleotides and 3.2 μM for sequencing reactions. Oligonucleotides designed for Stratagene Quick change^{TM} site directed mutagenesis (Section 2.3.5) were diluted to a stock of 100 μg/ml, using 125 ng/μl per PCR reaction. Oligos used in this work are listed in Tables 2.13 to 2.19.

Key:	GCTA	mutation
	GCTA	introduced restriction site
	F/R	Forward or Reverse respectively

Table 2.13: List of oligonucleotides used for sequencing and amplification of the *cdc37* gene

Name	Sequence	Direction	Bp's	Description
PW *cdc37* #1	CATGCCATGGCAATTGAT TACAGC	F	24	Sequence starting at base pair 1 of *cdc37* ORF
PW *cdc37* #2	ATCCGAAGGAGATTCCTT	R	28	Sequence starting at base pair 240 of *cdc37* ORF
PW *cdc37* #3	AACATAAGGATCCGAAG GAA	R	21	Sequence starting at base pair 249 of *cdc37* ORF
PW *cdc37* #4	CGATGAATCGTAGGCTCT TGT	F	21	Sequence starting at base pair 190 of *cdc37* ORF
PW *cdc37* #5	CCGAATTCATATGGCAAT TGATACAGC	F	28	Sequence starting at base pair 1 of *cdc37* ORF
PW *cdc37* #6	GCAGCCCGGGTAAGACA ATTGAGGAAT	R	28	Sequence starting at base pair 1401 of *cdc37* ORF
PW *cdc37* #11	GATCGTCGACATGGCAAT TGATTACAGC	F	28	Sequence starting at base pair 1 of *cdc37* ORF
PW *cdc37* #12	TCCATCACCATCCCGGAG	F	18	Sequence starting at base pair 1147 of *cdc37* ORF
PW *cdc37* #14	TTGGGCTTTGATTCCACC	F	18	Sequence starting at base pair 526 of *cdc37* ORF
PW *cdc37* #15	TCTTCCTCATCTGCCAAG	R	18	Sequence starting at base pair 839 of *cdc37* ORF

68

Table 2.14: List of oligonucleotides used for mutating the *cdc37* gene

Name	Sequence	Direction	Bp's	Comments/Purpose
cdc37-184 #2	GATGCTATTCTTTTGGAA GATTTTAATGCTGAACTA GAAGG	F	41	Overlap PCR to mutate alanine 275 to aspartic acid
cdc37-184 #3	CCTTCTAGTTCAGCATTA AAATCTTCCAAAAGAATA GCATC	R	41	Overlap PCR to mutate alanine 275 to aspartic acid
cdc37-J-1 #3	CCCTTCTAGTTCAGCGTT AAAGGCTTCC	F	28	Overlap PCR to recapitulate *cdc37-J* mutation #1
cdc37-J-1 #2	GGAAGCCTTTAACGCTGA ACTAGAAGGG	R	28	Overlap PCR to recapitulate *cdc37-J* mutation #1
cdc37-J-2 #3	CAAAAGGGCTTGAAGAA CATATTGTTTTGC	F	30	Overlap PCR to recapitulate *cdc37-J* mutation #2
cdc37-J-2 #2	GCAAAACAATATGTTCTT CAAGCCCTTTTG	R	30	Overlap PCR to recapitulate *cdc37-J* mutation #2
cdc37-J-3 #3	GGTCCAAGCTGTCCGCAA TAGCTGAT	F	27	Overlap PCR to recapitulate *cdc37-J* mutation #3
cdc37-J-3 #2	GATCAGCTATTGCGGACA GCTTGGACC	R	27	Overlap PCR to recapitulate *cdc37-J* mutation #3
cdc37-13-H #3	CCGATGAAGAATGATCA CCAAAATC	R	25	Overlap PCR to recapitulate *cdc37-13* mutation #H
dc37-13-H #2	GATTTTGGTGATCATTCT TCATCGG	F	25	Overlap PCR to recapitulate *cdc37-13* mutation #H
cdc37-13-L #3	CATCTTCCGACTTATTCT CATTG	R	23	Overlap PCR to recapitulate *cdc37-13* mutation #L
cdc37-13-L #2	CAATGAGAATAAGTCGG AAGATG	F	23	Overlap PCR to recapitulate *cdc37-13* mutation #L
cdc37 NdeI 3'	GCAGCATATGTTAAGAC AATTGAGGAAT	R	28	Introduce *NdeI* site at 3' end of *cdc37*
cdc37-S14A #2	GGATAAGCTAGAACTTGC TGATGACAGTGACATTG	F	35	Overlap PCR to mutate serine 14 to alanine
cdc37-S14A #3	CAATGTCACTGTCATCAG CAAGTTCTAGCTTATCC	R	35	Overlap PCR to mutate serine 14 to alanine
cdc37- S14E #2	CAATGTCACTGTCATCTT GAAGTTCTAGCTTATCC	R	35	Overlap PCR to mutate serine 14 to glutamic acid
cdc37-S14E #3	GGATAAGCTAGAACTTCA AGATGACAGTGACATTG	F	35	Overlap PCR to mutate serine 14 to glutamic acid

Table 2.14 continued: List of oligonucleotides used for mutating the *cdc37* gene

Name	SEQUENCE	Direction	Bp's	Comments/Purpose
cdc37-S17A **#2**	GAACTTAGTGATGAC<u>GCT</u> GACATTGAAGTTCATCC	F	35	Overlap PCR to mutate serine 17 to alanine
cdc37-S17A **#3**	GGATGAACTTCAATGTC<u>A</u> <u>GC</u>GTCATCACTAAGTTC	R	35	Overlap PCR to mutate serine 17 to alanine
cdc37-S6A	GAATTCATATGGCAATTG ATTACGCTAAGTGGGATA AGC	F	39	Mutate serine 6 to alanine
cdc37-S1417A **#2**	GGATAAGCTAGAACTT<u>GC</u> <u>T</u>GATGAC<u>GCT</u>GACATTGA GTTCATC	F	44	Overlap PCR to mutate serine 14 and 17 to alanine
cdc37-S1417A **#3**	GATGAACTTCAATGTC<u>AG</u> <u>C</u>GTCATC<u>AGC</u>AAGTTCCT AGCTTATCC	R	44	Overlap PCR to mutate serine 14 and 17 to alanine
cdc37-S14L **#2**	GGATAAGCTAGAACTT<u>TT</u> <u>A</u>GATGACAGTGACATTG	F	35	Overlap PCR to mutate serine 14 to leucine
cdc37-S14L **#3**	CAATGTCACTGTCATC<u>TA</u> AAAGTTCTAGCTTATCC	R	35	Overlap PCR to mutate serine 14 to leucine
cd37-220 **#2**	GATTTTCCCGGCGCT<u>TAA</u> GAGCAAGC	F	30	Overlap PCR to introduce stop codon to residue 220
cdc37-220 **#3**	GCTTGCTC<u>TTA</u>AGCGCCG GGAAAATC	R	30	Overlap PCR to introduce stop codon to residue 220
cdc37-220 xmaI	CGCGCGCCCGGG<u>TTA</u>TT <u>A</u>AGCGCCGGGAAAATC	R	34	Introduce 2 stop codons and *XmaI* site at residue 220
cdc37-190 **#2**	GGAGCCTGAA<u>TAA</u>CCTA AAAGAC	F	24	Overlap PCR to introduce stop codon to residue 190
cdc37-190 **#3**	GTCTTTTTAGG<u>TTA</u>TTCA GGCTCC	R	24	Overlap PCR to introduce stop codon to residue 190
cdc37-190 xmaI	CGCGCGCCCGGG<u>TTA</u>TT <u>A</u>TTCAGGCTCCTTTTTC	R	34	Introduce 2 stop codons and an *XmaI* site at residue 190
cdc37-155 **#2**	GCTAGAAGATGCA<u>TAA</u>A AGGAGTATG	F	26	Overlap PCR to introduce stop codon to residue 155
cdc37-155 **#3**	CATACTCCTT<u>TTA</u>TGCAT CTTCTAGC	R	26	Overlap PCR to introduce stop codon to residue 155
cdc37-155 xmaI	CGCGCGCCCGGG<u>TTA</u>TT <u>A</u>TGCATCTTCTAGCAAC	R	34	Introduce 2 stop codons and *XmaI* site at residue 155
cdc37-360 **#2**	GCCATCAGCAAA<u>TAA</u>CA AGCTGAAAGCG	F	28	Overlap PCR to introduce a stop codon to residue 120
cdc37-360 **#3**	GCTTTCAGCTTG<u>TTA</u>TTT GCTGATGG	R	26	Overlap PCR to introduce a stop codon to residue 120
cdc37-360 xmaI	GCTTTCCCCGGG<u>TTA</u>TTT GCTGATGG	R	26	Introduce a stop codon and an *XmaI* site at residue 120

Table 2.15: List of oligonucleotides used in the construction of the random mutagenesis (RM) template to generate temperature-sensitive mutants of the *cdc37* gene

Name	Sequence	Direction	Bp's	Comments/Purpose
RM *EcoRI* 5'	GCACGAATTCTTTTTCTG CTTACGGGTGTGC	F	31	Introduce *EcoRI* site to 5' of 300bp of *cdc37* 3' flanking sequence
RM *EcoRI* 3'	GCACGAATTCAGCACTA TGGAGTGAAGTTTGAGG G	R	35	Introduce *EcoRI* site to 3' of 300bp of *cdc37* 3' flanking sequence
RM *BglII* 3'	GCCTGTGTAAACCCCGTT TCAGATCTATTCCGTTCA TTTCC	R	41	Introduce *Bgl* II site to 3' of *cdc37*
RM *PacI* 5'	GAACGGAATTTAATTAA AACGGGGTTTACACAGG C	F	35	Introduce a *PacI* site at the 5' of *cdc37* plus 300 bp of 5' flanking sequence
HRC RM *cdc37* 5'	GGTTTACACAGGCTTTTG G	F	19	PCR amplification of fragments for homologous recombination
HRC RM *cdc37* 3'	GGCCATTTGGACTACATA TT	R	20	PCR amplification of fragments for homologous recombination
G-418 1379 5'	TTTTCGCCTCGACATCAT CT	F	20	Sequence staring at 1379 in template from 5'
ET Seq 1 *cdc37*	GTACTCCAATCAAAGGCA TC	R	20	Sequence starting at base pair 21013 of *cdc37* cosmid c9b6
ET Seq 2 *cdc37*	CTATTCTTTTGGAAGCCT TTAAT	F	23	Sequence starting at base pair 22107 of *cdc37* cosmid c9b6
ET Seq 3 *cdc37*	GTTAGGAGGAAAAGACT CAAAAG	R	23	Sequence starting at base pair 22464 of *cdc37* cosmid c9b6
ET Seq 4 *cdc37*	AAATATTACGCGTTTATT GTAGC	F	23	Sequence starting at base pair 20412 of *cdc37* cosmid c9b6
ET Seq 5 *cdc37*	GTGGGAAGTAATAGAGT ATTCGT	F	24	Sequence starting at base pair 20332 of *cdc37* cosmid c9b6
PW *cdc37* #16	GTCAAATCGACGAGAAT A	F	18	Sequence starting at base pair 20751 of *cdc37* cosmid c9b6
PW *cdc37* #17	AAATCAATAAATAAAATT TCA	R	21	Sequence starting at base pair 22758 of *cdc37* cosmid c9b6

Table 2.16: List of oligonucleotides used for amplifying, mutating and sequencing of the p50 gene

Name	Sequence	Direction	Bp's	Comments/Purpose
GST-p50 *BamH1* 5'	GCATGGATCCATGGTGG ACTACAGCG	F	26	Introduce 5' *BamH1* site to p50 to clone into pGEX6P-1
GST-p50 *Xho1* 3'	GCATCTCGAGCACACTG ACATCCTTCTC	R	28	Introduce 3' *Xho1* site to p50 to clone into pGEX6P-1
mycx6 3' *BamH1* used	GCATGGATCCTTACTATT ACAAGTCCTCTTCAG	R	33	Introduce *BamH1* site into myc tag to insert into pREP
mycx6 5' BamH1	GCATGGATCCATGGAGC AAAAGCTC	F	25	Introduce *BamH1* site into myc tag to insert into pREP
p50 *BamH1* 3'(minus STOP codon)	GCATGGATCCCACACTG ACATCCTTCTC	R	28	Introduce *BamH1* site into p50 and remove 3' stop codon to insert myc tag
p50 Forward primer 2	GTAAATACCAAGCCCGA GAAGAC	F	23	Sequence from bp 384 of p50 ORF
p50 Reverse primer 1	CTGTCTTAATCTTAGTGA AGAACTGC	R	26	Sequence from bp 704 of p50 ORF

Table12.17: List of oligonucleotides used for work with pREP vectors

Name	Sequence	Direction	Bp's	Comments/Purpose
pREP81 HA 5'	GCTGCATGCCTGCAGGTC GATCGACTCTAGAGG	F	34	introduce N-terminal HA tag and retain *Nde1* at 5' of pREP81
pREP81 HA 3'	GCATCATATGAGCGTAG TCTGGGACGTCGTATGGG TAATTAACAAAGCGACTA TAAGTCAG	R	62	introduce N-terminal HA tag and retain *Nde1* at 3' of pREP81
nmt 3'	GCAGCTGAATGCCCATAG T	R	20	Sequence starting at bp 100 from *Nde1* site
nmt 5'	GGAATCCGCAATCATCAA TTG	F	22	Sequence starting at bp 100 from *Nde1* site

Table 2.18: List of oligonucleotides used for amplifying, mutating and sequencing of the *swo1* gene

Name	Sequence	Direction	Bp's	Comments/Purpose
swo1 Nde1 5'	GATGCTAGCATATGTCGAACACAGAAAC	F	28	Introduce *Nde1* at 5' of p50
swo1 Xma1 3'	CTGACCCGGGTTAATCGACTTCCTCCATC	R	29	Introduce *Xma1* at 5' of p50

Table 2.19: List of oligonucleotides used in the construction of plasmids for the yeast two-hybrid

Name	Sequence	Direction	Bp's	Comments/Purpose
pADC Y2H CP	GAAATTCGCTTATTTAGAAGTGTC	R	24	Colony PCR of *swo1* in pADC vectors, sequence starting at bp 189 from *PvuII* site
pBDC Y2H CP	GATGGGCATTAATTCTAGTCAT	R	22	Colony PCR *swo1* in pBDC vectors, sequence starting at bp 207 from *PvuII* site
cdc37 **Y2H** **Sma1-5'**	CTGACCCGGGATGGCAATTGATTACAGC	F	28	Introduce *Sma1* at 5' of *cdc37* to insert into yeast two-hybrid vectors
Cdc37 Y2H **Sma1-3' minus** **STOP codon**	ACGTCCCGGGAGACAATTGAGGAATAGTC	R	29	Introduce *Sma1* and remove STOP codon at 3' of cdc37 to insert into yeast two-hybrid vectors
Y2H *swo1 Sma1* **5'**	CTAGCCCGGGATGTCGAACACAG	F	23	Introduce *Sma1* at 5' of *swo1* to insert into yeast two-hybrid vectors
Y2H *swo1 Sma1* **3' minus STOP** **codon**	CTGACCCGGGATCGACTTCCTCCATC	R	26	Introduce *Sma1* and remove STOP codon at 5' of *swo1* to insert into yeast two-hybrid vectors
Y2H p50 3' ***Sma1* minus** **STOP codon**	GCATCCCGGGCACACTGACATCCTTCTC	R	28	Introduce *Sma1* and remove STOP codon at 3' of p50 to insert into yeast two-hybrid vectors
Y2H p50 3' ***Sma1* plus** **STOP codon**	GCATCCCGGGTCACACACTGACATCCTTCTC	R	31	Introduce *Sma1* at 3' of p50 to insert into yeast two-hybrid vectors.
Y2H p50 5' ***Sma1***	GCATCCCGGGATGGTGGACTACAGCG	F	26	Introduce *Sma1* at 5' of p50 to insert into yeast two-hybrid vectors

2.3.2 PCR reactions

2.3.2.1 Sequencing PCR reactions

Add in reaction:
DNA	100-200 ng	
Primer	3.2 µM	
Terminator Dye (Version 3.1)	4 µl	
dH$_2$O	to a total volume of 20 µl	

Reaction:
Step 1	94 °C	3 minutes
Step 2	96 °C	30 seconds
Step 3	50 °C	15 seconds
Step 4	60 °C	4 minutes
Repeat Steps 2 to 4 30 times		
Extension	60 °C	1 minute

2.3.2.2 Standard PCR amplification reactions

Add in reaction:
DNA	50 ng
Primer 1	1 µl of 10 µM stock
Primer 2	1 µl of 10 µM stock
Buffer	2 µl of 10x stock
dNTPs	0.4 µl of 100 µM stock
Polymerase	0.1 µl taq/0.2 µl Pfu
dH$_2$O	add to total volume of 20 µl

Reaction:
Step 1	94 °C	3 minutes
Step 2	94 °C	90 seconds
Step 3	variable	variable
Step 4	72°C	3 minutes
Repeat Steps 2 to 4 30 times		
Extension	72 °C	3 minutes

2.3.3   PCR polymerase

For analytical applications Taq DNA polymerase (Roche #1-146-173) was used. *Pfu* DNA polymerase (Promega #M7741) was used for proofreading ability during PCR reactions where accurate replication of sequence was essential.

2.3.4   Directed mutagenesis

Directed mutagenesis was used to change specific base pairs to substitute codons, utilizing the most frequently occurring arrangement of base pairs for that residues in *S. pombe* according to (Forsburg, 1994). Oligos were designed incorporating the desired mutation. Standard PCR reactions were carried out using designated oligos. Products were purified (Section 2.2.8), restriction digested (Section 2.2.6) and ligated into appropriate vectors.

2.3.5   Site directed PCR mutagenesis

The Stratagene Quick change™ site directed mutagenesis XL (#200517) kit was used to introduce specific mutations by following the protocol provided by the manufacturer.

2.3.6   Directed mutagenesis by overlap PCR

Site directed mutagenesis to change specific codons was performed by overlap PCR as shown schematically in Diagram 2.4.

2.3.7   PCR purification

PCR amplified fragments were purified using the QIAquick gel extraction kit (# 28704) substituting Buffer QG for Buffer PBS according to the instructions by the manufacturer.

Diagram 2.4: Directed mutagenesis by overlap PCR; **A** Standard PCR reactions were carried out with primer combinations 1 & 3 and 2 & 4 using *pfu* polymerase. The red star indicates the mutation being introduced. **B** PCR products were run on a DNA gel and bands cut out manually. **C** PCR products from both reactions were transferred into a single tube and covered with dH_2O. Samples were incubated for 30 minutes at 37 °C. The eppendorf was inverted to mix contents. **D & E** 15 µl was used as a template for a second Standard PCR reaction with oligonucleotides 1 and 4. The final product was run on a DNA gel, purified and then cloned as appropriate.
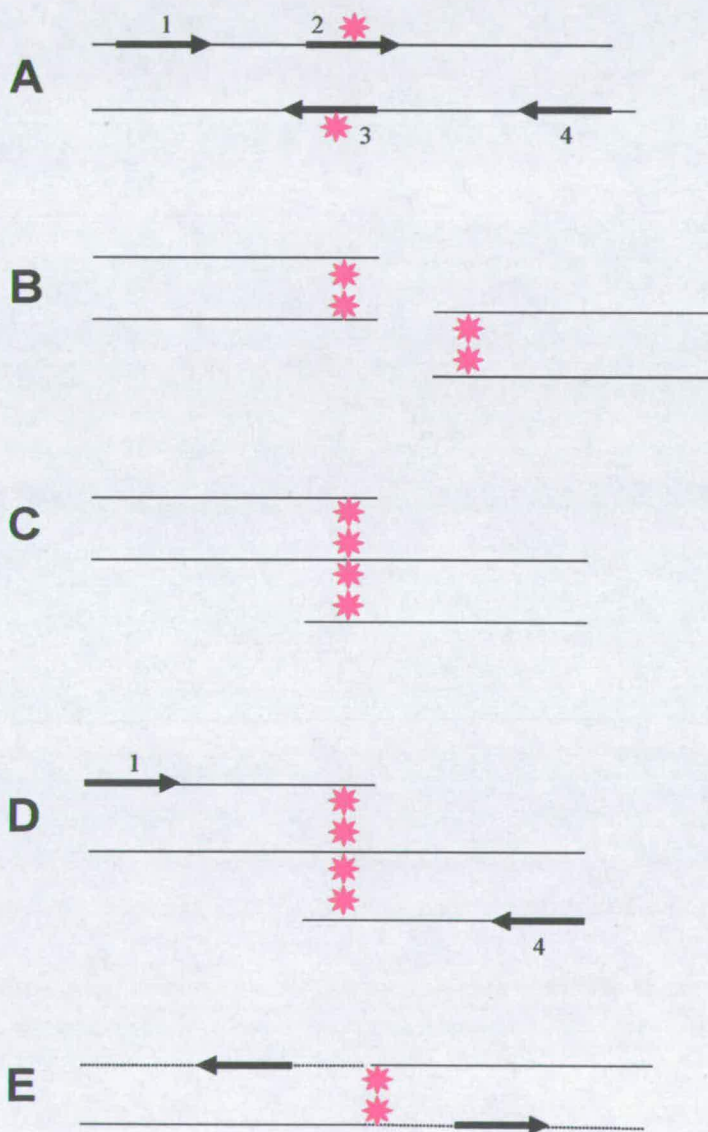
2.3.8   Purification of DNA sequencing reactions

To purify DNA sequencing reactions for analysis, 3 μl 3 M sodium acetate pH5.8, 62.5 μl ethanol and 14.5 μl dH$_2$O were added to samples once the PCR sequencing reaction had been carried out. Reactions were incubated at −80 °C for 15 minutes. Samples were then centrifuged at 4 °C for 30 minutes at 11,000 xg and then rinsed with 70% ethanol. DNA was dried in a speed vacuum for 10 minutes then sent for sequence analysis.

2.4   Bacterial Methods

2.4.1   *E. coli* Strains

The *E. coli* strain DH5α was used for general molecular cloning and the strain BL21 for the expression of GST fusion proteins.

2.4.2   Media

Luria Broth (LB):   10 g/l bacto-tryptone
                                     5 g/l bacto-yeast extract
                                     5 g/l NaCl
                                     pH7 (NaOH) and stored at room temperature

Solid LB: Add agar to a final concentration of 2%

2.4.3   Antibiotics

Ampicillin stock: 100 mg/ml in dH$_2$0 (Sigma-Aldrich #A9518)

                             Working concentration 0.5 μl/ml

Kanamycin stock: 50 mg/ml in dH$_2$0 (Sigma-Aldrich #0879)

                             Working concentration 0.5 μl/ml

2.4.4   Blue-white selection of *E. coli* colonies using x-gal

Identification of positive clones from the Stratagene Quick change™ site directed mutagenesis by blue-white selection required the addition of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (x-gal) (Amersham #US10077) to media of ~60 °C. A stock solution of 50 mg/ml was added to media to a final concentration of 40 µg/ml.

2.4.5   Making and transforming competent *E. coli*

2.4.5.1   Making *E. coli* competent for heat shock transformation

Retransformation of plasmids was carried out by heat shock transformation of DH5α as described by (Inoue et al., 1990). Competent *E .coli* stocks were made by inoculating 250 ml of SOB media in a 2 litre conical flask and incubating overnight at 18 °C shaking at 200 rpm.

SOB:	2% tryptone
	0.5% yeast extract
	10 mM NaCl
	2.5 mM KCl
	10 mM MgCl$_2$
	10 mM MgSO$_4$

At an OD$_{600}$ of 0.6 the culture was placed on ice for 10 minutes. Cells were centrifuged at 4 °C for 10 minutes at 5000 xg. The pellet was then resuspended in 80 ml of ice cold TB and incubated on ice for 10 minutes. Cultures were then spun down at 5000 xg at 4 °C for 10 minutes and resuspend in 18.6 ml of ice cold TB and 1.4 ml of DMSO. The cells were then distributed out as 200 µl aliquots in eppendorfs which were snap frozen in liquid nitrogen and store at –80 °C.

TB:	10 mM Pipes
	15 mM CaCl$_2$
	250 mM KCl
	55 mM MnCl$_2$

2.4.5.2 Heat shock transformation of DNA plasmids into competent *E. coli*

To transform plasmids by heat shock, aliquots of competent DH5α were thawed on ice and 100 μl of *E. coli* were added to DNA. Samples were mixed well and incubated on ice for 30 minutes. Aliquots were heat shocked at 42 °C for 90 seconds and then kept on ice for 2 minutes. 1 ml of LB was added to each reaction which was then incubated at 37 °C shaking at 200 rpm for 30-45 minutes. 200 μl of cells were then plated on LB agar plus appropriate antibiotics and incubated overnight at 37 °C.

2.4.5.3 Making competent *E. coli* for transformation by electroporation

Electroporation was used for DNA requiring high efficiency transformation. Competent DH5α or BL21 *E. coli* were made by picking 10 individual colonies from a freshly streaked plate and inoculating a 10 ml LB culture. Cells were shaken at 200 rpm overnight at 37 °C. The 10 ml cell culture was then used to inoculate 1 litre of LB. The culture was allowed to grow up to an OD_{600} of 0.35-0.4. *E. coli* were then incubated on ice for 1 hour. All of the following steps were carried out at 4 °C. The cell culture was centrifuged for 15 minutes at 5000 xg, supernatant was discarded and the pellet was resuspended in 2 ml of ice cold 10% glycerol. Cells were spun at 5000 xg for 15 minutes and resuspended in 25 ml of ice cold 10 % glycerol. This step was then repeated twice. Cells were resuspended in 1 ml of ice cold 10% glycerol and centrifuged at 5000 xg for 15 minutes. This step was repeated a second time. 50 μl aliquots were made, snap frozen in liquid nitrogen and store at −80 °C.

2.4.5.4 Electroporation transformation of DNA plasmids and ligations into competent DH5α or BL21 *E. coli*

For electroporation transformation, aliquots were thawed on ice and 50 μl of ice cold 10% glycerol was added. An appropriate amount of target DNA was placed into a pre-chilled electroporation cuvette with cells and mixed thoroughly. Cuvettes were chilled on ice for 5 minutes then shocked at 1.8 kV, 25 μFd capacitance, 200 Ω

resistance on a Bio-Rad Gene-Pulser. 900 μl of LB was immediately added to each sample. Cells are transferred to eppendorfs and grown up over 1 hour at 37 °C. Bacteria were then plated onto LB-agar plates plus appropriate antibiotics and incubated at 37 °C overnight.

2.5   Yeast

2.5.1   *S. pombe* stains

A list of the *S. pombe* strains used in this work are shown in Table 2.20.

2.5.2   *S. pombe* media for vegetative growth

S. pombe strains were grown on Yeast Extract (YE) a complex medium containing all necessary amino acids and that inhibits conjugation and sporulation. YE media contains thiamine which represses the *nmt1* promoter of pREP vectors.

Table 2.20: List of *S. pombe* strains used in this work.

Strain	Genotype
ED1021	*leu1-32 h⁻*
ED1022	*leu1-32 h⁺*
ED1088	*ura4-D18 h⁻*
ED1090	*ura4-D18 leu1-32 h⁻*
ED1526	*ade6-M21 ura4-D18 leu1-32 his1-102 cdc37::his1⁺ pREP82-cdc37⁺*
ED1537	*swo1-2HA ura4-D18 leu1-32* (Munoz and Jimenez, 1999)
ED1538	*leu1-32 ura4-D18 h⁻ cdc37-681* (temperature-sensitive mutant) (Tatebe and Shiozaki, 2003)
ED1560	*leu1-32 h⁺ swo1-26* (Hsp90 temperature-sensitive mutant) (Aligue et al., 1994)
ED1565	*ura4-D18 leu1-32 G-418 h⁻ cdc37::G418* cassette (*cdc37-184* temperature-sensitive mutant)
ED1566	*ura4-D18 leu1-32 G-418 h⁻ cdc37::G418* cassette (*cdc37-13* temperature-sensitive mutant)
ED1567	*ura4-D18 leu1-32 G-418 h⁻ cdc37::G418* cassette (*cdc37-J* temperature-sensitive mutant)
ED0824	*Cdc10-129 leu1-32 h⁻* (temperature-sensitive mutant) (Nurse et al., 1976)

YE: 5 g/l Oxoid yeast extract
                               30 g/l glucose
                               120 mg/l adenine, histidine, leucine, lysine
                               hydrochloride and uracil

For vegetative growth Edinburgh Minimal Media (EMM) was used which enabled
selective growth of strains by the addition of appropriate amino acids and expression
of pREP plasmids due to the absence of thiamine.

Emm: 3 g/l potassium hydrogen phthalate
                               2.2 g/l Na_2HPO_4
                               5 g/l NaCl
                               2% (w/v) glucose
                               20 ml/l salts (from a 50x stock)
                               1 ml/l vitamins (1000x stock)
                               0.1 ml/l minerals (10,000x stock)

Salts (50x):   52.5 g/l $MgCl_2 \cdot 6H_2O$
                               0.735 mg/l $CaCl_2 \cdot 2g/lH_2O$
                               50 g/l KCl
                               2 g/l Na_2SO_4

Vitamins (1000x):   1 g/l panthothenic acid
                               10 g/l nicotinic acid
                                      10 g/l myo-inositol
                               10 mg/l biotin

Minerals (10,000x):   5 g/l boric acid
                               4 g/l $MnSO_4$
                               4 g/l $ZnSO_4 \cdot 7H_2O$
                               2 g/l $FeCl_2 \cdot 6H_2O$
                               0.4 g/l molybdic acid
                               1 g/l KI
                               0.4 g/l $CuSO_4 \cdot 5H_2O$
                               10 g/l citric acid

2.5.3. Amino acids

Table 2.21: Amino acids added to EMM in this work are outlined below:

Amino Acid	Manufacturer Sigma-Aldrich #	Working Concentration
Adenine	A-9126	3.75 g/L
Leucine	U-0750	7.5 g/L
Uracil	H-8125	3.75 g/L
Histidine	L-8000	7.5 g/L
Thiamine	T-4625	15 g/L

2.5.4   5FOA media

In the presence of 5' fluoro-2'-deoxyuridine (5FOA), Ura^+ strains generate a toxic metabolite which is lethal and results in cell death (Grimm et al., 1988). 5FOA (Toronto Research Chemicals Inc. #595000) was added to melted EMM-agar media at 55 °C at 1 mg/ml to make plates for the plasmid shuffle assay (Section 2.7.1).

2.5.5   G418

The PFA6a-kanMX6 plasmid (Bahler et al., 1998) contains a G418 cassette that expresses resistance in *S. pombe* against Geneticin (G418 sulphate) (Gibco #11811-031). G418 was dissolved into molten YE media (~60 °C) at a concentration of 1 g/L.

2.5.6   Phloxin-B

S. pombe cells that are dead are permeable to Phloxin B which stains them red. Phloxin B (Sigma-Aldrich #P-2759) was added to melted agar media (~60 °C) at a final concentration of 1.75 µg/ml.

2.5.7   Storage of *S. pombe* strains

Yeast strains were stored in the short term on plates at 4 °C sealed with parafilm. *S. pombe* strains were stored for the long term by mixing 1.4 ml of cells grown in selection media with 0.6 ml of autoclaved glycerol. Samples were frozen at –80 °C in 1.8 ml Nunc Cryotube™ Vials (#368632).

2.5.8   Waking up *S. pombe* strains

S. pombe strains were woken up by scraping a small amount of frozen stock onto a sterile pipette tip. Samples were then placed on a plate containing media that would select for its growth and incubated at a suitable temperature for 2-3 days. Using a toothpick, cells were streaked out to enable single colony formation. Three single colonies per strain were then patched onto different selection media and incubated at appropriate temperatures for growth to ensure correct genotype.

2.6   Transformation of DNA into *S. pombe* protocols

2.6.1   Transformation by electroporation of plasmid DNA into *S. pombe*

DNA plasmids were transformed into *S. pombe* strains by electroporation. 10 ml of appropriate media was inoculated with a swab of cells on a sterile toothpick. Precultures were incubated at an appropriate temperature for 2 days. A suitable amount of preculture was used to inoculate 200 ml of liquid media. Cultures were incubated at a given temperature, shaking at 200 rpm overnight. At an OD_{600} of 0.4-0.5 cells were harvested by centrifugation at 5000 xg for 5 minutes. Cells were then washed 3 times with 20 ml ice cold 1.2 M Sorbitol, spinning at 5000 xg for 3 minutes between steps and consolidating each culture. Finally, the pellet was resuspended in 2 ml of ice cold 1.2 M sorbitol. 0.2 ml of cells was added to approximately 0.5 µg of plasmid DNA in a sterile cuvette and stored on ice. Each cuvette was pulsed at; 2.25 kV, 200, 25 uF. 0.5 ml of ice cold 1.2 M Sorbitol was immediately added to the transformation. 0.2 ml of transformed cells was spread on EMM plates plus suitable

amino acids for selection. Plates were incubated at an appropriate temperature for 3-4 days until single colonies were formed.

2.6.2 Lithium acetate based transformation for PCR based gene targeting in *S. pombe*

Transformation of PCR fragments for homologous integration into the chromosomal DNA was performed using the Lithium Acetate method as described by Bahler., *et al* (1998). Precultures were grown for two days in suitable media at an appropriate temperature. A 200 ml culture was inoculated with preculture and grown at an appropriate temperature shaking at 200 rpm overnight. At an OD_{600} of 0.4-0.5 cells were harvested at 5000 xg for 5 minutes. Supernatant was discarded and the pellet was washed in an equal volume of dH_2O. Cells were then spun down at 5000 xg for 5 minutes and pellets were resuspended in 1 ml of dH_2O by vortexing. Samples were transferred to a 1.5ml eppendorf and spun for 5 minutes at 5000 xg. 1 ml of LiAc/TE was added, cells were then spun down at 5000 xg for 5 minutes. 600 μl of LiAc/TE was added to resuspend the pellet. 100μl of cells were added to 2 μl sheared Herrings Testes DNA (10mg/ml) and 10 μl of target DNA for transformation. Mix was left to stand at room temperature for 10 minutes. 260 μl of 40% PEG/LiAc/TE was added and mixed gently. Samples were incubated at 32 °C for 30-60 minutes. 43 μl of DMSO was then added and samples were heat shocked for 5 minutes at 42 °C. Cells were then spun down at room temperature for 5 minutes at 5000 xg and washed once with dH_2O. The pellet was resuspended in 0.5 ml of dH_2O and plated on YE. Plates were incubated for 18 hours at an appropriate temperature, and then replicated onto selection media. New replica plates were incubated at an appropriate temperature for 3-4 days until single colonies appeared.

10x LiAc: 1 M LiAc pH7.5

10x TE: 0.1 M Tris-HCl pH7.5
                                   0.01 M EDTA

LiAc/TE:	10 ml 10xLiAc
	10 ml 10xTE
	80 ml dH$_2$0

40%PEG/LiAc/TE:	16 ml 50% PEG 4000
	2 ml 10xLiAc
	2 ml 10xTE
	10 ml dH$_2$0

2.7 Experimental techniques with S. *pombe*

2.7.1 Plasmid shuffle assay

ED1526 is a strain deleted for the endogenous *cdc37* gene and cell viability is maintained by the presence of wildtype *cdc37* on pREP82 that contains the *ura4$^+$* gene. The ability of different alleles and *cdc37* mutants to sustain cell viability was determined by expression from pREP 1, 41 or 81 in ED1526 by plasmid shuffle assay as shown in Diagram 2.5.

2.7.2 Serial dilutions of S. *pombe*

S. pombe precultures in suitable media were incubated overnight at an appropriate temperature. Precultures were adjusted to a final OD$_{600}$ of 0.5 in either dH$_2$0, YE or EMM. 1:4 serial dilutions were made of cells to a total of 5 dilutions which were then spotted as 5 μl onto appropriate media and incubated at a suitable temperature for 4 days.

2.7.3 Preparation of chromosomal DNA from S. *pombe*

A preculture was set up for 2 days, then used to inoculate a 100 ml culture which was grown to stationary phase (OD$_{595}$ 2 or 3). Cells were centrifuged at 5000 xg for 10 minutes. Pellets were resuspended in 1.5 ml of CPS buffer.

Diagram 2.5: Plasmid shuffle assay to determine the ability of *cdc37* mutants to sustain cell viability. **A** ED1526 *cdc37::his1*[+] *ade6-M21 ura4-D18 leu1-32 his1-102* pREP82-*cdc37*[+] was **B** transformed with *leu*[+] plasmids (pREP1, pREP41 or pREP81) expressing wildtype or mutant *cdc37*. Cells were precultured overnight in EMM containing uracil to promote loss of the *ura4*[+] plasmid pREP82-*cdc37*[+]. OD$_{600}$ was adjusted to 0.5 and serial dilutions were carried out and spotted onto MM+adenine+uracil plates with and without 5FOA. Plates were incubated at 25, 28 32 and 36 °C for 4 days. **C** 5FOA selects against *ura4*+ cells but allows *ura4*^{-} cells to grow. Strains carrying a functional *cdc37* construct expressed from pREP1 or pREP81 are able to grow in the absence of pREP82-*cdc37*[+] and are 5FOA resistant. Strains with a non-functional *cdc37* construct cannot loose the pREP82-*cdc37*[+] are lethal on 5FOA.
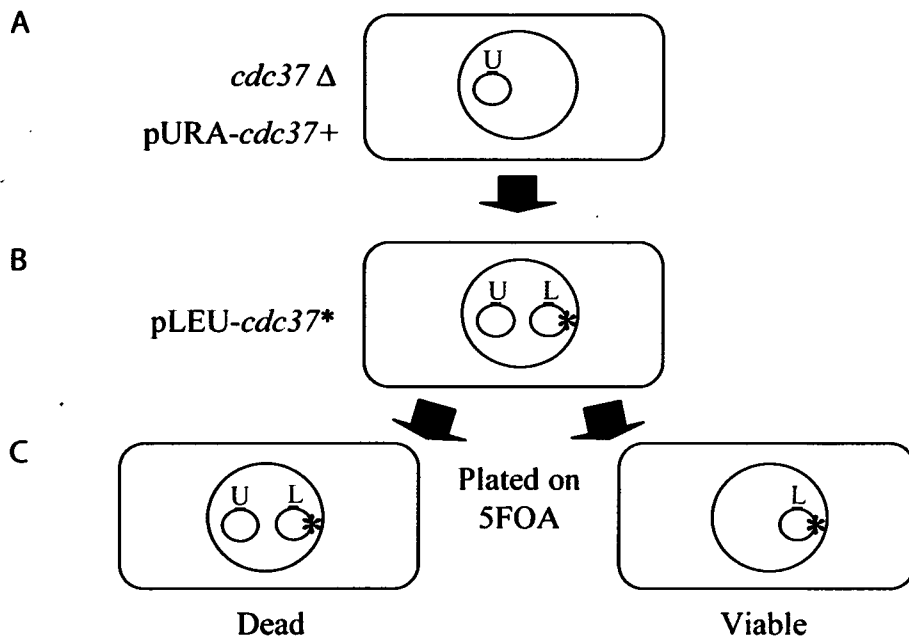
CPS buffer:	50 mM citrate phosphate buffer pH5.6
	1.2 M sorbitol
	0.1% (v/v) β-mercaptoethanol

15 mg of zymolyase-20T (ICN #320921) was added and samples were incubated at 37 °C for 5-30 minutes, until cell walls were fully digested. Cells were centrifuged at 5000 xg for 5 minutes and pellets were resuspended in 15 ml of 5x TE buffer (50 mM Tris-HCL pH7.0, 5 mM EDTA). 1.5 ml of 10% SDS was added and mixed thoroughly. Samples were then incubated 65 °C for 5 minutes. 5 ml of 5 M KOAc pH5.6 was added and incubated on ice for 30 minutes. Preps were then centrifuged for 15 minutes at 8000 xg. 1 ml of ice cold ethanol was added for every 20 ml of cells and mixed. Centrifugation was carried out for 10 minutes at 4 °C at 11,000 xg and pellets were resuspended in 3 ml of 5x TE buffer. RNase A was added to a final concentration of 100 µg/ml and incubated at 37 °C for 2 hours. Phenol-chloroform extraction (Section 2.2.9) was then used to purify DNA which was resuspended in a final volume of 200 µl of dH_2O.

2.7.4   Crossing of *S. pombe* strains

Strains of opposite mating types, h⁻ and h⁺, were grown on selective media plates. Each strain was patched independently and in combination of Spa plates containing leucine and mixed with a drop of dH_2O. Plates were incubated at a suitable temperature (usually 28 °C) for 1-2 days. Spa plates starve the cells of nitrogen and induce sporulation.

Spa media:	1% (w/v) glucose
	7.3 mM KH_2PO_4
	1 ml/l vitamins (1000x stock)
	45 mg/l adenine, histidine, lysine hydrochloride, uracil and leucine
For solid Spa media for plates:	3% Difco Bacto Agar

2.7.4.1 Random sporulation analysis of crosses between S. pombe strains

Successful crosses were incubated in a 1:50 dilution of Helicase, a snail gut digestive enzyme, from a 1:10 stock solution overnight at 28 °C. Helicase acts to enzymatically break down the asci wall and release the spores. Spores were centrifuged at 11,000 xg for 20 seconds, resuspended in 1 ml of dH_2O, spun again and resuspended in 500 µl of dH_2O. 50µl of each sample was plated and streaked into appropriate media and incubated at a suitable temperature for 2 days to observe growth. Plates were then replicated to selection media and different temperatures to determine the genotype of progeny.

2.7.4.2 Dissection of S. pombe tetrads derived from crosses

Asci are composed of 4 spores that can be dissected so that the genotype of each individual spore can be determined. A swab of the cross material was patched onto a thinly poured YE plate and then streaked using a sterile loop and dH_2O. Asci were picked using the tetrad dissector and placed at designated locations. Asci were incubated overnight at 4 °C and then shift to 28 °C for 3 to 4 hours. Using the tetrad dissector, each of the four spores from the asci were isolated and placed at designated locations. Spores were then incubated at an appropriate temperature for 2-3 days and then replicated to appropriate temperatures on suitable selection media to determine genotypes.

2.7.4.3 Culturing S. pombe temperature-sensitive mutant strains

S. pombe Temperature-sensitive mutant strains were cultured as 200 ml YE cultures shaking overnight at 28 °C. Cultures were diluted to an OD_{600} of 0.1 with media pre-warmed at 28 °C. Cell cultures were then split into two separate flasks and incubated shaking at 28 and 36 °C.

2.8   *S. pombe* cytology and immunofluorescence

2.8.1   Staining of *S. pombe* cells with calcofluor

Calcofluor was used to stain the walls and septa of *S. pombe* cells. 1 ml of cell culture (OD_{600} 0.4-0.5) were added to 100 µl of formaldehyde and incubated on a rotating wheel for 15 minutes at room temperature. Cells were then washed three times in 250 µl of dH_2O spinning at 10,000 xg at each step for 1 minute. Finally, cells were resuspended in 50 µl of dH_2O. 2 µl of cells were mixed with 1 µl of Calcofluor (Sigma-Aldrich F3543) (1 mg/ml) on a microscope slide and sealed with a cover slip and nail varnish. Cells were visualized immediately under a 63x oil objective Axioskop 2 lens.

2.8.2   Staining of *S. pombe* cells with DAPI

The DNA content of *S. pombe* cell culture (OD_{600} 0.4-0.5) was visualized by staining with 4',6-Diamidino-2-phenylindole (DAPI). 100 µl of formaldehyde was added to 1 ml of cells and incubated at room temperature for 15 minutes on a rotating wheel. Cells were then washed three times in 1 ml of dH_2O spinning each step at 11,000 xg for 1 minute. Cells were resuspended in a final volume of 50 µl of dH_2O. 2 µl of cells were mixed on a slide with 1 µl of DAPI (50 µg/ml) (Sigma-Aldrich D9542) and covered with a cover slip which was sealed nail varnish. Cells were viewed immediately under a 63x oil Axioskop 2 objective lens.

2.8.3   Staining of *S. pombe* cells with Phalloidin-conjugated Rhodamine

Phalloidin-conjuagted rhodamine was used for staining actin in *S. pombe* cells according to (Chang et al., 1996). 1 ml of cell culture (OD_{600} 0.4-0.5) was mixed with 100 µl of formaldehyde and placed on a rotating wheel for 10 minutes at room temperature. Cells were washed 3 times with 1 ml of PEM spinning cells at 5000 xg for 10 seconds between washes. 1 ml of PEM plus 1% Triton-X-100 was then added and incubated at room temperature for 2 minutes. Cells were resuspended in a final

volume of 50 µl of PEM. 2 µl of cells were mixed with 1 µl of 1:15 dilution of Phalloidin-TRITC (Sigma-Aldrich P1951). Phalloidin-TRITC is a fluorescent that enables visualisation of actin under UV.

PEM: 100 mM Pipes (pH7)
                                        1 mM MgSO$_4$
                                        1 mM EGTA

2.8.4   Immunofluorescence staining of *S. pombe* cells

Staining of *S. pombe* cells using antibodies was carried out by harvesting 40 ml of cells at an OD$_{595}$ of 0.5-0.8 by filtration onto Millipore HVLP 0.45μm membranes (Fischer #FDR-295-050Q) and fixed by submerging into -80°C chilled 100 % methanol and shaking vigorously. Tubes were incubated at -80°C and for more than 2 hours. Filters were removed and cells pelleted by centrifugation for 5 min at 5000 xg. Cells were resuspended in 1 ml of 100% methanol and stored at -80 °C. 50-100 μl of fixed cells were transferred into a fresh eppendorf tube. Cells were washed twice in 1 ml of PEM, using spinning at 5000 xg for 1 minute. Pellets were resuspended in 1 ml of PEMS with 0.2 mg/ml zymolyase-20T and incubated at 37 °C for 1 hour. Samples were washed twice with 1 ml of PEMS, spinning 5000 xg for 1 minute and resuspended in 1 ml PEMS/1% Triton X-100 for 30 seconds. Cells were then harvested at 5000 xg for 1 minute and washed twice in 1 ml of PEM. The cells were then resuspended in 1 ml of PEMBAL and incubated at room temperature on a rotating wheel for >30 min. Approximately 5 μl of cells were removed and resuspended in 30 μl PEMBAL plus an appropriate dilution of primary antibody. Primary antibody incubation was carried out overnight rotating at room temperature. Cells were then washed three times; harvesting cells at 5000 xg for 1 minute and incubating for 10 minutes in 1 ml PEMBAL. The pellet was finally resuspended in 30 μl of PEMBAL containing a 1:300 dilution of appropriate Alexa 2° antibody and incubated at room temperature for > 6 hours. Cells were washed 3 times in 1 ml of PEMBAL being resuspended in a final volume of 50 µl. 2-3 µl of cells are placed on a slide and sealed with a cover slip and nail varnish for immediate observation under an oil Axioskop 2 63x objective lens.

| PEMS: | 1.2 M sorbitol in 1X PEM |
| | to 200 ml final with sterile dH$_2$O |

PEMBAL:	1 % BSA (Sigma-Aldrich A7638, globulin free)
	0.1 % NaN$_3$
	100 mM lysine hydrochloride
	in PEM

2.8.5 Measuring Cell Length of *S. pombe*

Cells were stained with calcofluor and viewed under Axioskop 2 fluorescent microscope (Zeiss) with a 63x objective lens. Photographs were taken of fields of cells with a digital camera (Princeton Instruments) and a minimum of 200 cells per sample were measured using the IPLab scientific imaging software (scanalytics).

2.8.6 Counting *S. pombe* cell number by Coulter Counter

The number of *S. pombe* cells in a sample can be determined using a Coulter counter. A preculture was incubated for 2 days and used to inoculate a 200 ml culture that was incubated overnight at an appropriate temperature shaking at 200 rpm. 100 µl of cells were placed into a sterile Coulter counter vial containing 10 ml of filtered Isoton II and were mixed. Samples were sonicated at 5% for 10 seconds and then mixed thoroughly. Vials were placed into the Coulter counter and 500 µl of each sample was counted twice and the average number of cells per ml was calculated.

2.8.7 Analysis of DNA content of *S. pombe* by FACS

Flow cytometry by Fluorescent activated cell sorting (FACS) was used to determine whether cells contain a replicated or unreplicated genome by staining the DNA content with propidium iodide (Alfa et al., 1993). Stains were grown up at a suitable temperature and harvested at 2000rpm for 5 minutes to yield 5 x 10^6 cells per sample. Pellets were resuspended in 1 ml of cold 70% ethanol and stored at 4 °C. For each sample the cell number per ml was determined by Coulter counter Section 2.8.6

An appropriate number of cells were spun down at 5000 xg for 5 minutes and resuspended in 1 ml of 0.1 M HCl plus 2 mg/ml pepsin. Samples were incubated at room temperature for 1 hour. Cells were centrifuged at 5000 xg for 5 minutes and washed in 1.5 ml 50 mM sodium citrate (pH7). Pellets were resuspended in 500 µl of 50 mM sodium citrate (pH7) containing 0.1 mg/ml RNase A. Cells were incubated at 37 °C overnight. 500 µl of 50 mM Na citrate containing 4µg/ml propidium iodide (Sigma-Aldrich #P4864) was added to each sample. Cells were sonicated at 5% for 10 seconds and immediately processed in a FACScan (Becton Dickson).

2.9   Protein extraction from *S. pombe*

2.9.1   Small scale protein extraction using a Ribolyser

A preculture was used to inoculate a 200 ml culture of *S. pombe* cells that was cultured to an OD_{600} of 0.4. 40ml of cells were harvested by centrifugation at 5000 xg for 3 minutes. Pellets were resuspended in 10 ml of ice-cold STOP buffer or dH_2O, centrifuged at 5000 xg for 3 minutes and the supernatant was discarded.

For **denatured** protein extracts pellets were resuspended in 100 µl of Buffer A and for **native** extracts pellets were resuspended in 100 µl of buffer B.

STOP buffer:

150 mM NaCl
50 mM NaF
10 mM EDTA
1 mM NaN_3

Buffer A:

10 mM sodium phosphate buffer pH7.0
100 µl of Complete Inhibitors (x10stock) (Roche #187-3580)
1% Triton X-100
0.1% SDS
1 mM EDTA
150 mM NaCl
1 mM PMSF
dH_2O to a total volume of 1ml

Buffer B: Buffer A
                                   No SDS
                                   100 mM NaCl

Cells were added to pre-cooled ribolyser matrix tubes (HYBAID #RYM-6011) and vortexed for 2 seconds. Samples were then placed into a ribolyser machine and set to run for 20 seconds at 4.0 g.

For **denatured** extracts, 100 μl of 2x SDS loading buffer was added and vortexed for 2 seconds. Tubes were heated to 95 °C for 5 minutes and allowed to cool for 1 minute. The tube bottom was pierced with a hot needle and the contents were drained into a fresh 1.5 ml eppendorf by centrifuging at 5000 xg for 1 minute. Protein preps were stored at -80 °C and spun for 3 minutes at 11,000 xg prior to loading on gel.

For **native** extracts, 100 μl of buffer B was added to the tubes and vortexed for 2 seconds. The base of the tubes was punctured, placed in a 1.5ml eppendorf and spun at 5000 xg for 1 minute. Samples were then centrifuged at 11,000 xg for 15 minutes at 4 °C to generate low speed soluble extracts. The supernatant was transferred to a new eppendorf and stored at -80 °C.

2.9.2   Small scale native protein extraction using glass beads

This protocol was used to make protein from *S. pombe* to look at protein-protein interactions. 50 ml of cell culture of OD_{600} 0.4 were harvested at 5000 xg and washed once in 20 ml of STOP buffer. Supernatant was removed and pellets were resuspended in 100 μl of appropriate lysis buffer. Two samples were added to 1 ml of autoclaved acid washed 425-600 micron glass beads (Sigma-Aldrich G8772). Tubes were vortexed three times for 20 seconds with 1 minute breaks on ice. The bottom of the tubes were punctured, placed in an eppendorf and spun at 5000 xg for 1 minute. 200 μl of lysis buffer was added to the beads and vortexed for 2 seconds. Samples were then centrifuged again at 5000 xg for 1 minute and the supernatant was

transferred to a new tube. Samples were centrifuged at 11,000 xg to remove insoluble material, placed into a fresh chilled tube and stored at -80 °C.

2.9.3 Small scale native protein extraction using filtration and glass beads

Protein preparation for size exclusion chromatography were made by harvesting 50 ml of cell culture of an OD_{600} 0.4 by filtration over a 0.45µm milipore filter (Fisher #FDR-295-050Q). Filters were placed in an eppendorf and washed with 1 ml of cold dH_2O. Tubes were then spun at 12,000 xg for 10 of seconds. The filter paper was removed and centrifuged again at 8000 xg for 1 minute. The supernatant was removed and pellets were resuspended in 100 µl of appropriate lysis buffer. Two samples were added to 200 µl of autoclaved acid washed 425-600 micron glass beads (Sigma-Aldrich G8772). Samples were then treated as described in 2.5.3.

2.9.4 Quantification of protein concentration using the Bio-Rad protein assay

The protein concentration of *S. pombe* protein preparations was determined using the Bio-Rad protein assay kit (#500-0006) following the manufacturers instructions.

2.9.5 Immunoprecipitation (IP)

Protein A Sepharose CL-4B beads (Amersham 17-0780-01) were rehydrated with 1 ml of lysis buffer and incubated for 10 minutes on ice. Beads were centrifugation at 5000 xg for 3 minutes at 4 °C, washed three times in 1 ml of lysis buffer and then finally resuspended in an equal volume of buffer to beads. For each IP, 30 µl of beads were transferred into a chilled eppendorf. An appropriate amount of antibody was added and the volume in each tube was adjusted to 500 µl using lysis buffer. Samples were incubated for 1 hour at 4 °C on a rotating wheel. Samples were then spun down at 5000 xg at 4 °C for 1 minute and washed 3 times with 1 ml of lysis buffer. Two protein preparations (Section 2.9.2) added to each IP, adjusting the volume to 1ml

with lysis buffer. 50 µl of native protein prep is retained as an input control for analysis of IPs by SDS PAGE and western blot. Samples were incubated for 1 hour on a rotating wheel at 4 °C. IP's were washed by centrifugation at 5000 xg for 1 minute at 4 °C, resuspended in 1ml of lysis buffer and transferred to a new chilled tube. Beads were washed another three times and transferred to a new tube on the fourth wash. Supernatant was discarded and 30 µl of 2x SDS loading buffer was added to each IP and boiled for 5 minutes. Samples were stored at -80 °C.

2.9.6 Treatment of protein preparations with phosphatase

For treatment of *S. pombe* Cdc37 with phosphatases, Cdc37 was immunoprecipitated (IP) as described in Section 2.9.5, using 2.5µl of Cdc37 antibody and 500 µg of *S. pombe* whole cell protein extracts. All IPs were pooled and divided into 4 separate eppendorfs. Samples were treated in a total liquid volume of 40 µl with either Lambda protein phosphatase λ-PPase (#PO753S NEB) or Calf intestinal phosphatase (CIP) (NEB #M0290) as shown below:

1. 1x buffer and 2 mM $MnCl_2$
2. 1x buffer, 2 mM $MnCl_2$ and 800 units of Lambda phosphatase or CIP
3. 1x buffer, 2 mM $MnCl_2$, 800 units of Lambda phosphatase or CIP and phosphatase inhibitors (250 mM NaF, 4 mM Na-orthovanadate, 10 mM EDTA (Watson et al., 2001))
4. 1x buffer, 2 mM $MnCl_2$, 800 units of Lambda phosphatase or CIP and heat inactivated at 65°C for 1 hour in the presence of 50 mM EDTA

The first three conditions were incubated at 30 °C for 1 hour. Samples were centrifuged at 5000 xg and supernatant removed. The beads were washed 4 times with appropriate lysis buffer and boiled for 5 minutes in 2x SDS loading buffer. Samples were then analysed by SDS PAGE.

2.9.7 Size exclusion chromatography (SEC)

Protein samples of *S. pombe* were made as described in Section 2.9.3. Small scale size exclusion chromatography was carried out using 250 µg of protein at a 1 µg/µl concentration on a superpose 6 column (Amersham). Large scale size exclusion chromatography used 12 ml of protein sample at a 1 µg/µl concentration on a sephacryl s-300 HR 26/60 column (Amersham). Small scale size exclusion chromatography was run at a rate of 0.2 ml per minute giving 0.5 ml fractions, while large scale ran at 0.5 ml per minute resulting in 3 ml fractions. The following buffer was used to run the columns:

SEC Buffer:

20 mM Hepes KOH pH7.9
3 mM $MgCl_2$
150 mM KCl
10% glycerol
1 mM DTT
20 mM molybdate

Fractions were collected and run on large acrylamide gels for western blot or Coomassie staining analysis.

2.9.8 TCA Precipitation of size exclusion chromatography fractions

Protein samples were concentrated after size exclusion chromatography by TCA precipitation. 200 µl of TCA was added to each sample on ice. Tubes were vortexed and incubated on ice for 30 minutes. Samples were centrifuged at 4 °C at 11,000 xg for 15 minutes. Supernatant was removed by aspiration and pellets resuspended in 1 ml of acetone chilled at -80 °C. Samples were vortexed and centrifuged at 4 °C at 11,000 xg for 15 minutes. Supernatant was discarded and pellets were allowed to air dry for 15-30 minutes. 50 µl of 2x SDS Sample Buffer was added to run on SDS PAGE for analysis by western blot or Coomassie staining. If samples turned orange then 1-2 µl of 1.5 M Tris was added to adjust the pH.

2.9.9 GST pull-down

2.9.9.1 Making recombinant GST protein

The *E. coli* strain BL21 was transformed (Section 2.4.5.3) with constructs of the pGEX-1 vector. Single colonies were picked and precultured in 25 ml of LB plus ampicillin at 37 °C shaking at 200 rpm overnight. A 500 ml culture of LB plus ampicillin was inoculated with the preculture and incubated at 37 °C shaking at 200 rpm for approximately 1.5 hours until OD_{600} 0.5 was obtained. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM to induce expression of the pGEX-1 construct. The culture was incubated at 30 °C shaking at 200 rpm for 3.5 hours. Cells were harvested by centrifugation at 4 °C at 8000 xg for 10 minutes. Pellet was resuspended in 5 ml of ice cold lysis buffer.

Lysis Buffer:
500 mM NaCl
0.5% NP-40
50 mM Tris-HCl pH7.6
5 mM EDTA
5 mM EGTA
1x Complete Inhibitors

Cells were sonicated at Drive 20.5, auto 4.9, 6 times for 30 seconds with 30 second breaks. Sonicated cells were spun down at 16,000 xg for 15 minutes at 4 °C. Supernatant was transferred to a new tube and incubated with 200 µl of glutathione-sepharose 4B (Amersham 17-0756-01) beads being rocked gently at 4 °C for 10 minutes. Samples were pelleted by spinning at 5000 xg for 1 minute, supernatant was removed and beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of cold TEE buffer.

TEE Buffer:
200 mM Tris pH8
5 mM EDTA
5 mM EGTA

A volume of cold elution buffer was then added and mixed thoroughly by inverting.

Elution Buffer:	200 mM Tris-HCl pH8
	5 mM EDTA
	5 mM EGTA
	50 mM Glutathione

Samples were centrifuged at 5000 xg for 1 minute and supernatant was transferred to a fresh tube. This was repeated a total of three times, combining each elution step. An aliquot of the GST (fusion) protein was then run against a known amount of BSA for quantification on SDS PAGE and stained with Coomassie.

Dialysis was carried out to remove the glutathione b placing samples in snake skin dialysis tubing (Pierce 68035 3500 MWCO) and incubated at 4 °C in Dialysis buffer being gently rotated in solution with a stirrer. Dialysis was carried out for 4 hours, after which the dialysis buffer was replaced and incubated for another 4 hours.

Dialysis Buffer:	150 mM NaCl
	20 mM Tris-HCl pH7.6
	1 mM EDTA
	1 mM EGTA

Samples were placed into new eppendorfs, sterile glycerol was added to a final concentration of 20% and stored at -80 °C. All samples were then quantified using the Bio-rad protein assay (Section 2.9.4).

2.9.9.2   GST pull-down experiment

Glutathione-sepharose 4B beads were washed three times in appropriate buffer, e.g. PBS, Buffer R or TEG, and centrifuged at 5000 xg for 1 minute. 1-3 µg of GST or GST fusion protein was incubated with glutathione-sepharose 4B beads in 500 µl of appropriate buffer at 4 °C on a rotating wheel for 30 minutes. Beads were washed twice in designated buffer, centrifuging samples at 5000 xg for 1 minute. 500 µg of native *S. pombe* protein extract was added to each tube and samples were incubated at 4 °C for 2 hours on a rotating wheel. Samples were washed 4 times with appropriate buffer centrifuging at 5000 xg for 1 minute. Beads were then either treated with

PreScission™ Protease (Section 2.9.9.3) or resuspended in 40 µl of 2x SDS loading buffer and boiled for 5 minutes prior to loading on SDS PAGE.

Buffer R: 20 mM Hepes
                             150 mM NaCl
                             0.5 mM EDTA
                             10% glycerol
                             0.1% Triton-X 100
                             1 mM DTT
                             10x Complete Inhibitors (100 µl per ml)

TEG: 25 mM Tris pH 7.4
                             15 mM EGTA
                             10% glycerol
                             1 mM DTT

2.9.9.3   PreScission™ Protease treatment of GST fusion proteins

PreScission™ Protease (Amersham #27-0843-01) was used to cleave recombinant protein from its' GST tag. GST fusion protein bound to glutathione-sepharose 4B beads were resuspended in 50 µl of cleavage buffer and 1 µl of PreScission™ Protease (2 units). Reactions were allowed to run for 4 hours at 5 °C.

Cleavage Buffer: 50 mM Tris pH7
                             150 mM NaCl
                             1 mM dithiothreitol

Samples were centrifuged at 5000 xg for 3 minutes and the supernatant was transferred to a new tube. 40 µl of 2x SDS loading buffer was added to both the beads and the supernatant. Samples were boiled for 5 minutes, centrifuged at 5000 xg and loaded onto SDS PAGE.

2.9.10   SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE gels were made as shown in Table 2.22 and run in Running buffer in Bio-Rad cells. High percentage separating gels were used for observing proteins of low

molecular weight and low percentage gels gave better resolution for higher molecular weight proteins.

Running Buffer: 25 mM Tris
                                      250 mM Glycine
                                      0.1% SDS

Table 2.22: Components used to pour SDS PAGE gel for analysis of protein

	Separating Gel	Separating Gel	Stacking Gel
	7%	12%	5%
1.5 M Tris-HCL pH8.8	2.5 ml	2.5 ml·	-
0.5 M Tris-HCL pH6.8	-	-	2.5 ml
Acrylamide solution (30%)	2.5 ml	4 ml	1.33ml
10% (w/v) SDS	100 µl	100 µl	100 µl
dH$_2$0	4.85 ml	3.35 ml	6.1 ml
10% (w/v) AMPS	50 µl	50 µl	50 µl
TEMED (add last)	5 µl	5 µl	10 µl
	10 ml	10 ml	10 ml

For protein gels a SDS-PAGE standards broad range marker was used (Bio-Rad 161-0317) and for western gels a broad range pre-stained protein marker (NEB P7708) was used. Protein samples were treated as follows prior to loading on gels:

Denatured: Centrifuge for 3 minutes at 12,000 xg and load 10 µl

Native: Add equal quantities of 2x SDS loading buffer and protein extract.

                      Heat at 95 °C for 5 minutes then spin for 3 minutes at 12,000 xg load 10 µl.

2.9.11   Coomassie Staining of Protein Gels

For visualisation of protein on a SDS PAGE gel directly, gels were stained with Coomassie for 1 hour.

Coomassie:	0.1% Brilliant Blue R250
	(Sigma-Aldrich B7920)
	50% Methanol
	10% Acetic Acid

Gels were destained by incubating for 1-2 hours on a shaker at room temperature.

Destain:	10% Acetic Acid
	30% methanol
	dH_2O.

2.9.12 Western blotting and immunoprobing of SDS PAGE gels

The stacking gel was removed and the separating gel was placed in chilled transfer buffer on a shaker for 10 minutes. A PVDF membrane (Bio-Rad PVDF #162-0177) was dipped in methanol for a few seconds, submerged in dH_2O for 1-2 minutes then incubated in chilled transfer buffer on a shaker for 10 minutes. The transfer of protein from small gels was carried out according to manufacturer's instructions and run for 1 hour at 100 volts. The transfer of large gels was carried out using the Bio-rad semi-dry transfer system as set out by the manufacturers manual and run for 1 hour at 20 milliamps. Membranes were then placed into 100 ml of PBST/5% "Marvel" dried milk powder on a shaker for 1 hour at room temperature.

Membranes were rinsed with 2 changes of PBST, then 1 x 15 minutes PBST and 2 x 5 minutes PBST at room temperature on a shaker. The membrane was then incubated with 10 ml of PBST plus primary antibody for one hour at room temperature or 4 °C overnight. Membrane was washed in PBST as previously described and incubated in 10 ml of PBST plus secondary antibody on a shaker for 1 hour at room temperature. PBST washes were then repeated again. ECL Detection solutions I and II (Amersham #RPN2108) were added in equal volumes and placed on the membrane for 1 minute. Membranes were then exposed to Hyperfilm ECL (Amersham RPN3103k) and developed.

2.9.13   Assay for Cdc2 kinase activity

The kinase activity of Cdc2 was assayed in temperature-sensitive mutants by its ability to phosphorylate Histone H1 *in vitro*. 2.5×10^8 cells were harvested by centrifugation at 5000 xg for 5 minutes at 4 °C. Pellets were washed once with 20 ml of STOP buffer and resuspended in 20 μl of HB15 buffer by vortexing. Samples were added to 1ml of ice cold acid washed glass beads and vortexed for 20 seconds 4 times with 1 minute breaks on ice. 500 μl of ice-cold HB15 buffer was added, mixed well and 500 μl of supernatant was removed and placed into a new eppendorf. Extracts were centrifuged at 12,000 xg for 1 minute at 4 °C. The supernatant was placed into a fresh chilled tube and the protein concentration of each sample determined by Bradford assay (Section 2.9.4) and adjusted to 0.5 μg/μl with ice cold HB15 buffer.

Cdc2 was immunoprecipitated from native *S. pombe* protein extracts using 10 μl of p13^{Suc1} beads (Upstate 14-122) (1:1 v/v ratio with lysis buffer) per sample. Beads were washed twice in HB15 buffer and resuspended in 250 μl of HB15 buffer. 70 μg of *S. pombe* protein was transferred to each tube and IPs were incubated at 4 °C for 30 minutes. Beads were washed four times with HB15 buffer and resuspended in 6 μl of HB5 buffer and mixed. Samples were incubated at room temperature for 15 minutes. 10 μl of KIN buffer was then added and mixed gently. Reactions were incubated at room temperature for 10 minutes and stopped by the addition of 2x SDS loading buffer. Samples were then run on a 12% SDS PAGE, stained with Coomassie and then dried. The dried gel was place din a phosphor-imager cassette overnight and processed using the STORM phosphor-imager.

HB15 buffer:

1 x Complete
60 mM β-glycerophosphate
15 mM ρ-nitrophenylphosphate
25 mM MOPS(pH7.2)
15mM EGTA
15 mM MgCl$_2$
1 mM DTT
0.1 mM Na$_3$VO$_4$ (pH8)
1% Triton X-100

HB5 buffer: Buffer HB15
                                     Plus 5 mM EGTA

KIN buffer: 200 µM ATP
                                     2 mg/ml of histone H1
                                     40 µCi of [γ-^{32}P] ATP

2.9.14   Antibodies

Antibodies used in this work are listed in Table 2.23.

2.10   Protocols for *S. cerevisiae*

2.10.1   Media

2.10.1.1   YPD medium and YPD agar medium

YPD: 20 g/L Difco peptone
                                     10 g/L Yeast extract
                                     20 g/L Agar (for plates only)

pH was adjusted to 5.8 and autoclaved. Once cooled to 55 °C, dextrose was added to 2%.

2.10.1.2   SD Medium and SD medium plates

SD: 6.7 g yeast nitrogen base without amino acids
                                     20 g agar (plates only)
                                     850 ml H$_2$0
                                     100 ml of appropriate sterile 10x drop out
solution

pH was adjusted to 5.8 and allowed to cool to 55 °C before adding the appropriate sterile carbon source usually dextrose to 2%.

Table 2.23: Antibodies used in this work are listed below.

Name	Poly/monoclonal	Origin	Reference
Anti-*S. pombe* Cdc37 antibody	polyclonal	Raised against full length *S. pombe* Cdc37 in Rabbit	
12CA5 Anti-HA	Monoclonal	Raised in mouse	Roche #1-583-816
Anti-PSTAIR	Monoclonal	Raised in mouse against synthetic 16 amino acid oligopeptide containing the $p34^{cdc2}$ (cdk1) PSTAIR sequence	Sigma-Aldrich #P7962
Anti-phosphor-tyrosine 15 cdc2	Polyclonal	Raised in rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Tyr15 of human cdc2.	NEB #9111
anti-cdc2 Y63.2	Monoclonal	Raised in mice against *S. pombe* Cdc2	kind gift from Paul Nurse lab
anti-cdc13 6F 10/11	Monoclonal	Raised in mice against *S. pombe* Cdc13	kind gift from Paul Nurse lab
anti–rabbit IgG antibody Alexfluor [680]		For immunofluorescence	Molecular Probes
Anti-mouse	IgG HRP linked		Amersham NA931V
Anti-rabbit	IgG HRP linked		Amersham NA935V
Anti-rat	IgG HRP linked		Amersham NA934V

2.10.2   Nutrients

2.10.2.1   Carbon Sources

Added to SD media: 40% dextrose

20% glucose

2.10.2.2   Dropout supplements

Table 2.24: Amino acids used to supplement media for growth of *S. cerevisiae*

Supplement	Sigma-Aldrich #	Working concentration
L-Histidine	H-9511	20 mg/L
L-Leucine	L-1512	100 mg/ml
L-Tryptophan	T-0254	20 mg/ml
L-Uracil	U-0750	20 mg/ml
L-Adenine		20 mg/ml

2.10.3   Transformation of *S. cerevisiae*

2.10.3.1   Lithium Acetate transformation of plasmid DNA into *S. cerevisiae*

S. cerevisiae was transformed using the Lithium acetate based method (Gietz et al., 1992). A preculture was grown in 50 ml of YPD, 30 ml of which was used to inoculate 300 ml of YPD and was cultured at 30 °C overnight shaking at 230-270 rpm. At an OD_{600} of 0.4-6 cells were centrifuged at 5000 xg for 5 minutes at room temperature and supernatant was removed. Pellets were washed in an equal volume of dH_20 or 1x TE and mixed by vortexing. Samples were centrifuged at 5000 xg for 5 minutes and resuspended in 1.5 ml of 1x TE/LiAc. 0.1 µg of plasmid and 0.1 mg of sheared ss herring DNA was added to 0.1 ml of yeast cells in a new tube. 0.6 ml of sterile TE/PEG/LiAc was added and samples were incubated at 30 °C for 30 minutes

shaking at 200 rpm. 70 µl of DMSO was added and cells were heat shocked for 15 minutes at 42 °C. Tubes were then placed on ice for 1-2 minutes. Cells were then centrifuged at 11,000 xg at room temperature for 5 seconds. Supernatant was discarded and pellet was resuspended in 0.5 ml of sterile 1x TE buffer. 100 µl of transformed cells were plated on SD-agar media with appropriate nutrients and incubated at 30 °C until single colonies appeared.

2.10.4 Maintaining *S. cerevisiae* strains

2.10.4.1 Storage of *S. cerevisiae*

Strains of *S. cerevisiae* were stored on selection plates at 4 °C for the short term. For long term storage of *S. cerevisiae* strains were mixed with 1.4 ml of preculture grown in selection media with 0.6ml of autoclaved glycerol. Samples were frozen at to –80 °C in 1.8ml Nunc Cryotube™ Vials (#368632).

2.10.4.2 Waking up *S. cerevisiae* strains

S. cerevisiae strains were woken up by scraping up a small amount of frozen stock with a sterile pipette tip and placing it on a selection plate. Plates were incubated at a suitable temperature for 2-3 days. Cells were then streaked out using a sterile toothpick and incubated at an appropriate temperature until single colonies had appeared. Single colonies were patched out on to different selection media and incubated at an appropriate temperature for growth to ensure correct genotype.

2.10.4.3   *S. cerevisiae* **strains used**

<u>AH109:</u>

System	GAL4 2H-3
Genotype	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gla80Δ, LYS2::GAL1 ₍UAS₎-GAL1 ₍TATA₎-HIS3, MEL1 GAL2 ₍UAS₎-GAL2 ₍TATA₎-ADE2, URA3::MEL1 ₍UAS₎-MEL1 ₍TATA₎-lacZ*
Reporters:	HIS3, ADE2, lacZ, MEL1
Transformation Markers:	trp1, leu2
Source:	Holtz unpublished

2.10.5   Yeast two-hybrid assay

The Yeast two-hybrid system was used to study protein-protein interactions and is detailed schematically in Diagram 2.6.

2.10.5.1   Colony lift filter yeast two-hybrid assay

Strains were grown up as patches overnight on selective agar plates. An autoclaved Whatmann #5 (7cm diameter) filter paper (#1005070) was pre-soaked in 2.5-5ml of Z buffer and X-gal in a Petri dish. Another sterile filter paper was placed over the yeast colonies to transfer cells and then submerged in liquid Nitrogen for 10 seconds. The filter was removed and allowed to thaw at room temperature. The filter paper was then placed colony side up into a Petri dish on top of a filter paper pre-soaked in Z buffer. Samples were incubated at 30 °C for 20 minutes to 1 hour. If colonies turned blue this would suggest an interaction between the two candidate proteins.

Z-Buffer:	16.1 g/L $Na_2HPO_4 \cdot 7H_2O$ 5.5 g/L $NaH_2PO_4 \cdot H_2O$ 0.75 g/L KCl 0.246 g/L $MgSO_4 \cdot 7H_2O$
X-gal stock:	20 mg/ml

Z-Buffer/X-gal Solution: 100 ml Z-Buffer
0.27 ml β-mercaptoethanol
1.67 ml X-gal stock

Diagram 2.6: The yeast two-hybrid system is used to identify protein-protein interactions between candidate proteins, shown here as A and B. **A** The candidate A protein is tagged with a GAL4 DNA binding domain and **B** candidate B with the GAL4 activation domain. **C** If the two candidate proteins physically bind, then the DNA binding domain and activating domain come together and enable expression of the reporter gene.

Chapter 3:

Identification of the essential domains of the molecular chaperone protein Cdc37 in *S. pombe*

3.0 Identification of the essential domains of *S. pombe* Cdc37

3.1 Introduction

The expression of mutant alleles in a cell lacking the endogenous gene to determine their ability to sustain cell viability is a valuable tool for identifying essential domains of a candidate protein. The *S. pombe cdc37* gene was mutagenised both randomly and by directed mutagenesis to generate various mutants to identify which domains are crucial for cell viability. Three domains have been identified in the human Cdc37 homologue using limited proteolysis and peptide analysis: an N-terminal region consisting of amino acids 1-126, a middle region composed of residues 128-282 and a C-terminal portion of amino acids 283-378 (Shao et al., 2003). By aligning human and *S. pombe* Cdc37 sequences these three domains were mapped onto the *S. pombe* protein as indicated in Figure 3.1. Crystallisation studies of the human Cdc37 protein comprising amino acids 148 to 347 identified structural features of the middle and C-terminal domains (Roe et al., 2004). A 6-helix bundle (amino acids 148-245) was identified, connected to a small 3-helix bundle (residues 316-347) by a long single helix (residues 246 to 286) and a disordered polypeptide chain (amino acids 309-315) (Roe et al., 2004). These features have also been mapped onto *S. pombe* Cdc37 as shown in Figure 3.2.

At present there is no known role for the C-terminal domain of Cdc37, although functions for the N-terminal and middle regions have been identified. The N-terminal domain of human, rat and *S. cerevisiae* Cdc37 contains conserved serine residues that are important for function and are phosphorylated by casein kinase II (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003). Phosphorylation of these conserved serine residues is important for the binding of client proteins by the molecular chaperone Cdc37 (Miyata and Nishida, 2004). The N-terminal domain of

Figure 3.1: The protein sequence of **A** Human Cdc37 was aligned with **B** *S. pombe* Cdc37. Domains identified in human Cdc37 by limited proteolysis and peptide analysis (Shao et al., 2003) and the Hsp90-binding (purple) and homodimerisation (blue) domains (Roe et al., 2004) were mapped onto the *S. pombe* Cdc37. These represent the domains referred to for *S. pombe* Cdc37 in this Chapter.
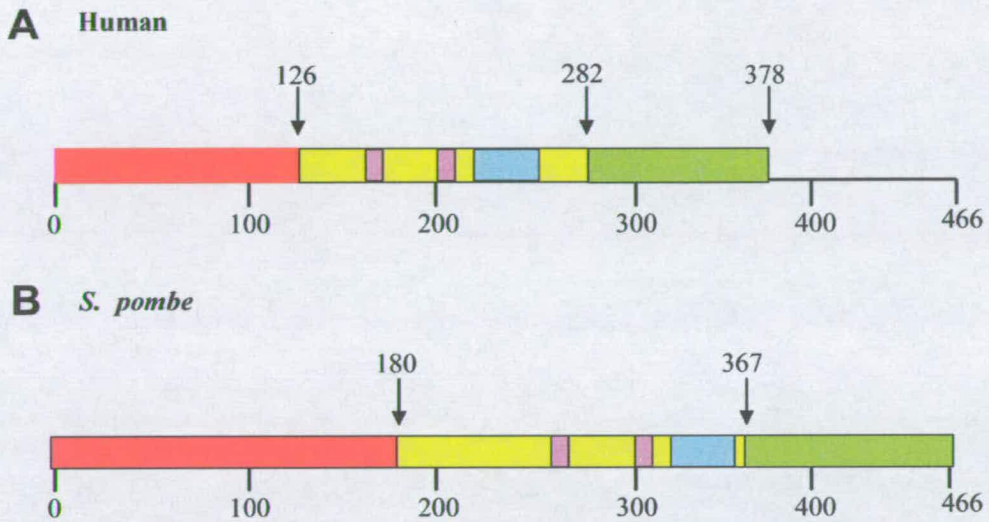
Figure 3.2: Alignment of the structures identified in the human Cdc37 protein with the *S. pombe* Cdc37 protein. Amino acids 148 to 347 of human Cdc37 were crystallized and the structural features determined for this region (Roe et al., 2004). These features were mapped onto the *S. pombe* Cdc37 protein sequence by alignment.
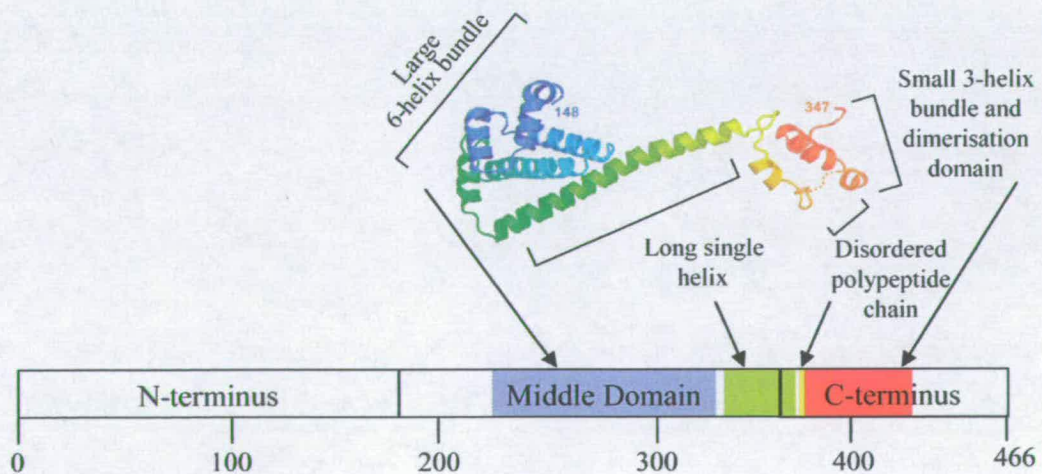
Cdc37 binds client proteins as shown for the eIF2α kinase interacting with human Cdc37 (Shao et al., 2003). The middle domain of human Cdc37 contains the homodimerisation binding domain (amino acids 218 to 255) and the regions responsible for interactions with the co-chaperone Hsp90. A complex between the interacting domains of yeast Hsp90 and human Cdc37 has been crystallised and its structure determined (Roe et al., 2004). Amino acids 164-170 and 204-208 of human Cdc37 were found to form a hydrophobic patch that interacts with the N-terminal region of yeast Hsp90 (Roe et al., 2004). The Hsp90-binding and homodimerisation binding sites identified in human Cdc37 have also been mapped by alignment onto the *S. pombe* Cdc37 protein as shown in Figure 3.1.

This chapter describes the creation and analysis of a range of *cdc37* mutants with the aim of identifying the essential domains of this molecular chaperone that are essential for cell viability. Mutants of *cdc37* were generated by *in vitro* pentapeptide transposition random mutagenesis and directed mutagenesis to produce a range of truncation and five amino acid in-frame insertion mutants throughout the Cdc37 protein. These *cdc37* mutants were expressed in the *S. pombe* strain ED1526, deleted for the endogenous gene and kept alive by expression of the plasmid *pREP82-cdc37⁺*. The ability of these *cdc37* mutants to substitute for wildtype Cdc37 and to sustain cell viability in *S. pombe* was assessed in ED1526 using the plasmid shuffle assay.

3.2 Random mutational analysis of *S. pombe cdc37* using *in vitro* pentapeptide transposition

3.2.1 *In vitro* pentapeptide transposition of *cdc37*

Random mutagenesis by *in vitro* pentapeptide transposition was carried out using the GPS™-LS Linker Scanning System from NEB to generate a range of *S. pombe cdc37* mutants (described in detail in Materials and Methods: Section 2.2.13). A transposon/transprimer is randomly inserted by *In vitro* pentapeptide transposition mutagenesis and its subsequent removal leaves a 15 bp insertion in the target DNA, producing an in-frame insertion of five codons. Insertion into the target DNA of less

than 15 bp, results in a frameshift and/or the introduction of a stop codon which creates a truncation mutant.

The target DNA, the *cdc37* gene, was restriction digested with *NdeI* and *XmaI* and purified from the pREP81 vector. Removal of the vector ensured that *in vitro* pentapeptide transposition insertions only occurred in the target gene to improve mutagenesis efficiency. The small scale individual analysis protocol, as described by the manufacturer, was carried out using the GPS5 donor DNA and the TnsABC* Transposase to randomly insert a Tn7-based Transprimer into *cdc37* DNA fragments. Once fragments containing insertions had been identified and ligated into the pREP81 vector, the transprimer was removed by digestion with the restriction enzyme *PmeI*, leaving only the *in vitro* pentapeptide transposition insert. Plasmids were allowed to recircularise in the presence of DNA ligase and were then sequenced to determine the pattern of inserted nucleotides.

3.2.2 Mutations obtained by *in vitro* pentapeptide transposition

A total of 14 *cdc37* mutants were generated by *in vitro* pentapeptide mutagenesis. A summary of the mutants detailing their insertions is given in Table 3.1; Figure 3.3 is a schematic diagram to show the location of the insertions within the *cdc37* gene. Three categories of mutations were identified; in-frame insertions of five codons, in-frame stop codons and insertions that produced frameshifts leading to a stop codon. Two of the mutants, I120 and I386, were obtained by an Honours student, Katie Single.

3.2.3 Directed mutagenesis to generate truncation mutants of *cdc37*

A range of *cdc37* truncation mutants had been generated by *in vitro* pentapeptide transposition mutagenesis. To generate even coverage of truncation mutants across the Cdc37 protein, directed mutagenesis was carried out. Overlap PCR and site-directed mutagenesis were employed to create truncation mutants introducing stop codons at specific locations in the full length *cdc37* gene as detailed in Table 3.2.

Table 3.1: Table of *cdc37* mutations generated by *in vitro* pentapeptide mutagenesis detailing the insertion size, location and type of mutant generated.

Name	Size of insert (bp)	Location of first insertion codon	Mutation Type	Location of Stop codon	Codon changes (in red)
37	14	37	Stop codon	40	RQRDGV∘T
60	14	60	Stop codon	61	MAMV∘TPIRRL
I120	15	120	In-frame		IKDACLNNAE
250	13	250	Frameshift	260	EDGGLNIQEVCQY•
I252	15	252	In-frame		GKKCLNKKVANI
264	14	264	Frameshift	272	SSSGFKPTKSF•
273	16	273	Frameshift	282	HLNCLNIKYLGR•
321	13	321	Frameshift	332	NGLRCLNINIFSKN•
351	14	351	Stop codon	354	VRIQYV∘T
385	13	385	Frameshift	395	SITVFKHHPGRFY•
I386	15	386	In-frame		SITIPVFKHPEA
412	13	412	Frameshift	422	NLQMFKHTSSYDQ•
428	13	428	Stop codon	429	KINV∘TYC
I422	15	422	In-frame		LDKMFKHKIVVL

Figure 3.3: A schematic diagram to illustrate the location of mutations obtained by *in vitro* pentapeptide mutagenesis and directed mutagenesis within the Cdc37 protein.
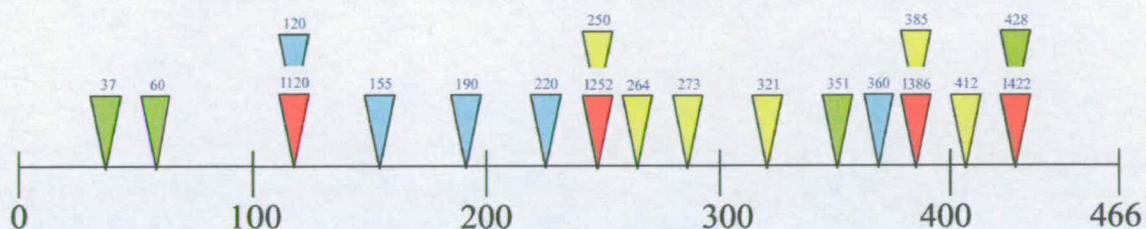
Insertions introducing a frame-shift and resulting in a stop codon
Insertions introducing a stop codon
In-frame insertions of 5 amino acids
Directed mutagenesis introducing a stop codon

420   Denotes the codon location of the insertion

Table 3.2: Truncation mutants generated by directed mutagenesis.

Name	Method	STOP codon location
cdc37-120	Site directed	120
cdc37-155	Overlap PCR	155
cdc37-190	Overlap PCR	190
cdc37-220	Overlap PCR	220

These truncation mutants were also generated by directed mutagenesis using standard PCR amplification with a mutagenised 3' primer that introduced a stop codon followed by an *Xma1* restriction site for cloning, to remove all downstream *cdc37* sequence. This was to ensure that read-through of the stop codon did not occur and that a truncated Cdc37 protein was produced.

3.3   Ability of *cdc37* mutants to sustain cell viability

A range of *cdc37* mutants had been generated, described in the previous section, and their ability to sustain cell viability in *S. pombe* was assayed by plasmid shuffle in the strain ED1526. All *cdc37* mutants were expressed from pREP81. Expression of wildtype Cdc37 from pREP81 produces the Cdc37 protein at a level very similar to endogenous Cdc37 (Figure 3.4). This system was therefore used to test the ability of *cdc37* mutants to sustain viability in the plasmid shuffle assay.

3.3.1   Ability of *cdc37* in-frame insertion mutants to sustain cell viability

In vitro pentapeptide mutagenesis of the *cdc37 gene* generated four mutants with in-frame insertions of five amino acids. These mutants were expressed from pREP81 in the plasmid shuffle assay to determine their ability to support growth and to see whether any mutant phenotypes resulted. Expression of *cdc37-I120*, *cdc37-I386* and *cdc37-I422* resulted in cells that were wildtype in appearance, supporting growth as well as *pREP81-cdc37* expression (Figure 3.5). The five amino acid insertions in *cdc37-I120*, *cdc37-I386* and *cdc37-I422* start at codons 120, 422 and 386 respectively. These data show that insertions in the N- and C- terminal domains do not dramatically affect essential Cdc37 function in *S. pombe*. In contrast, the mutant *cdc37-I252* was unable to sustain cell viability and no growth was observed at any temperature in the plasmid shuffle. Cells were heterogeneous in phenotype, being elongated, round, small and bowling pin shaped, all characteristic of depletion of Cdc37 from cells as described by (Westwood et al., 2004). The *cdc37-I252* mutant has a five amino acid insertion in the middle domain starting at residue 252 which disrupts the essential function of the molecular chaperone Cdc37, so that it could not

Figure 3.4: Comparison of Cdc37 protein levels of endogenous Cdc37 in the *cdc37*⁺ strain ED1022 and expression of *pREP81-cdc37* in the *cdc37Δ* strain ED1526. Western blots were carried out with anti-*S. pombe* Cdc37 antibody to determine Cdc37 protein levels and anti-TAT1 antibody (a kind gift from Keith Gull) as a loading control.
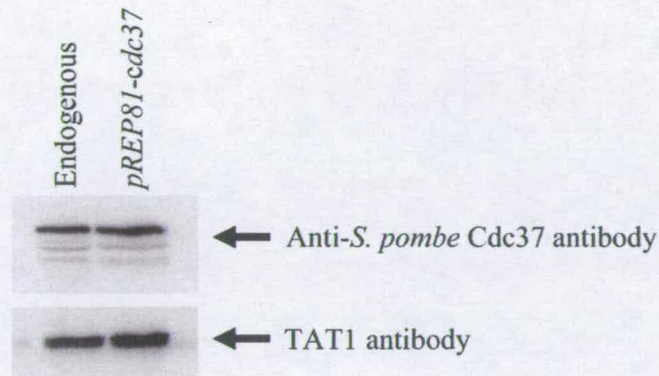
Figure 3.5: A schematic diagram of the location of the five amino acid insertion sites within the Cdc37 protein. Cdc37 mutants were expressed from pREP81 in ED1526 to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C (shown here at 28 °C, behaviour did not differ at other temperatures).
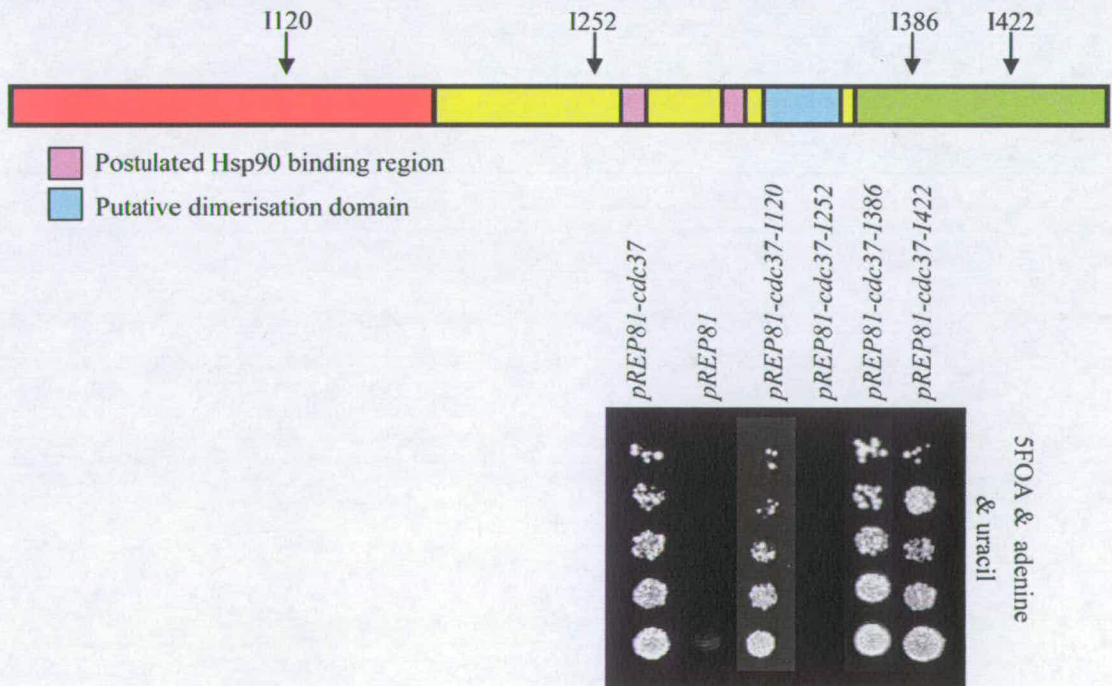
be substituted for wildtype Cdc37. According to the alignment of the human and *S. pombe* Cdc37 proteins shown in Figures 3.1 and 3.2, the five amino acid insertion of *cdc37-I252* is located at the edge of the postulated Hsp90 binding domain, within the 6-helix bundle of the middle domain (Roe et al., 2004) and may disrupt structure in this region abrogating Cdc37 function.

3.3.2   Ability of *cdc37* truncation mutants to sustain cell viability

Truncation mutants of *cdc37* were generated as previously described, and their ability to sustain cell viability was assayed using the plasmid shuffle system. All truncation mutants were expressed in the *S. pombe* strain ED1526 from pREP81. Expression of Cdc37 mutants, *cdc37-428, cdc37-412, cdc37-385, cdc37-360* (formally called *cdc37-1*) and *cdc37-351*, truncated in the C-terminal domain, were able to sustain cell viability supporting growth as well as expression of *pREP81-Cdc37* (Figure 3.6). These data indicate that the C-terminal domain is not essential for Cdc37 function and cell viability in *S. pombe*. Shorter truncation mutants of Cdc37, *cdc37-321, cdc37-273, cdc37-264* and *cdc37-250*, deleted in the middle domain, were unable to compensate for loss of full length Cdc37 and could not sustain growth at any temperature (Figure 3.6). Cells phenotypically resembled those observed with expression of only the pREP81 vector consisting of morphologically heterogeneous non-dividing cells. Interestingly, Cdc37 truncation mutants *cdc37-190* and *cdc37-220* were able to support a low level of growth when expressed from pREP81 (Figure 3.6). This result was surprising, considering *cdc37-190* and *cdc37-220* only express 40% and 47% of full length Cdc37 protein respectively. These Cdc37 truncation mutants lack the majority of the middle and all of the C-terminal domains, indicating that these domains are not essential for Cdc37 function and cell viability. Expression from pREP81 of Cdc37 mutants, *cdc37-37, cdc37-60, cdc37-120* and *cdc37-155*, truncated in the N-terminal domain, were unable to sustain growth.

Figure 3.6: A schematic diagram of the Cdc37 truncation mutants expressed from pREP81 in the *cdc37Δ* strain ED1526. Strains were plated onto 5FOA to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C (shown here at 28 °C, behaviour did not differ at other temperatures).
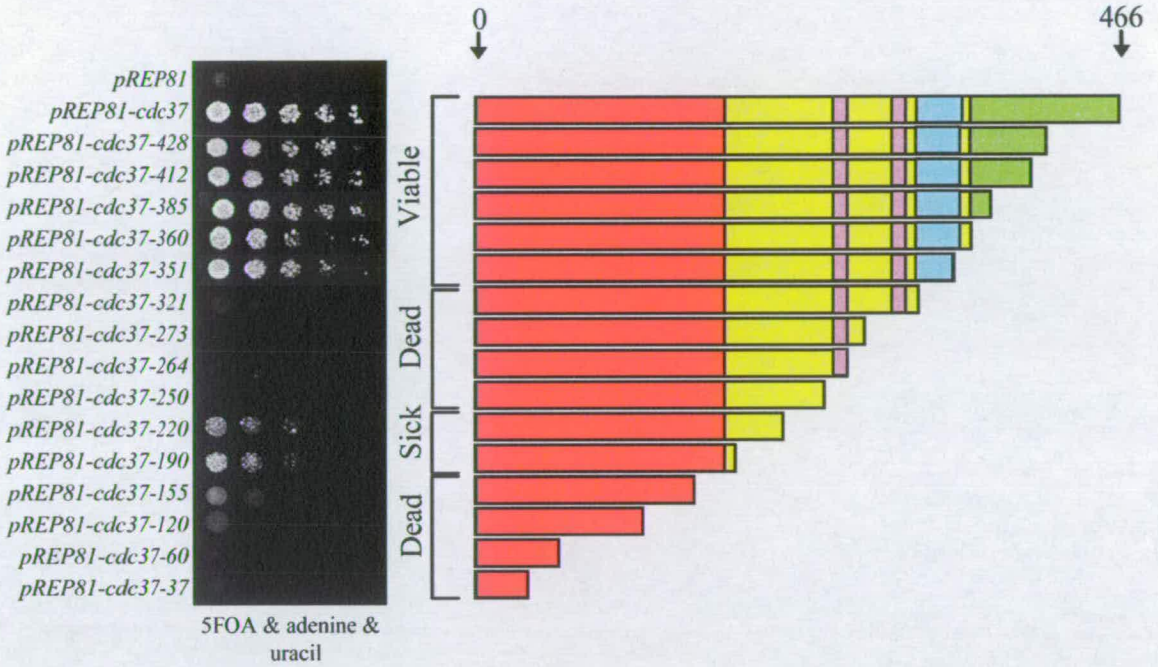
Figure 3.7: Western blot of *S. pombe cdc37⁺* strain Ed1090 expressing truncation mutants from pREP81. Protein levels of truncations compared to endogenous Cdc37 were ascertained by western blot with anti-*S. pombe* Cdc37 antibody. * indicates proteolytic truncation of endogenous Cdc37.
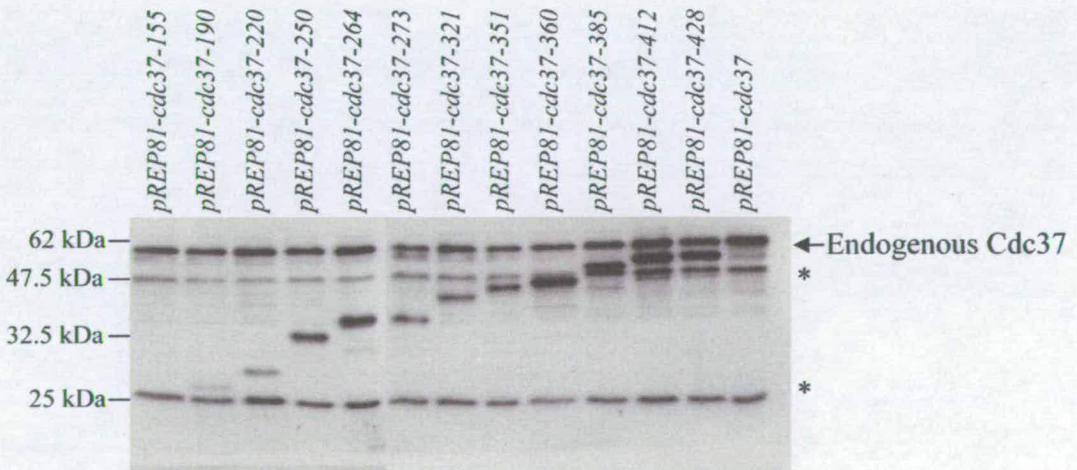
The protein levels of Cdc37 truncation mutants expressed from pREP81 were studied to determine whether reduced protein levels might be the cause for the observed phenotypes in the plasmid shuffle assay. Reduced protein levels have been observed in *S. cerevisiae* for the Cdc37 truncation mutant *cdc37-1*, which is truncated at codon 360 (Gerber et al., 1995). *S. pombe* Cdc37 truncation mutants were expressed from pREP81 in the *cdc37⁺* strain ED1090 for comparison by western blot analysis to full length endogenous Cdc37. Whole cell protein extracts were made for each mutant and loaded equally onto SDS polyacrylamide gels. Western blot analysis was carried out using the anti-*S. pombe* Cdc37 antibody. All Cdc37 truncation mutants yielded a truncated protein that was detected by western blot except for *cdc37-155* (Figure 3.7). Mutants truncated within the C-terminal and middle domains produced protein at levels very similar to endogenous Cdc37. Mutants *cdc37-190* and *cdc37-220* were the only truncations that were detected by western blot at reduced levels compared to endogenous Cdc37. This may be due to low expression levels, reduced stability of mutant protein or poor recognition by the anti-*S. pombe* Cdc37 antibody whose recognition epitopes have not been fully characterised.

3.3.3   Ability of *cdc37* truncation mutants to sustain cell viability when overexpressed

Cdc37 truncation mutants were overexpressed in the plasmid shuffle assay from the pREP1 vector to determine the effect of increasing mutant protein levels on cell viability. Overexpression of the Cdc37 mutants *cdc37-321, cdc37-273, cdc37-264* and *cdc37-250*, truncated in the middle domain, supported growth as well as expression of *pREP1-cdc37* (Figure 3.8). This was a surprising result as these mutants were unable to maintain cell viability in the plasmid shuffle when expressed from pREP81. Cdc37 truncation mutants, *cdc37-190* and *cdc37-220*, which lack the majority of the middle and all the C-terminal domains, were able to sustain growth when overexpressed (Figure 3.8). With expression from pREP81, these two Cdc37 truncation mutants only supported limited growth, most likely due to their reduced protein levels (Figure 3.8). The Cdc37 mutant *cdc37-155*, truncated in the N-terminal domain was unable to support growth even when overexpressed (Figure 3.8).

The protein levels of Cdc37 truncation mutants overexpressed from pREP1 were assayed against endogenous Cdc37 protein levels to determine whether the resulting phenotypes were due to dramatically increased mutant protein levels. Overexpression of the truncation mutant's *cdc37-155*, *cdc37-190* and *cdc37-220* yielded protein that was detected by western blot with the anti-*S. pombe* Cdc37 antibody (Figure 3.9). These data indicate that the Cdc37 *S. pombe* antibody does recognise the *cdc37-155* protein sequence. Cdc37 truncation mutants were all expressed at a level much greater than endogenous Cdc37. The truncation mutants *cdc37-190* and *cdc37-220* may be able to support cell viability when overexpressed because protein levels were greater than endogenous Cdc37 protein levels; whereas protein levels were reduced compared to the endogenous Cdc37 protein when these mutants were expressed from pREP81. The mutant *cdc37-155* is expressed at a level much greater than the endogenous but cannot sustain cell viability, indicating that the essential function of Cdc37 is abolished in this mutant. Proteolysis of Cdc37 was observed in these experiments, most notably for *cdc37-190* and *cdc37-220* (Figure 3.9). This phenomenon may be a reflection of the stability of the truncation mutant when overexpressed in this *S. pombe* strain.

3.4   Cdc37 mutants do not exhibit any dominant negative effect

All *cdc37* mutants previously described were overexpressed from pREP1 in the *S. pombe cdc37*[+] strain ED1090 to look for any dominant negative effect. A dominant negative effect may occur when a mutant version of a protein inhibits the endogenous protein in some manner negatively affecting the cell. Overexpression in ED1090 of the Cdc37 truncation and in-frame insertion mutants did not negatively effect growth or alter morphology at 25, 28, 32 and 36 °C, except for the mutant *cdc37-321*, whose overexpression resulted in slightly elongated cells (data not shown). The *cdc37-321* truncation mutant is truncated in the 6-helix bundle around the homodimerisation domain identified in human Cdc37 (Roe et al., 2004). A truncation in this region may

Figure 3.8: A schematic diagram of the Cdc37 truncation mutants overexpressed from pREP1 in the *cdc37Δ* strain ED1526. Strains were plated onto 5FOA to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C (shown here at 28 °C, behaviour did not differ at other temperatures).
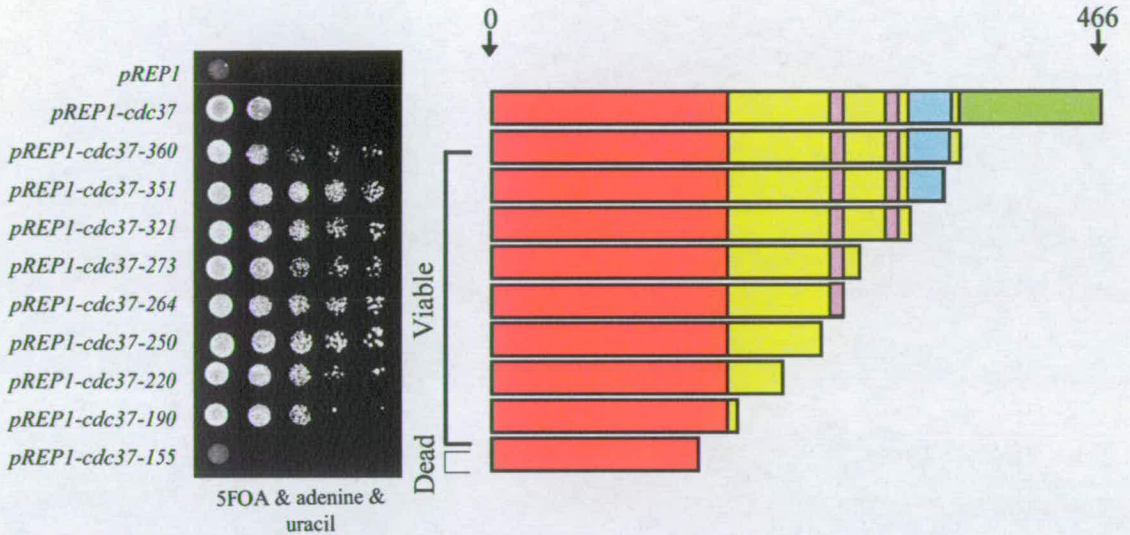
Figure 3.9: Western blot of *S. pombe* wildtype strain Ed1090 overexpressing truncation mutants *pREP1-cdc37-155*, *pREP1-cdc37-190* and *pREP1-cdc37-220*. Protein levels of truncations were compared to endogenous Cdc37 by western blot with Cdc37 antibody. * indicates proteolytic truncation of endogenous Cdc37.
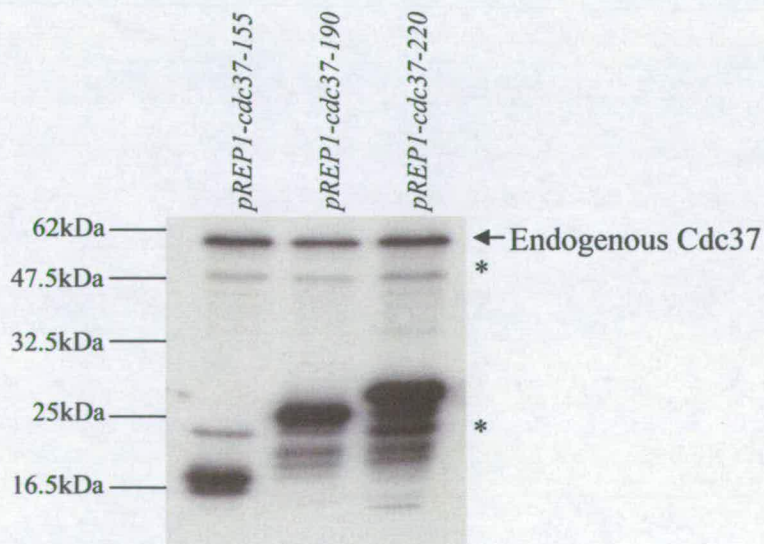
negatively affect structure and function of Cdc37, but still permit a level of dimerisation, titrating endogenous Cdc37 away and preventing proper function.

3.5 Discussion

A range of truncation and in-frame insertion mutants of *cdc37* were generated using *in vitro* pentapeptide transposition mutagenesis and directed mutagenesis and were tested for their ability to support cell viability. The aim of these experiments was to identify the domains essential for Cdc37 to sustain cell viability in *S. pombe*. The domains identified in human Cdc37 (Shao et al., 2003) were mapped onto *S. pombe* Cdc37 and used as a guide line for the predicted domains of the *S. pombe* Cdc37 protein.

The in-frame insertion of five amino acids into the C-terminal or N-terminal region in mutant's *cdc37-I120*, *cdc37-I386* and *cdc37-I422* did not negatively affect Cdc37 function. The mutant *cdc37-I252* containing an in-frame insertion at residue 252 in the middle domain abolished Cdc37 function and was unable to sustain viability. The introduction of five extra amino acids in *cdc37-I252* may have significantly altered the conformation of Cdc37, inhibiting function of the molecular chaperone, as the insertion lies in the large 6-helix bundle (Roe et al., 2004). An insertion at this point in Cdc37 may prevent proper folding and conformation from being achieved thus abrogating function. Alternatively, the insertion may lie in or near to an essential functional domain that is negatively affected by the presence of five extra amino acids. The alignment of human and *S. pombe* Cdc37 indicates that the *cdc37-I252* insertion is located between the two regions identified as important for Hsp90 binding, just upstream of the putative dimerisation region (Roe et al., 2004) and may negatively affect these interactions.

In accordance with observations in *S. cerevisiae* (Lee et al., 2002), the C-terminal domain of *S. pombe* Cdc37 was found to be dispensable for essential function. In *S. cerevisiae* the Cdc37 truncation mutant Cdc37$^{1-388}$, which lacks the C-terminal domain, was able to restore v-src activity as well as full length Cdc37 in the *S.*

*cerevisiae sti1*Δ mutant (Lee et al., 2002). Interestingly, the shorter truncation mutant, *cdc37-1*, truncated at codon 360, supports cell viability in a *cdc37*Δ in a temperature dependent manner (Gerber et al., 1995). Overexpression of this *S. cerevisiae* truncation mutant is able to support a greater level of growth at the restrictive temperature (Gerber et al., 1995).

In *S. cerevisiae* the truncation mutant Cdc37[1-355] which lacks the latter part of the middle domain and the entire C-terminal domain was unable to restore cellular viability in a *cdc37*Δ strain whether expressed at low or high level (Lee et al., 2002). In contrast, the equivalent *S. pombe* mutants, *cdc37-321, cdc37-273, cdc37-264* and *cdc37-250*, which are truncated within the middle domain, around the putative Hsp90-binding and homodimerisation domains, were able to sustain cellular viability, but only when overexpressed. These mutants were lethal in the plasmid shuffle with expression from pREP81, most likely due to reduced function as protein levels were very similar to endogenous Cdc37. Increasing the protein level of these truncation mutants, may raise the overall function above a threshold level so that cell viability is maintained. Reduced function of these Cdc37 truncation mutants may be because the N-terminal domain is titrated away from carrying out essential roles by the aberrant middle domain attempting, for example to interact with Hsp90 or homodimerise. Alternatively, the folding of Cdc37 may be perturbed, as these mutants are truncated in the 6-helix bundle identified in human Cdc37 (Roe et al., 2004), which could negatively affect protein structure. Disruption of the protein structure in this region might affect essential functions and/or interactions of Cdc37, possibly disrupting the N-terminal domain and its interactions with client proteins.

Truncation mutants *cdc37-190* and *cdc37-220*, which lack the majority of the middle and all of the C-terminal domains which include the Hsp90-binding and homodimerisation regions, were able to sustain cell viability when overexpressed. Expression of these mutants from pREP81 supported limited growth, but overexpression of *cdc37-190* and *cdc37-220* was able to sustain growth as well as expression of *pREP1-cdc37*. These results differ from observations in *S. cerevisiae* which showed that overexpression of the truncation mutants Cdc37[1-148] and Cdc37[1-

, also truncated around the N-terminal and middle domain boundary, permitted slow growth in a temperature dependent manner in a *cdc37Δ* strain (Lee et al., 2002). Protein levels of *S. pombe cdc37-190* and *cdc37-220* expressed from pRP81 were reduced compared to endogenous Cdc37, which may explain why these mutants could only support limited growth. Growth was fully restored when these Cdc37 truncation mutants were overexpressed, increasing protein levels above endogenous Cdc37. These data indicate that only the N-terminal domain is required for the essential function of Cdc37 in *S. pombe*, providing their protein levels are high enough.

Expression of Cdc37 mutants truncated within the N-terminal domain from pREP81 were unable to sustain cell viability. Cell viability could not be maintained by *cdc37-155*, even when protein levels were greater then endogenous Cdc37. Truncations from the C-terminus into the N-terminal domain of Cdc37 completely abolish essential function. The N-terminal domain appears to be the domain essential for Cdc37 function to sustain cell viability. N-terminal truncation mutants of *S. pombe* Cdc37, lacking the first 20 or 40 amino acids, were unable to maintain cell viability even when over-expressed on pREP1 in a plasmid shuffle (from Dr Ina Martin personal communication). The highly conserved N-terminal domain is essential for Cdc37 function as it interact directly with the client proteins (Shao et al., 2003).

In summary, the middle and C-terminal domains of *S. pombe* Cdc37 are completely dispensable for growth, when protein levels are not a limiting factor. Expression of only the N-terminal domain is sufficient to maintain essential Cdc37 function. This is surprising as these mutants do not contain the Hsp90-binding or homodimerisation domains, indicating that these functions may not be essential for Cdc37 in *S. pombe*. Truncations within the N-terminal domain were unable to sustain growth at low or high expression levels.

Chapter 4:

Mutational Analysis of the N-terminal

Domain of *S. pombe* Cdc37

4.0   Mutational Analysis of the N-terminal Domain of S. pombe Cdc37

4.1   Introduction

The first 40 amino acids of Cdc37 are the most highly conserved across species (Figure 4.1) and have been the focus of many mutational studies. Mutation of serine 14 in *S. cerevisiae* to a leucine gave rise to *cdc37-34* a temperature-sensitive mutant (Dey et al., 1996). Further mutational analysis of the conserved N-terminal serine residues identified serines 14 and 17 of *S. cerevisiae* (Bandhakavi et al., 2003) and serine 13 of rat (Miyata and Nishida, 2004) and human (Shao et al., 2003) (equivalent to *S. cerevisiae* serine 14) as important sites of phosphorylation.

Human Cdc37 is phosphorylated *in vivo* and mutating serine 13 to alanine inhibits this (Shao et al., 2003). A recombinant C-terminal truncation of human Cdc37 was phosphorylated *in vitro* by casein kinase II (CKII) (Shao et al., 2003). However, CKII was unable to phosphorylate N-terminal deletions of cdc37 *in vitro* (Miyata and Nishida, 2004) or mutant versions of human Cdc37 where serine 13 was substituted with an alanine (Miyata and Nishida, 2004). Cdc37 serine 13 in rat and human and serines 14 and 17 in *S. cerevisiae* are the major sites of phosphorylation by CKII. Phosphorylation of these N-terminal serine residues is important for Cdc37 function (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003). This has lead to the formation of the hypothesis that CKII and Cdc37 are involved in a feedback loop of activation, whereby CKII activates Cdc37 by phosphorylation and Cdc37 maintains CKII activation (Bandhakavi et al., 2003). When cells were treated with TBB, a specific inhibitor of CKII, Cdc37 phosphorylation was reduced and protein levels of the Cdc37 clients MOK and Raf-1 decreased (Miyata and Nishida, 2004). The non-phosphorylated form of human Cdc37 was also found to have reduced binding affinity for clients Akt, aurora-B, cdk4 and MRK (Miyata and Nishida, 2004).

Figure 4.1: An alignment of the first 40 amino acids of human, *S. cerevisiae* and *S. pombe* Cdc37. Serine residues that are conserved among Cdc37 homologues and sites of phosphorylation in human and *S. cerevisiae* Cdc37 are highlighted in pink.
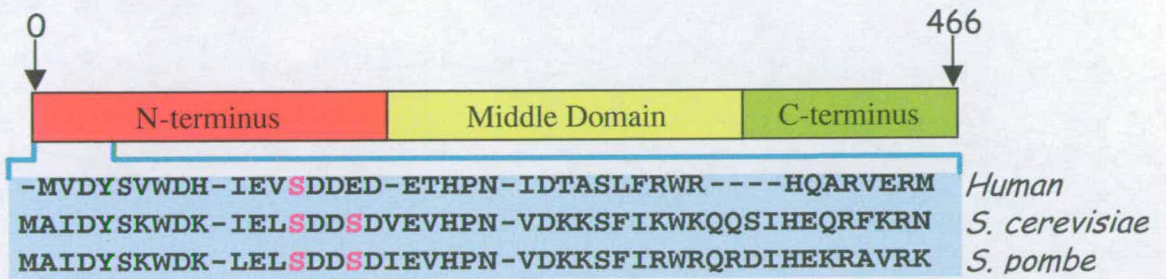
This chapter describes how a mutation, equivalent to the *S. cerevisiae* temperature-sensitive mutation *cdc37-34,* was introduced into the *S. pombe cdc37* gene with the aim of generating a temperature-sensitive mutant. However, this mutation proved to be lethal in *S. pombe* and this prompted a more detailed investigation of the conserved N-terminal serine residues of Cdc37 with the aim of identifying putative phosphorylation sites. Further work was also carried out to develop an assay to determine the phosphorylation status of *S. pombe* Cdc37.

4.2   Mutational analysis of the N-terminal serine residues of *S. pombe* cdc37

4.2.1   Generating a *S. pombe* mutation equivalent to the *S. cerevisiae* temperature-sensitive mutant *cdc37-34*

The temperature-sensitive mutant *cdc37-34* in *S. cerevisiae* was isolated by its ability to relieve the toxic effect of over-expressed $pp60^{v-src}$ (Dey et al., 1996). The temperature-sensitive mutant *cdc37-34* contains a single point mutation of serine 14 to leucine (Dey et al., 1996). Mutation of the equivalent serine 14 in *S. pombe* to leucine was carried out using Stratagene Quick change™ site directed mutagenesis (Section 2.3.5) on the *cdc37* gene in the vector pREP81 generating the mutant *cdc37-S14L*. The mutant *cdc37-S14L* was expressed from pREP81 by plasmid shuffle assay to determine its ability to sustain cell viability at different temperatures. *pREP81-cdc37-S14L* was unable to promote growth at 25, 28, 32 or 36 °C (Figure 4.2), and cells were morphologically heterogeneous, being long, rounded and bowling pin shaped, characteristic of depletion of Cdc37 from cells as described in (Westwood et al., 2004).

Lethality of the *cdc37-S14L* mutant expressed from pREP81 may be due to instability of the mutant protein, as observed with some truncation mutants described in Chapter 3. Therefore *Cdc37-S14L* was overexpressed from pREP1 in the *S. pombe* strain ED1526 and assayed by plasmid shuffle to determine whether increasing the level of

Figure 4.2: Expression of the mutants *cdc37-S14L* and *cdc37-S1417A* from pREP81 to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C.
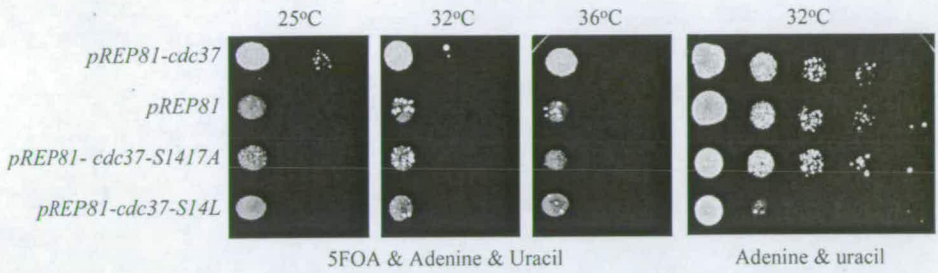
Figure 4.3: Overexpression of the mutants *cdc37-S14L* and *cdc37-S14A* from pREP1 to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C. **A** Strains were plated as serial dilutions and incubated at different temperatures for 4 days and **B** the cellular morphology of those strains on 5FOA.
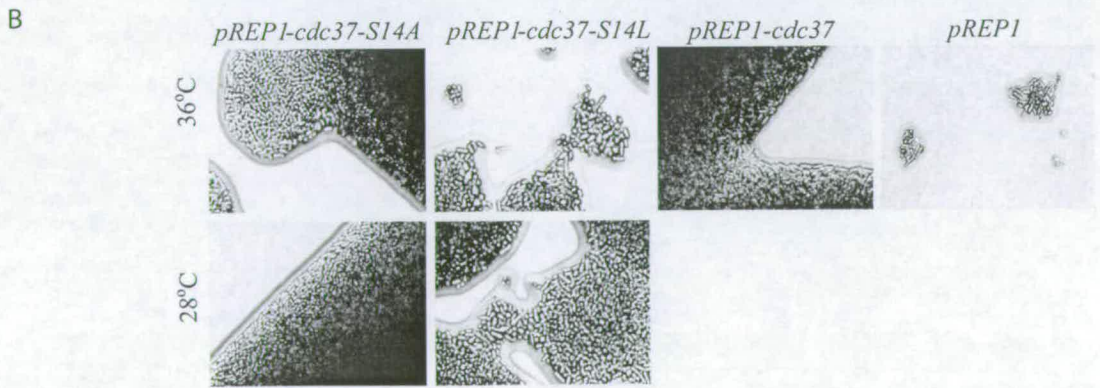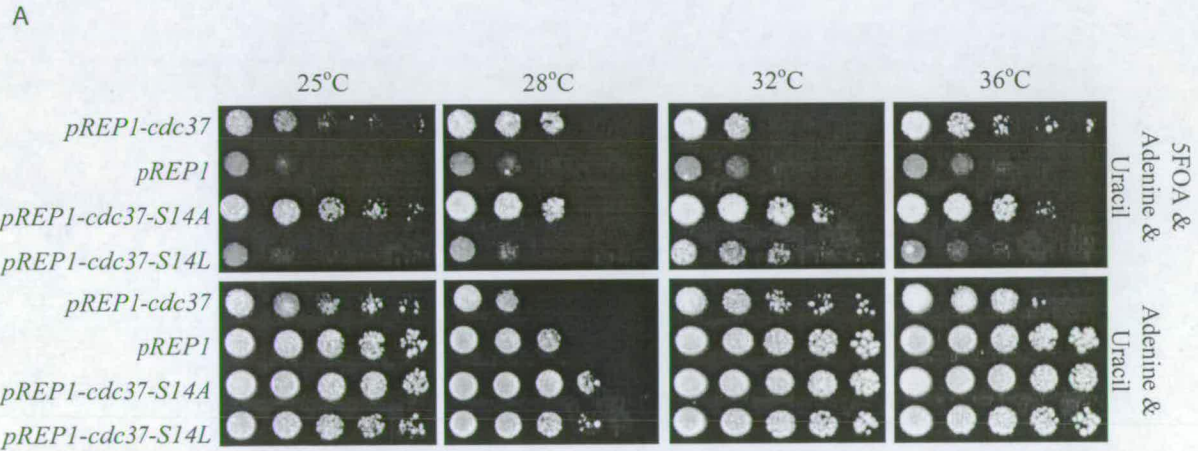
mutant protein could sustain cell viability. Overexpression of *cdc37-S14L* by plasmid shuffle assay resulted in a weak temperature-sensitive phenotype (Figure 4.3). A low level of growth was observed at 25, 28 and 32 °C but not at 36 °C. At all temperatures cells were morphologically heterogeneous, displaying phenotypes characteristic of depletion of Cdc37 from cells as described in (Westwood et al., 2004). These data indicate that Cdc37 function is not completely abolished by the mutation of serine 14 to leucine. Increasing the protein levels of this mutant, raising the level of activity above a threshold in the cell can sustain viability in a temperature dependent manner.

4.2.2 Mutational analysis of the extreme N-terminal serine residues of Cdc37

The first two N-terminal serine residues (6 and 14) are conserved in all Cdc37 homologues, and yeasts contain a third conserved serine residue at amino acid 17. The *S. cerevisiae* serine residues 14 and 17 and the rat and human serine 13 are phosphorylated by CKII and are important for Cdc37 function (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003). To investigate further the role of N-terminal serine residues in *S. pombe* Cdc37, mutational studies were carried out to determine whether these serine residues are of functional importance. Site directed mutagenesis by overlap PCR (Section 2.3.5) was used to mutate serines 14 and 17 to alanine, both independently and together. The mutation of serine 6 to alanine was introduced using a modified 5' oligonucleotide in a standard PCR reaction (Section 2.3.3.2). Mutants were transformed into the *S. pombe* strain ED1526 and expressed from pREP81 to determine their ability to sustain cell viability by plasmid shuffle assay. Independent mutations of serine residues 6 and 17 to alanine did not affect cell viability at any temperature (Figure 4.4) and cells were phenotypically wildtype in appearance. *cdc37-S1417A* (Figure 4.2) and *cdc37-S14A* (Figure 4.4) were unable to maintain cell viability at 25, 28, 32 and 36 °C. Both of these mutants gave rise to a population of cells that were either small and round or 4-5 fold longer than wildtype cells. These data indicate that serine 14 is important for Cdc37 to maintain cell viability in *S. pombe*, but serines 6 and 17 are not.

Figure 4.4: Expression of *cdc37* mutants *cdc37-S6A*, *cdc37-S14A* and *cdc37-S17A* from pREP81 to determine their ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C.
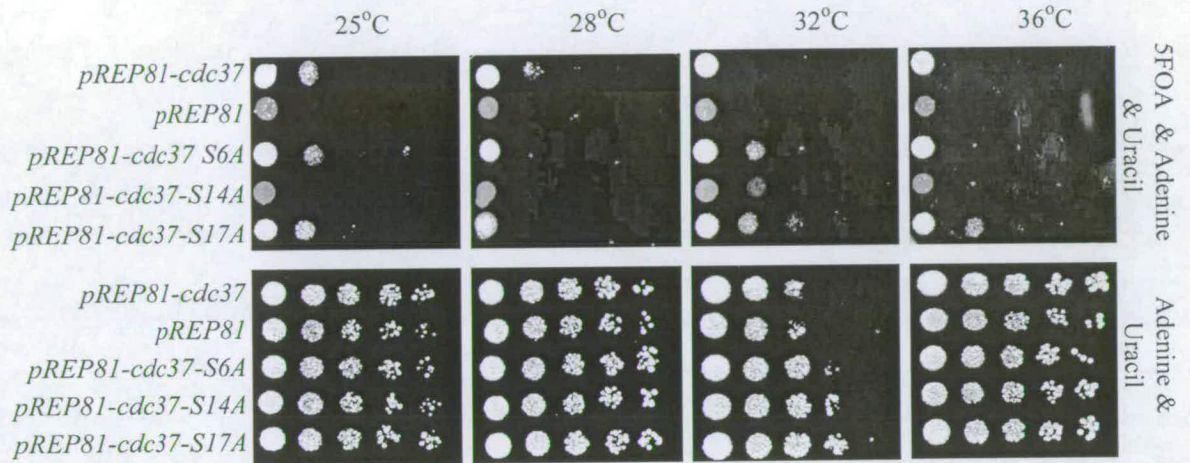
Cdc37-S14A was overexpressed to establish whether raising mutant protein levels could sustain cellular viability by plasmid shuffle assay, as overexpression of *cdc37-S14L* by plasmid shuffle enabled growth in a temperature dependent manner. Overexpression of *cdc37-S14A* from pREP1 in ED1526 was able to support growth as well as expression of *pREP1-cdc37* and cells were phenotypically wildtype in appearance at 25, 28, 32 and 36 °C (Figure 4.3). Therefore, mutating serine 14 to alanine, does not completely abrogate Cdc37 function, as increasing the mutant protein level which raises the overall cellular level of *Cdc37-S14A* activity sustains cell viability.

4.2.3 Mimicking phosphorylation of Cdc37 by mutating serine 14 to glutamic acid

Phosphorylation of rat Cdc37 by CKII *in vitro* was inhibited by mutating serine 13 (Miyata and Nishida, 2004). CKII preferentially phosphorylates motifs that are enriched up to four codons away on either side of the phosphorylatable residue with glutamic acid and aspartic acid (Kuenzel et al., 1987; Songyang et al., 1996). Specifically, CKII was found to favour peptides with aspartic acid at positions −3 and +2 (Songyang et al., 1996). Serine 14 in *S. pombe* aligns with *S. cerevisiae* serine 14 and rat and human serine 13, residues known to be phosphorylated by CKII. *S. pombe* Cdc37 serine 14 is a candidate for CKII phosphorylation as this codon is flanked on either side by acidic residues. Serine 14 has a glutamic acid at -2 and 3 aspartic acid amino acid residues at +1, +2 and +4.

Serine 14 was substituted for a negatively charged glutamic acid residue which can in some instances mimic for a phosphoserine, as seen with Wis1 (Samejima et al., 1997). Overlap PCR was used to mutate Serine 14 to glutamic acid producing *pREP81-cdc37-S14E*. This mutant allele was expressed from pREP81 by plasmid shuffle assay to determine its ability to sustain cell viability. Expression of *cdc37-S14E* from pREP81 was able to support a low level of growth, greater than observed for the expression of just the *pREP81* vector (Figure 4.5A). Expression of the mutant *cdc37-S14E* in the plasmid shuffle produced cells that were phenotypically heterogeneous at

all temperatures, being elongated, rounded and bowling pin shaped cells (Figure 4.5B). Although cells were morphologically aberrant in appearance, the mutation of serine 14 to glutamic acid supported a greater level of growth than observed with cdc37-S14L and cdc37-S14A expressed from pREP81 in the plasmid shuffle assay. Glutamic acid may not be an effective mimic for phosphoserine which is why cells expressing the cdc37-S14E mutants are phenotypically abnormal in appearance. These data indicate that Cdc37 serine 14 may be a site of phosphorylation in *S. pombe*.

The effect of increasing cdc37-S14E protein levels was investigated, as it had been previously shown that increasing the levels of certain Cdc37 mutant proteins within the cell can promote a greater level of growth. cdc37-S14E was over-expressed from pREP1 in ED1526 by plasmid shuffle to observe the ability of this mutant construct to sustain cell viability with increased protein levels. Expression of *pREP1-cdc37-S14E* was able to support growth in the plasmid shuffle as well as expression of *pREP1-cdc37* (Figure 4.6). Cells were phenotypically aberrant compared to those expressing *pREP1-cdc37*, being slightly rounder and smaller in appearance compared to a wildtype cell. Overexpression of cdc37-S14E does not affect the level of growth but does negatively affect cell morphology. These data indicate that glutamic acid may be a poor mimic for Cdc37 phosphoserine at residue 14 in this instance, inhibiting optimum function of this molecular chaperone.

4.3   Overexpression of Cdc37 serine mutants to test for any dominant negative effect

cdc37-S14A, cdc37-S14E, cdc37-S14L and cdc37-S1417A, described collectively as serine 14 mutants, were overexpressed in the cdc37[+] strain ED1090 to observe whether they produced a dominant negative effect. Serine 14 mutants were expressed from pREP1 in ED1090 and incubated at 28 and 36 °C. All strains were able to promote growth and did not exhibit any dominant negative effect. All strains were phenotypically wildtype in appearance at both temperatures.

Figure 4.5: Expression of the mutant *cdc37-S14E* from pREP81 to determine its ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C. **A** Growth of strains plated as serial dilutions on media with and without 5FOA and **B** **the c**ellular morphology of strains in the plasmid shuffle assay.
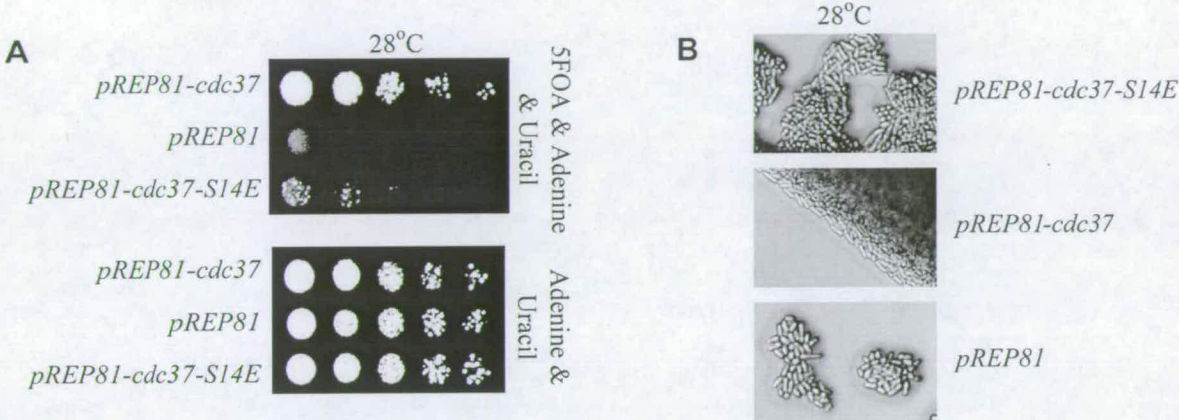
Figure 4.6: Overexpression of the mutant *cdc37-S14E* from pREP1 to determine its ability to sustain cell viability by plasmid shuffle assay at 25, 28, 32 and 36 °C.
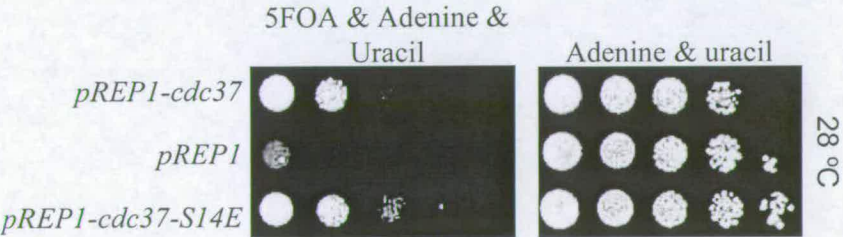
4.4   Protein levels in Cdc37 serine 14 mutants

The protein levels of serine 14 mutants of Cdc37 expressed from pREP81 were assayed to test whether phenotypes observed in the plasmid shuffle were due to specific mutations or reduced expression of Cdc37 mutants. Cdc37 and serine 14 mutants were N-terminally tagged with HA to assay protein levels by western blot, as serine mutants are single point mutations that cannot be distinguished by electrophoretic mobility from endogenous Cdc37. Whole cell extracts of denatured protein were made of each mutant and run on SDS PAGE. Western blot with an anti-HA antibody was carried out to observe Cdc37 serine mutant protein levels. The TAT1 antibody was also used in western blots to detect for *S. pombe* tubulin as a loading control. Western blot results indicate that serine 14 mutants do not differ in protein level from wildtype Cdc37 (Figure 4.7) indicating that observed phenotypes are not due to reduced mutant protein.

To verify that the N-terminal HA-tag did not have any negative effect on Cdc37 and serine 14 mutants, constructs were tested in the plasmid shuffle assay expressed from pREP81 to observe their affect on cell viability. *pREP81-HA-cdc37* behaved like wildtype in the plasmid shuffle with no obvious defects in phenotype (Figure 4.8). At 25, 28, 32 and 36 °C expression of *pREP81-HA-cdc37-S14A* and *pREP81-HA-cdc37-S14L* was unable to support growth and cells were heterogeneous in phenotype as previously described for these mutants without the N-terminal tag (data not shown). *pREP81-HA-cdc37-S14E* was able to support a low level of growth consisting of cells that were heterogeneous in phenotype as was previously observed for this mutant without the HA-tag. The phenotypes observed by plasmid shuffle for these mutants are the same as those seen in the absence of an N-terminal HA tag, indicating that the tag has no negative effect on Cdc37 function.

Figure 4.7: Comparison of protein levels of N-terminally HA-tagged Cdc37 and serine 14 mutants expressed from pREP81 in ED1526 at 28 °C. Whole cell extracts were made, run on SDS PAGE and western blot were carried out with anti-HA antibody and the TAT1 antibody as a loading control.
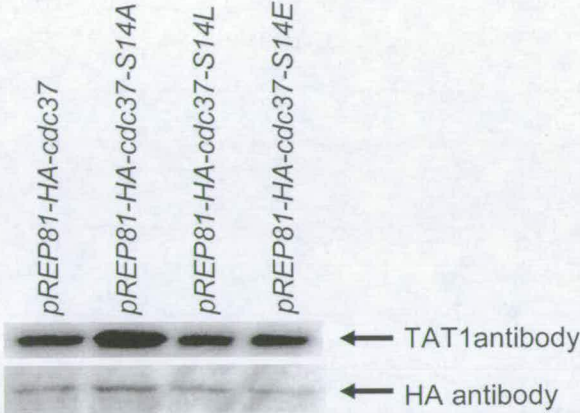
Figure 4.8: Expression of N-terminally HA-tagged *cdc37*, *S14A*, *S14L* and *S14E* from pREP81 to determine their ability to sustain cell viability by plasmid shuffle assay at 28, 32 and 36 °C.
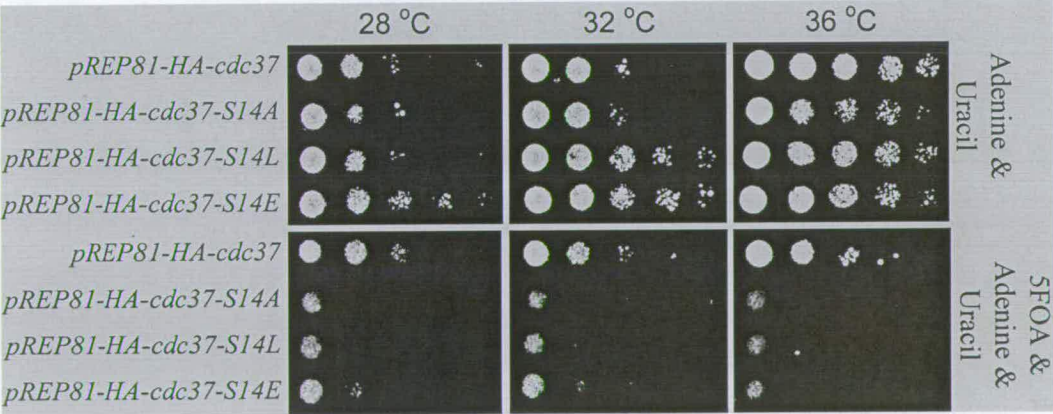
4.5 The mobility of Cdc37 and serine mutant proteins do not differ on SDS PAGE gels as a method of assaying phosphorylation status

To investigate further phosphorylation of Cdc37 in *S. pombe* a biochemical approach was taken. Phosphorylation can alter the mobility of a protein by gel electrophoresis by SDS PAGE, as has been shown for CKII (Litchfield et al., 1992). To date, no difference in mobility by gel electrophoresis due to phosphorylation has been reported for Cdc37 from rat, human and *S. cerevisiae* by western blot analysis. To determine whether the mobility of Cdc37 differed by gel electrophoresis due to phosphorylation, serine 14 mutants and wildtype Cdc37 protein samples were run by SDS PAGE for comparison. Wildtype Cdc37 and serine mutants *cdc37-S14A*, *cdc37-S14L*, *cdc37-S14E* and *cdc37-S1417A* were expressed from pREP1 in the *cdc37⁺* strain ED1090. Whole cell protein extracts were made and run on large SDS PAGE gels overnight at 4 °C to obtain high resolution. Western blot analysis was carried out using the anti-*S. pombe* Cdc37 antibody. The observed migration of Cdc37 and serine mutants did not differ as judged by western blot (Figure 4.9).

4.5.1 Treatment with phosphatase does not alter molecular weight of Cdc37

Overexpression of Cdc37 and serine 14 mutants is a crude technique to identify differences in mobility due to phosphorylation. A more elegant approach was undertaken, treating endogenous Cdc37 with phosphatase to determine whether dephosphorylation of Cdc37 can be observed by differences in mobility by SDS PAGE. Previously whole cell protein extracts were treated with both Lambda phosphatase and Calf intestinal phosphatase (CIP) and samples were run by SDS PAGE to look for any differences in mobility by gel electrophoresis due to phosphorylation. However, these experiments were unsuccessful in detecting any difference in mobility and were further complicated as CIP runs at exactly the same place as Cdc37 by SDS PAGE. To eliminate these issues and other factors in crude protein extracts that could affect the phosphatase reaction, Cdc37 was

Figure 4.9: Western blot with anti-*S. pombe* antibody of wildtype Cdc37 and serine mutants expressed from pREP1 in the *S. pombe* strain ED1090 to look at differences in mobility due to phosphorylation. SDS polyacrylamide gel was stained with Coomassie as a loading control.
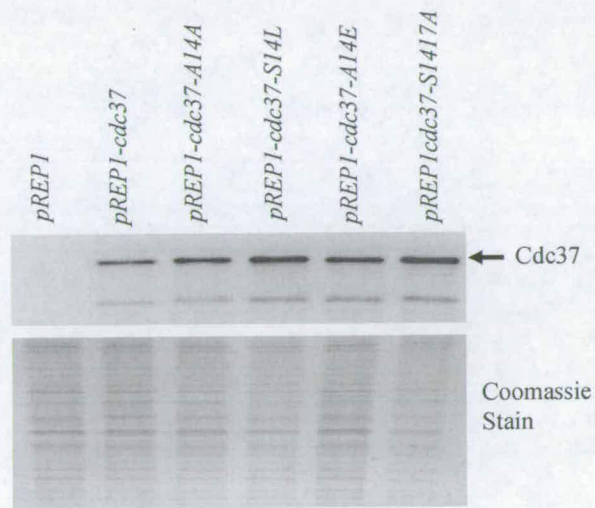
immunoprecipitated (Section 2.5.7) using the anti-*S. pombe* Cdc37 antibody and then treated with either Lambda phosphatase or CIP (Section 2.5.6). All samples were loaded onto a 10% polyacrylamide gel interspersed with untreated native protein extracts from ED1090 for accurate comparison of Cdc37 mobility. Gels were run overnight at 4 °C for high resolution and western blots were carried out with the anti-*S. pombe* Cdc37 antibody. No difference in the mobility of phosphatase treated Cdc37 compared to the endogenous Cdc37 was observed under any phosphatase treatment conditions (Figure 4.10 A & B). These data indicate that dephosphorylation of Cdc37 in *S. pombe* cannot be detected by differences in mobility by electrophoresis on SDS PAGE gels.

4.6   Discussion

Mutational analysis of serine 14 in *S. pombe* Cdc37 has shown that this residue is important for Cdc37 function to maintain cell viability. However, it has not been established how this amino acid contributes to Cdc37 function. Theses data indicate that phosphorylation of serine 14 in Cdc37 might occur as *pREP81-cdc37-S14E* was able to support a low of level of growth whereas *pREP81-cdc37-S14A* and *pREP81-cdc37-S14L* could not. The mutation of serine 14 to glutamic acid to mimic phosphorylation may not be a complete substitute and overexpression of this mutant was still unable to rescue the morphologically aberrant appearance of the cells. Attempts to determine the phosphorylation status of Cdc37 by SDS PAGE were unable to identify any difference in mobility between wildtype Cdc37 and serine mutants. Treatment of endogenous Cdc37 with phosphatases did not produce any difference in mobility by SDS PAGE.

Mutational analysis of the conserved N-terminal residues serines 6 and 17 to alanine did not affect cell viability in *S. pombe*, indicating that serines 6 and 17 did not affect Cdc37 function *in vivo*. In accordance with findings in *S. cerevisiae*, the *S. pombe* mutant *cdc37-S1417A* was unable to support growth by plasmid shuffle assay. In *S. cerevisiae, cdc37-S1417A* exhibited reduced growth with a doubling time of 10 hours compared to 90 minutes for a wildtype strain (Bandhakavi et al., 2003). The *S. pombe*

Figure 4.10 A: Lambda phosphatase treatment of immunoprecipitated endogenous Cdc37 from the *cdc37*⁺ strain ED1022 using anti-*S. pombe* Cdc37 antibodies. Western blot analysis was carried out with the anti-*S. pombe* Cdc37 antibody.
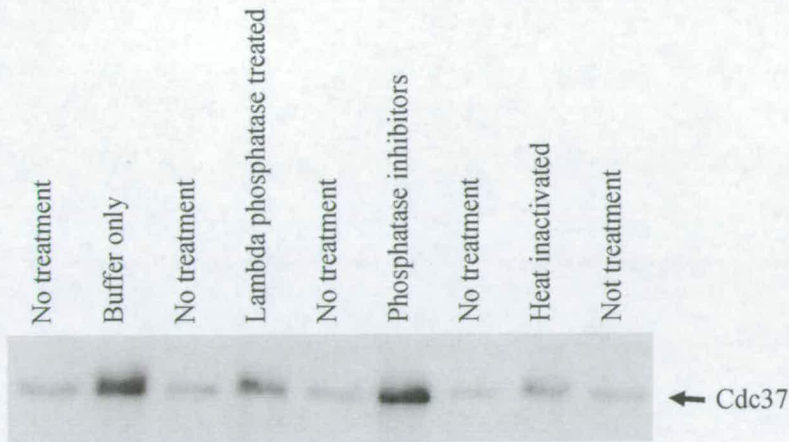
Figure 4.10 B: CIP phosphatase treatment of immunoprecipitated endogenous Cdc37 from the *cdc37*⁺ strain ED1022 using anti-*S. pombe* Cdc37 antibodies. Western blot analysis was carried out with the anti-*S. pombe* Cdc37 antibody.
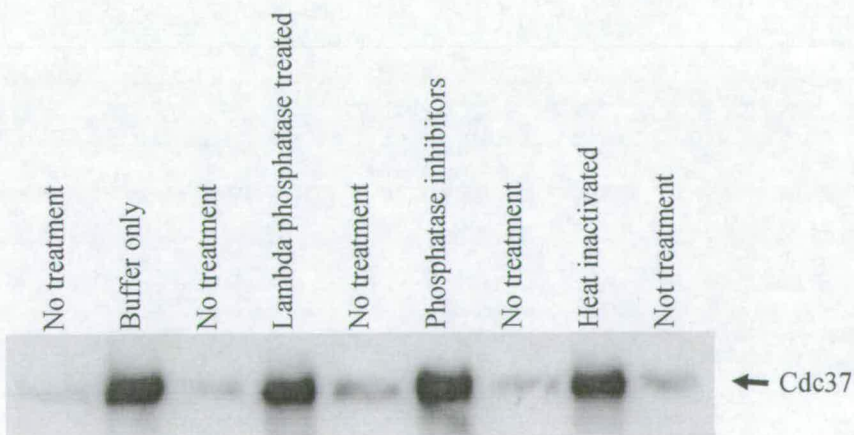
mutant *pREP81-cdc37-S1417A* cannot maintain cell viability by plasmid shuffle due to the mutation of serine 14 and not serine 17, as expression of *pREP81-cdc37-S17A* maintains cell viability by plasmid shuffle in *S. pombe* but *pREP81-cdc37-S14A* cannot. In *S. cerevisiae cdc37-S14A* had more severe growth and morphological defects than *cdc37-S17A*, although both are important sites of phosphorylation by CKII (Bandhakavi et al., 2003).

Consistent with findings in *S. cerevisiae* (Bandhakavi et al., 2003), mutating serine 14 to alanine of *S. pombe* Cdc37 abrogated function so that this mutant could not sustain cell viability in the plasmid shuffle when expressed from pREP81. Expression of *cdc37-S14A* in *S. pombe* was unable to sustain cell viability unless over-expressed from pREP1, when it could support growth as well as expression of *pREP1-cdc37*. Expression of Cdc37 with serine 14 substituted for leucine to recreate *S. cerevisiae* mutation *cdc37-34* in *S. pombe*, was lethal when expressed from pREP81, but supported growth in a temperature dependent manner when overexpressed. These findings indicate that serine 14 to alanine or leucine does not completely abolish Cdc37 function and overexpression of these Cdc37 mutants raises the level of mutant protein, increasing overall cellular activity which can sustain cell viability.

These data indicate that Serine 14 is important for Cdc37 function *in vivo*. The mechanism by which serine 14 is important for Cdc37 function to sustain cell viability is not understood, but there is a high probability from data in other systems that it is a vital site of phosphorylation (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003). Alternatively, mutating serine 14 to alanine or leucine may alter conformation, possibly causing instability of the chaperone protein. This may reduce Cdc37 function to a very low level that cannot sustain cell viability, unless the mutant is overexpressed raising essential function above a threshold level.

Expression of *cdc37-S14E* from pREP81 only partially restored the essential function required by Cdc37 to sustain cell viability. Over-expression of Serine 14 to glutamic acid is able to support significant growth but cells are morphologically aberrant. The equivalent *S. cerevisiae* Cdc37 mutant *S1417E* has a significantly greater growth rate

with a doubling time of 4 hours compared to 10 hours for S1417A (Bandhakavi et al., 2003). In *S. pombe pREP81-cdc37-S14E* was able to support greater growth than *pREP81-cdc37-S14A* and *pREP81-cdc37-S14L*. These data imply that glutamic acid can partially substitute for phosphoserine when *cdc37-S14E* is expressed from pREP81. This has also been observed in *S. cerevisiae* where morphological defects for *cdc37-S14,17E* were significantly fewer than observed for *cdc37-S14,17A* (Bandhakavi et al., 2003). However, in *S. pombe* overexpression of *cdc37-S14A* results in viable cells that were wildtype in appearance, but overexpression of *cdc37-S14E* supported growth of cells that are morphologically abnormal. Glutamic acid may not be a complete substitute for mimicking phosphorylation of Cdc37 serine 14 *in vivo* and is therefore unable to restore full function to the molecular chaperone. However, the ability of *cdc37-S14A* to support a wildtype phenotype in the plasmid shuffle when overexpressed indicates that if this amino acid is phosphorylated in *S. pombe*, it is not essential for Cdc37 function and cell viability.

Development of an assay to determine phosphorylation status of Cdc37 by SDS PAGE gel was not accomplished. No difference in Cdc37 mobility was observed between wildtype Cdc37, serine mutants and wildtype Cdc37 treated with phosphatases.

Chapter 5:

Temperature-sensitive mutants of

S. pombe cdc37

5.0   Temperature-sensitive mutants of *S. pombe cdc37*

5.1   Introduction

The concept of a temperature-sensitive mutant was first presented by (Horowitz and Leupold, 1951) for Neurospora. The first temperature-sensitive conditional lethal mutants identified in *S. pombe* were generated by mutagenic treatment with nitrous acid resulting in strains that grew at 25 but not at 37 °C (Bonatti et al., 1972). Temperature-sensitive generally refers to a heat-sensitive mutant with a low permissive temperature and a higher restrictive temperature, but cold-sensitive mutants also exist whose restrictive temperatures are lower then their permissive. Temperature-sensitive mutants contain a mutation within a gene that permits protein function at permissive temperatures but not at restrictive by causing instability and/or inactivation of the protein. Temperature-sensitive mutants are valuable tools for studying the cellular role of a protein by carrying out detailed analysis of the defects that arise at the restrictive temperature. Temperature-sensitive mutants of *CDC37* have been generated in *S. cerevisiae* and have been valuable in gaining a better understanding of the Cdc37 molecular chaperone in this system (Dey et al., 1996; Gerber et al., 1995; Mort-Bontemps-Soret et al., 2002; Reed, 1980; Reed, 1980; Valay et al., 1995).

Previous studies of Cdc37 in *S. pombe* showed that depletion of the Cdc37 protein in shut-off experiments resulted in a phenotypically heterogeneous cell population that ceased to divide after ~36 hours (Westwood et al., 2004). The aims of the present study were to generate *cdc37* temperature-sensitive mutants to observe the effect of rapid loss of Cdc37 function in *S. pombe*. In this study three *cdc37* temperature-sensitive mutants, *cdc37-184*, *cdc37-13* and *cdc37-J*, were generated and analysed in parallel with a fourth, *cdc37-681*, which was a kind gift from the Shiozaki Lab (Tatebe and Shiozaki, 2003). The mutant *cdc37-184* was generated by introducing the equivalent mutation to the *S. cerevisiae* temperature-sensitive mutant *cdc37-184* into the *S. pombe cdc37* gene. *cdc37-J* and *cdc37-13* were generated by random mutagenic

PCR of the *cdc37* gene. Detailed characterisation of these four temperature-sensitive mutants was carried out to determine the defects that occur in cells at the restrictive temperature and the findings are described in this Chapter.

5.2 Generating *cdc37* temperature-sensitive mutants in *S. pombe*

5.2.1 The temperature-sensitive mutant *cdc37-681*

The *S. pombe* temperature-sensitive mutant *cdc37-681* was a kind gift from the Shiozaki lab. This *cdc37* mutant was isolated as a suppressor of hyperactivation of the stress-activated MAP kinase pathway involving Spc1 (Tatebe and Shiozaki, 2003). Sequencing of *cdc37-681* identified the mutation of a proline substituted for a leucine at codon 285.

5.2.2 Random mutagenesis of *cdc37* by PCR to generate temperature-sensitive mutants

Random mutagenic PCR was used as an unbiased method of creating mutations in the *cdc37* gene that may yield proteins whose ability to function properly is temperature dependent. In order to facilitate mutagenesis of the *cdc37* gene that could then be substituted for the endogenous copy, a template was constructed for PCR amplification incorporating the *S. pombe cdc37* gene upstream of a G418 resistance cassette for selection (Bahler et al., 1998). This was flanked at either end by 300 bp of chromosomal *cdc37* flanking sequence for efficient homologous recombination into the *S. pombe* genome. The technique used is shown schematically in Figure 5.1. PCR amplification reactions (Section 2.3.2.2) were carried out on the template using a range of mutagenic PCR conditions to randomly introduce mutations throughout the *cdc37* gene with the aim of generating temperature-sensitive mutants. The mutagenic PCR conditions that were employed in reactions included varying the ratio of dNTPs, adding $MnCl_2$ and incorporating both variables in combination.

Figure 5.1: Random mutagenesis of the *cdc37* gene using mutagenic conditions in PCR reactions to generate *cdc37* temperature-sensitive mutants. **A** The *cdc37* gene plus 300 bp of the chromosomal flanking sequence at the 5' end was amplified by PCR using *pfu* polymerase from the cosmid c9b6 (Sanger Centre) introducing new restriction sites, *PacI* at the 5' and *BglII* at the 3' end. **B** PCR amplified fragments were cloned into the PFA6a-KanMX6 vector (Bahler et al., 1998) upstream of the G418 resistance cassette. 300 bp of *cdc37* 3' chromosomal flanking sequence was amplified by PCR introducing *EcoRI* restriction sites at both ends and was cloned downstream of the G418 cassette. **C** Mutagenic PCR amplification was carried out on the entire *cdc37*-G418 construct. **D** PCR amplified fragments were integrated into the genome of *S. pombe* by homologous recombination. **E** Stable transformants were selected on YE media containing G418 and temperature-sensitive mutants were identified by incubation of resulting colonies at different temperatures.

A Amplification by PCR from the cosmid c9b6 of the *cdc37* gene and 300 bp of 5' and 3' flanking sequence, introducing new unique restriction sites for cloning

B Construction of the template for PCR

C Amplification of the template under different mutagenic PCR conditions

D PCR fragments were transformed into *S. pombe* and stable homologous recombination was selected for on YE+G418 media

E Temperature-sensitive mutants were identified by observing growth and phenotypes at different temperatures

Altering the ratio of dNTPs in a PCR reaction can enhance the level of mutagenesis. For example, 10-fold higher levels of dGTP in the dNTP pool results in a more error prone PCR reaction (Takashi Toda, personal communication). By using this ratio of dNTPs there is a bias towards the incorrect incorporation of dGTP (Fromant et al., 1995). However, increasing any single dNTP in a PCR reaction above 4mM results in a significant reduction in product yield (Fromant et al., 1995). To generate *cdc37* temperature-sensitive mutants, PCR reactions were optimised to obtain sufficient yield in the presence of mutagenic ratios of dNTPs.

An alternative mutagenic procedure is the inclusion of $MnCl_2$ in reactions. $MnCl_2$ concentrations of 0.5mM (Cadwell and Joyce, 1992) and 0.25mM (Lin-Goerke et al., 1997) have been found to be mutagenic in PCR reactions, reducing the fidelity of the polymerase to introduce random nucleotide changes in PCR amplified fragments. These levels of $MnCl_2$ in a PCR reaction significantly reduce the amplification yield (Fromant et al., 1995), making optimization of the technique essential. $MnCl_2$ was used at concentrations considered mutagenic while generating a workable amount of PCR product in order to obtain *cdc37* temperature-sensitive mutants.

Oligonucleotides were designed to amplify by PCR from the outermost sequence of the template for optimum efficiency homologous recombination. *Taq* polymerase which has no proofreading capacity was used in PCR reactions to increase mutation frequency. 10μg of PCR amplified mutagenic DNA was produced for efficient transformation and recombination into the *S. pombe* genome (Bahler et al., 1998).

5.2.3   The generation of *cdc37* temperature-sensitive mutants by random mutagenic PCR

A total of three screens were carried out with the aim of generating temperature-sensitive mutants of *cdc37* in *S. pombe* by PCR amplification of the template (Section 5.2.2) under a range of mutagenic conditions detailed in Table 5.1. Mutagenic PCR reactions were purified by phenol-chloroform extraction and ethanol precipitation (Section 2.2.9), and resuspended in a final volume of 10μl dH$_2$0. 10 μg of amplified

DNA was transformed into the haploid *S. pombe* strain ED1090 using the lithium acetate transformation protocol (Bahler et al., 1998), to replace the endogenous *cdc37* gene. Transformed cells were plated directly onto YE media and incubated at 28 °C for 18 hours. Plates were then replicated onto YE plus G418 and incubated at 28 °C until stable single colony transformants appeared. Candidate temperature-sensitive mutants of *cdc37* were identified by replicating plates onto YE+G418 incubated at 28 °C and YE+G418+Phloxin-B plates incubated at 36 °C for 24 hours. Phloxin-B is only permeable to dead cells, staining them deep red for easy identification. Colonies were screened by eye for growth on plates at 28 °C and for dead, dark red Phloxin-B stained colonies at 36 °C, characteristic of temperature-sensitive mutant lethality.

The stability of candidate *cdc37* temperature-sensitive mutants was verified by patching mutants onto YE media and incubating at 28 °C, then replicating back onto YE media containing G418 and allowing stable transformants to grow up at 28 °C. All G418 resistant candidate *cdc37* temperature-sensitive mutants were then crossed with the temperature-sensitive mutant *cdc37-681* (Tatebe and Shiozaki, 2003) to determine whether the integration location of the mutagenic PCR fragment was at the *cdc37* locus (Section 2.7.4). The spores were pooled and treated with Helicase (Section 2.7.4.1), plated onto YE+G418 and incubated at 36 °C to observe whether wildtype colonies formed. Recombination events during meiosis mean that if cells contain a random insertion in an incorrect location, i.e. not at the *cdc37* locus, then a proportion of wildtype spores will result.

A total of 11,750 stable colonies were obtained from all three screens, from which two *cdc37* temperature-sensitive mutants were isolated, *cdc37-13* and *cdc37-J*. Both of these mutants were generated by the inclusion of $MnCl_2$ in PCR reactions. *cdc37-13* was obtained from a PCR reaction containing 0.1 mM $MnCl_2$ and cdc37-J from PCR reactions with the inclusion of 0.05 mM $MnCl_2$.

Table 5.1: Number of stable transformants and *cdc37* temperature-sensitive mutants obtained from different mutagenic PCR conditions during Screens 1, 2 and 3 to generate *cdc37* temperature-sensitive mutants by random mutagenic PCR.

Screen	PCR conditions	Number of transformations	Number of stable transformants	*cdc37* temperature-sensitive mutants
1	10x dGTP	1	1050	0
	0.3 mM MnCl$_2$	1	7	0
	0.3 mM MnCl$_2$ and 10x dGTP	1	33	0
	No mutagen	1	1425	0
2	10x dGTP	4	303	0
	0.2 mM MnCl$_2$	2	84	0
	0.1 mM MnCl$_2$	2	126	*cdc37-13*
3	0.2 mM MnCl$_2$	3	317	0
	0.15 mM MnCl$_2$	3	931	0
	0.1 mM MnCl$_2$	3	940	0
	0.05 mM MnCl$_2$	3	1826	*cdc37-J*
	0.025 mM MnCl$_2$	4	4708	0
Totals		**28**	**11750**	**2**

The low yield of *cdc37* temperature-sensitive mutants generated in these three screens might be due to inefficient targeting into the *cdc37* locus of DNA fragments amplified by random mutagenic PCR or inefficient mutagenic PCR conditions. To test the first possibility, the efficiency of homologous recombination into the *cdc37* locus was determined. 25 G418^R colonies were picked at random from Screen 2 and crossed with the temperature-sensitive mutant *cdc37-681*. Random spore analysis was carried out and resulting progeny were plated on YE and incubated at 28 °C. 20 single colonies were picked per cross, and their genotype determined by replicating onto YE media plus and minus G418 and incubating at 28 and 36 °C. Correct insertion of the *cdc37* PCR amplified fragment will result in spores being either *cdc37$^+$* and G-418 resistant or *cdc37-681* and G-418 sensitive. Incorrect insertion will produce progeny with other types of genotypes, including *cdc37-681* which is G-418 resistant and *cdc37$^+$* that is G-418 sensitive. Homologous integration of the PCR fragments into the *cdc37* locus occurred in 23 out of 25 colonies giving 92% efficiency of correct integration for this method and indicating this is not a limiting factor in generating *cdc37* temperature-sensitive mutants.

5.2.4 Directed mutagenesis to generate *cdc37* temperature-sensitive mutants

The temperature-sensitive mutant *cdc37-184* was identified in *S. cerevisiae* and arrests in START when incubated at the restrictive temperature (Valay et al., 1995). The mutant *cdc37-184* has a mutation of alanine at amino acid 275 to aspartic acid (Mort-Bontemps-Soret et al., 2002). Alanine 275 in *S. cerevisiae* aligns with alanine 287 in *S. pombe*. Directed overlap PCR mutagenesis was carried out on the *S. pombe cdc37* gene in the vector pREP81, to introduce the equivalent mutation to *S. cerevisiae cdc37-184* into the *S. pombe cdc37* gene. The ability of the *S. pombe cdc37-184* mutant expressed from pREP81 to sustain cell viability at different temperatures was determined by plasmid shuffle assay in the *cdc37Δ* strain ED1526 (Section 2.7.1). Expression of *cdc37-184* from pREP81 in the *cdc37Δ* strain sustained growth in a temperature dependent manner (Figure 5.2A). Cells expressing the *S. pombe cdc37-184* mutation were able to maintain growth at 25, 28 and 32 °C

Figure 5.2: Expression of the *S. pombe cdc37-184* mutant from *pREP81* in the strain ED1526 by plasmid shuffle assay to determine its ability to sustain cell viability at different temperatures. **A** Serial dilutions were plated on 5FOA media and incubated at different temperatures for 4 days. Cells at 36 °C expressing **B** *pREP81-cdc37-184* and **C** *pREP81-cdc37*.
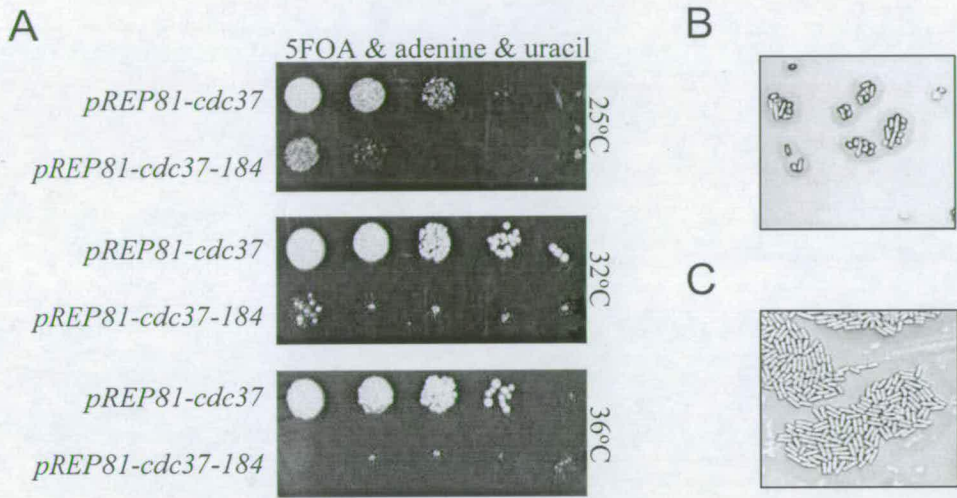
producing cells which were wildtype in appearance. At 36 °C, *cdc37-184* was unable to support growth in the plasmid shuffle assay. The cell population was heterogeneous in phenotype, consisting of elongated, short, rounded and bowling pin shaped cells (Figure 5.2B) characteristic of depletion of Cdc37 from cells (Westwood et al., 2004).

5.2.5 Integration of *cdc37-184* into the *S. pombe* chromosome

To study further the temperature-sensitive mutant *cdc37-184*, the mutation was introduced into the random mutagenesis PCR template so that it could be integrated into the chromosomal *cdc37* locus using the technique shown schematically in Figure 5.3. PCR amplification of the template was carried out with *Pfu* polymerase to minimise the occurrence of undesired mutations. PCR reactions were purified and transformed into *S. pombe* (described in Section 5.2.3) and a total of 815 stable transformants were obtained which were then replicated and incubated at 28 and 36 °C to identify colonies with a temperature-sensitive phenotype. Four candidate colonies were identified as possible temperature-sensitive mutants and were crossed with *cdc37-681* for verification of correct integration of *cdc37-184*. All four strains had correct integration of the *cdc37-184* fragment. These four *cdc37* temperature-sensitive mutants were able to form single colonies by streaking on YE plates and incubating at 25, 28 and 32 °C for 4 days, but not at 36 °C. Morphologically cells were elongated at 36 °C compared to the lower temperatures.

5.3 Characterisation of *cdc37* temperature-sensitive mutants

5.3.1 Identification of *cdc37* temperature-sensitive mutations

The *S. pombe* temperature-sensitive mutants *cdc37-J* and *cdc37-13* generated by random PCR mutagenesis were sequenced to identify the location and nature of their mutations within the *cdc37* gene. The mutant *cdc37-184* was also sequenced to verify the introduction of the mutation within the *cdc37* gene and to ensure the absence of other undesired mutations that might have occurred during PCR amplification. Genomic DNA was prepared from the temperature-sensitive mutants (Section 2.7.3)

Figure 5.3: Integration of the *cdc37-184* mutation into the *S. pombe* genome. The *cdc37-184* mutation was introduced into the random mutagenesis template **A** by restriction digestion from the *pREP81-cdc37-184* plasmid using unique restriction sites *Blp1* and *Swa1* and ligating into the equivalent restriction sites in the random mutagenesis template. **B** Standard PCR amplification was carried out and **C** fragments were then integrated into the genome of *S. pombe* by homologous recombination. **D** Stable transformants were selected for on media containing G418 and temperature-sensitive mutants were identified by incubation of resulting colonies at different temperatures.

A Insertion of the *cdc37-184* mutation ⋆ into the Template for PCR.

B PCR amplify fragments under standard PCR conditions

C PCR fragments transformed into *S. pombe*. Selected for stable homologous recombination on YE-G418 media

D Temperature-sensitive mutants were identified by observing growth and phenotype at different temperatures
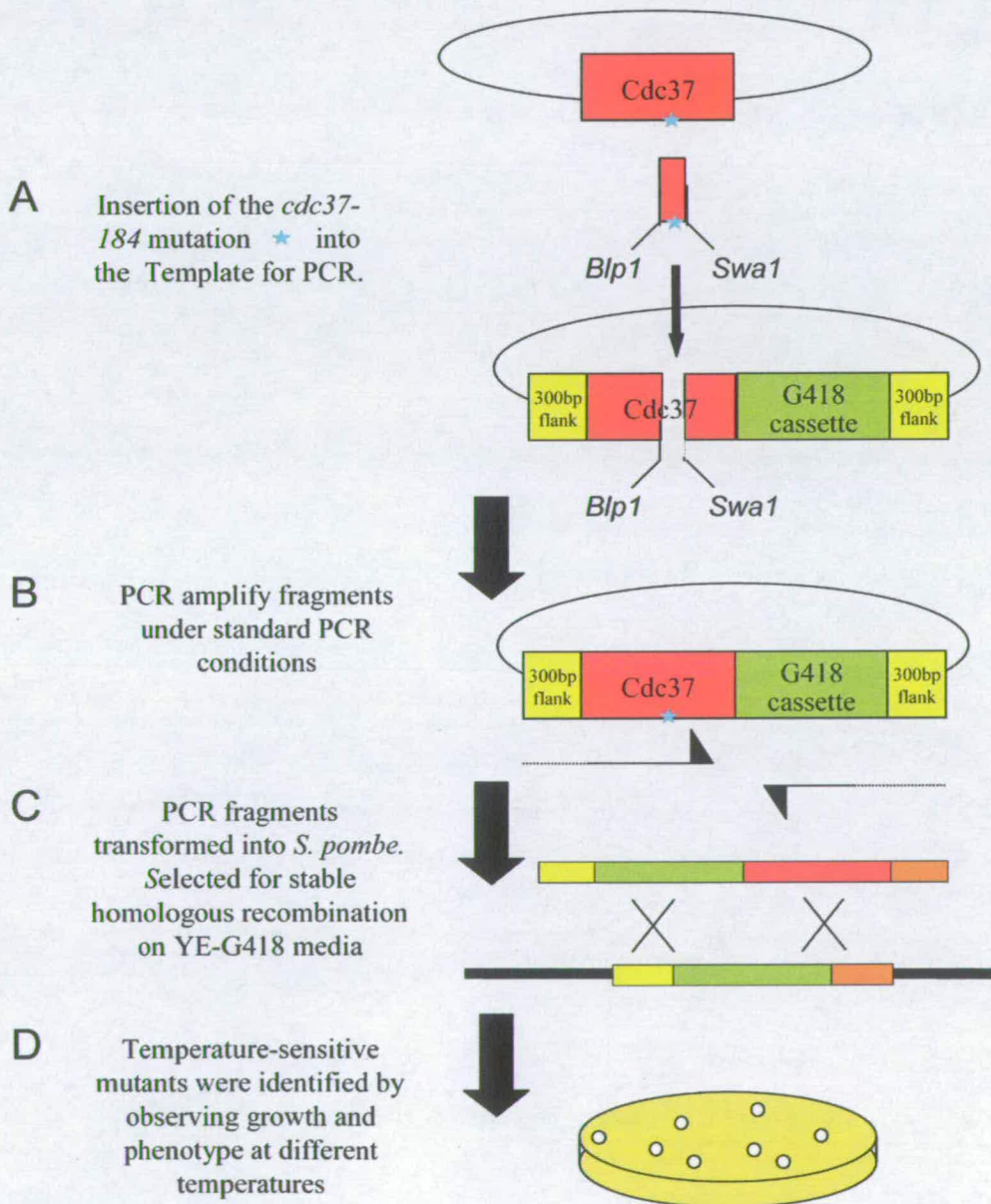
and each section of the *cdc37* gene was amplified in 3 separate PCR reactions. The 3 PCR reactions were then combined to reduce the possibility of a mutation introduced during one PCR reaction biasing sequencing results. Fragments were sequenced in the forward and reverse direction for precision over the entire *cdc37* gene and flanking sequence in duplicate to determine accurately the genomic sequence of each temperature-sensitive mutant.

The mutations identified in the *S. pombe cdc37* temperature-sensitive mutants *cdc37-184*, *cdc37-13* and *cdc37-J* are shown in Figure 5.4 with the *cdc37-681* mutation (Tatebe and Shiozaki, 2003) for comparison. Sequence determination of *cdc37-184* verified the substitution of an alanine at codon 275 to aspartic acid. No other mutations were identified in the *cdc37* gene or flanking regions for the *cdc37-184* mutant. Two mutations were identified in the open reading frame (ORF) of *cdc37-13*. Codon 237 had been mutated from glutamic acid to lysine and amino acid 261 was changed from a tyrosine to a histidine. *Cdc37-J* was found to contain three mutations in the ORF. Amino acid 286 had been mutated from leucine to methionine, residue 305 from histidine to leucine and codon 314 from arginine to glycine.

5.3.2 Identifying the mutations in *cdc37-J* and *cdc37-13* responsible for the temperature-sensitive phenotype

Temperature-sensitive mutants *cdc37-J* and *cdc37-13* each contain more than one nucleotide change. To determine which mutations were responsible for producing a temperature-sensitive phenotype, mutations were introduced into the *cdc37* gene both individually and in combination by overlap PCR. Each mutant was expressed from pREP81 in the *cdc37Δ* strain to observe their ability to sustain cell viability at different temperatures. However, the expression of all *cdc37-13* or *cdc37-J* (Figure 5.5) mutants at 25, 28, 32 and 36 °C was able to support growth as well as expression of *pREP81-cdc37* and cells were phenotypically wildtype in appearance. None of the *cdc37-13* or *cdc37-J* mutations, individually or in combination, permitted growth in a temperature dependent manner. These data show that the effect on growth and cell morphology of expression of integrated mutants *cdc37-13* and *cdc37-J* differs from

Figure 5.4: A schematic diagram to show the location of *cdc37* temperature-sensitive mutant mutations identified in *S. pombe* temperature-sensitive mutants *cdc37-681*, *cdc37-184*, *cdc37-13* and *cdc37-J*.

□ *cdc37-184* : Alanine 287 to aspartic acid

△ *cdc37-J* : mutation J1: Leucine 286 to methionine, J2: Histidine 305 to leucine, J3: Arganine 314 to glycine

◇ *cdc37-13* : mutation L: Glutamic acid 237 to lysine, mutation H: Tyrosine 261 to histidine

○ *cdc37-681* : Leucine 285 to proline

■ Postulated Hsp90 binding region
■ Putative dimerisation domain
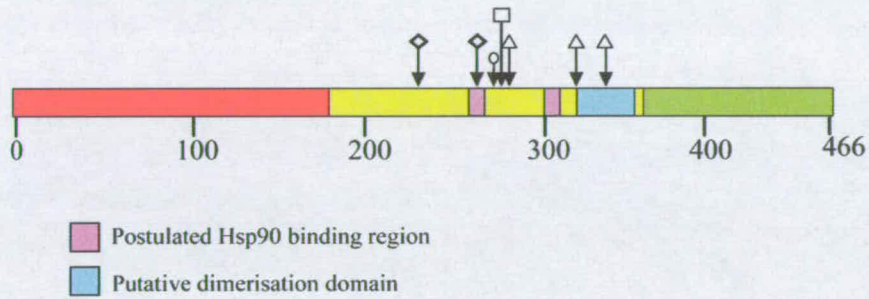
Figure 5.5: Expression of the *cdc37* gene containing mutations L and H identified in *cdc37-13* and mutations J1, J2 and J3 identified in *cdc37-J* from pREP81 by plasmid shuffle assay. The location and nature of these mutations identified in *cdc37-13* and *cdc37-J* are shown in Figure 5.4.
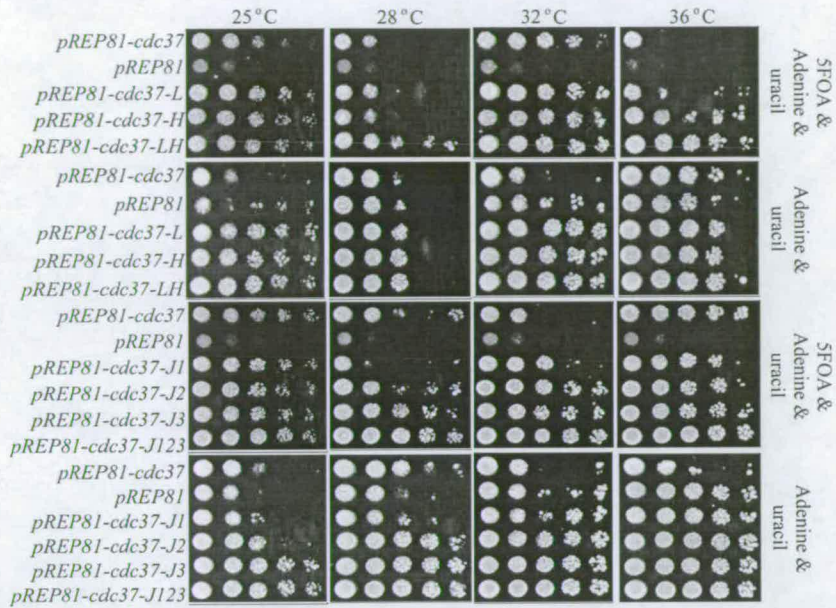
Figure 5.6: Expression of the *cdc37* gene containing mutations L and H identified in *cdc37-13* and mutations J1, J2 and J3 identified in *cdc37-J* from pREP1 by plasmid shuffle assay.
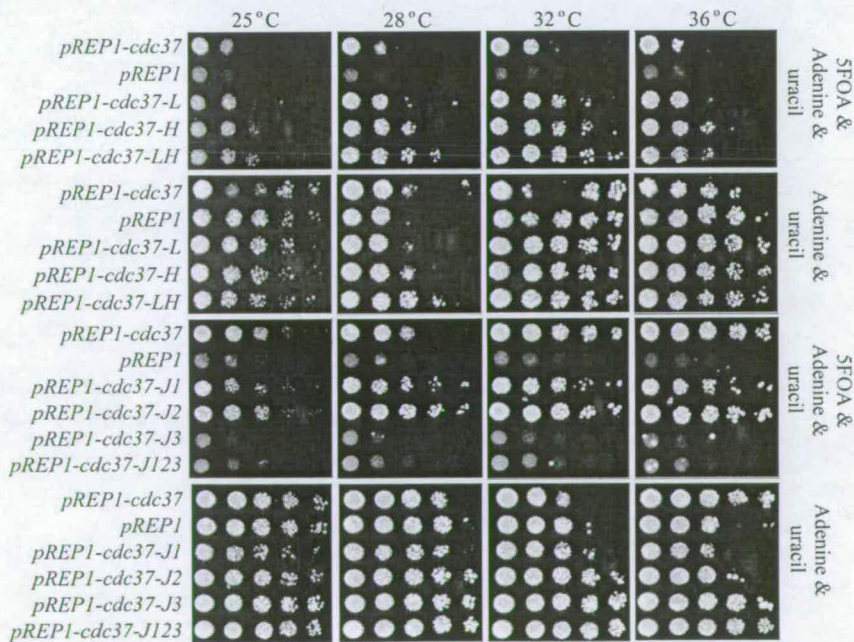
their expression from pREP81. This has also been seen with *cdc37-184* whose expression from pREP81 in the plasmid shuffle assay supports a morphologically heterogeneous cell population at the restrictive temperature, but the integrated mutation produces highly elongated cells. These experiments were unable to identify the mutations that cause the temperature-sensitive phenotype of *cdc37-J* and *cdc37-13*, possibly because Cdc37 protein levels from pREP81 may be greater than when the same mutant proteins were expressed as single copy integrants. This may raise the level of mutant protein and overall function above a threshold that can sustain cell viability. However, in Chapter 3, wildtype Cdc37 expressed from pREP81 produced protein at a level very similar to endogenous Cdc37, although subtle differences in protein levels may not be easily detectable by western blot.

The *cdc37-J* and *cdc37-13* mutant alleles described above were overexpressed from pREP1 in the *cdc37Δ* strain to examine the effect of increasing mutant protein levels on cell viability at different temperatures. Expression of mutants containing *cdc37-13* mutations and *cdc37-J1* and *cdc3-J2* permitted growth as well as expression of *pREP1-cdc37* at 25, 28, 32 and 36 °C and cells were morphologically wildtype in appearance (Figure 5.6). Expression of the mutants *cdc37-J3* and *cdc37-J123* from pREP1 were unable to sustain growth (Figure 5.6) at all temperatures and cells were heterogeneous in phenotype as observed in Cdc37 depletion experiments (Westwood et al., 2004). Overexpression of wildtype Cdc37 does not compromise cell viability, but overexpression of the mutants *cdc37-J3* and *cdc37-J123* results in lethality. Overexpression of certain alleles in *S. pombe* can be lethal, as observed with overexpression of *nda3* and *ubc4*, which encode for beta-tubulin and an ubiquitin conjugating enzyme respectively (Javerzat et al., 1996).

5.3.3   Overexpression of *cdc37* mutants to look for any dominant negative effect in the presence of endogenous Cdc37

Cdc37-184 and all *cdc37-13* and *cdc37-J* mutants described in the previous section were overexpressed from pREP1 in the *cdc37$^+$* strain ED1090 to look for any dominant negative effects. Mutants were transformed into *S. pombe* strain ED1090

and stable transformants were incubated at 28 and 36 °C. No dominant negative effect was observed for any mutant at either temperature. All cells were morphologically wildtype in appearance and successfully formed colonies (data not shown).

5.3.4 The ability of *cdc37* temperature-sensitive mutants to support growth at different temperatures

To determine accurately the permissive and restrictive temperature ranges of all four *cdc37* temperature-sensitive mutants, their ability to form single colonies at different temperatures was investigated. The *cdc37* temperature-sensitive mutant strains and a *cdc37*$^+$ strain ED1022 were streaked onto YE plates and incubated at 25, 28, 32 and 36 °C for 4 days. The *cdc37*$^+$ strain was able to form single colonies at all temperatures. All four *cdc37* temperature-sensitive mutants were able to form single colonies when incubated at 25, 28 and 32 °C (Figure 5.7). However, these mutant strains were unable to form single colonies at 36 °C.

The ability of *cdc37* temperature-sensitive mutants and the *cdc37*$^+$ strain to grow on media containing different chemical inhibitors was investigated using DL-methionine methylsolfonium chloride (MMS) (Sigma 64382) that induces DNA damage and 3-AT and Canavanine, that inhibit amino acid synthesis. Inhibitors of amino acid synthesis were used as it has previously been reported that Cdc37 is required for the amino acid starvation response in *S. cerevisiae* involving Gcn2 (Donze and Picard, 1999). 3-AT (Sigma A-8056) is an inhibitor of histidine biosynthesis and Canavanine (Sigma C1625) is a toxic analogue of arganine. Strains were streaked onto plates containing different concentrations of each inhibitor and incubated at different temperatures, but mutants showed equal sensitivity to chemical inhibitors as *cdc37*$^+$ strain ED1022.

Figure 5.7: *cdc37* temperature-sensitive mutants and the *cdc37⁺* strain ED1022 were streaked on YE plates and incubated at 25, 28, 32 and 36 °C for four days to determine whether these mutants could form single colonies.
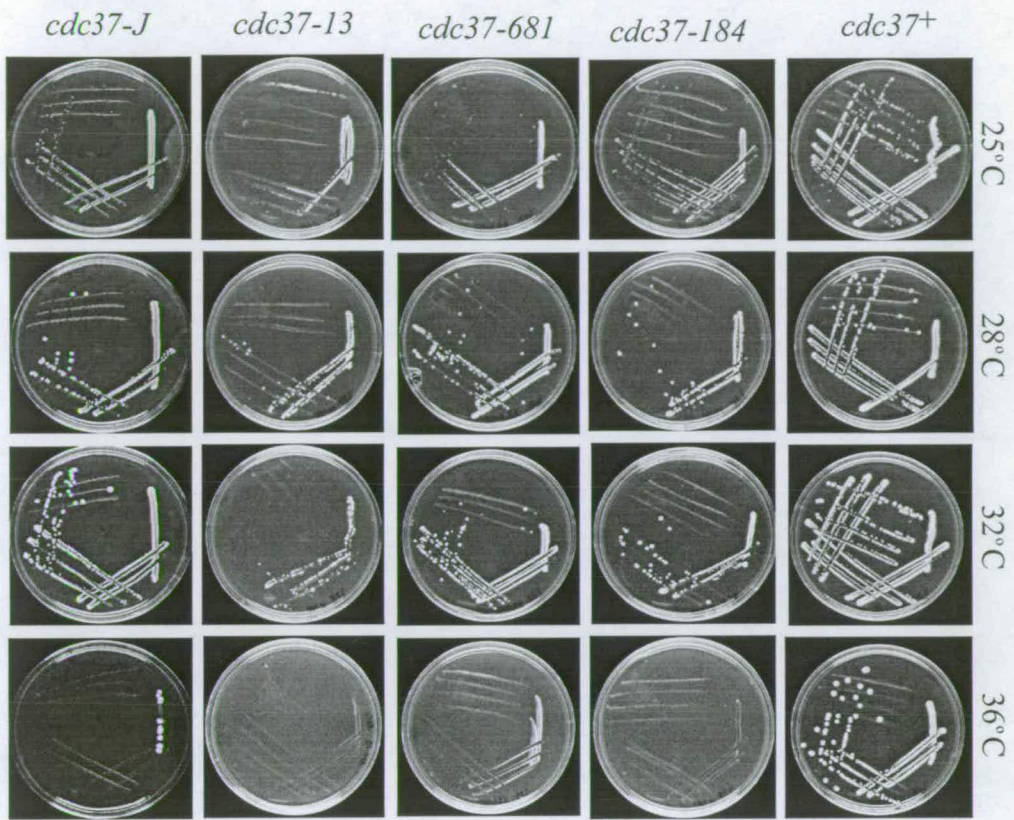
Figure 5.8: *cdc37-J* was plated as serial dilutions, diluted in dH₂0, on different media with and without G418 and incubated for 4 days at 36 °C.
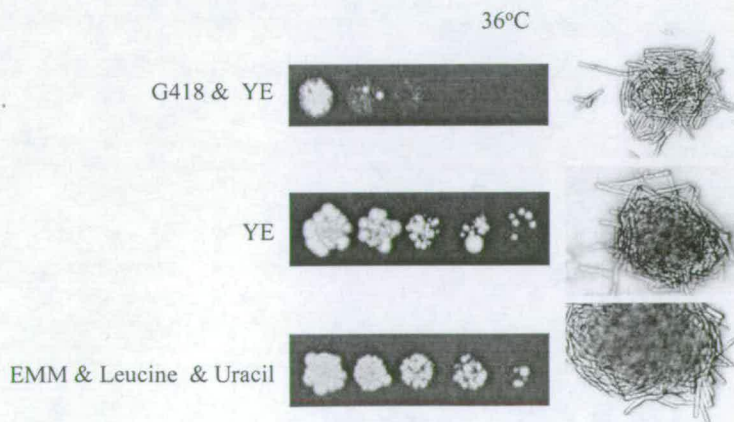
As previously stated, the mutant *cdc37-J* did not form single colonies when streaked on YE at 36 °C in the absence of G418, but it did support growth when plated as serial dilutions under these conditions, although cells were morphologically elongated. The ability of *cdc37-J* to form colonies when plated as serial dilutions may be due to the greater cell density, as a low level of colony formation is apparent on the initial inoculation line from streaking at 36 °C. Serial dilutions of *cdc37-J* at 36 °C did not grow successfully on media containing G418 (Figure 5.8) which binds the 80S ribosome complex and inhibits eukaryotic protein synthesis by disturbing its proofreading ability resulting in early termination and mistranslation. All subsequent characterisation studies of *cdc37-J* described here were carried out in the absence of G418.

To determine how quickly cell proliferation was affected in *cdc37* temperature-sensitive mutants after shift to the restrictive temperature of 36 °C, the increase in cell number in liquid cultures at 28 and 36 °C over a time course was investigated. *cdc37-681*, *cdc37-184*, *cdc37-J, cdc37-13* and the *cdc37$^+$* strain ED1022 were cultured at 28 °C, then split and kept at 28 °C or shifted to 36 °C, as described in Section 2.7.4.3. At set time points, two 100 μl samples were taken from each cell culture and processed for counting in a Coulter electronic particle counter (Section 2.8.6). The number of cells in cultures increased over the time course for the *cdc37$^+$* strain ED1022 at both 28 and 36 °C and for all four *cdc37* temperature-sensitive mutants at 28 °C (Figure 5.9). However, at 36 °C the rate of cell division for all *cdc37* temperature-sensitive mutants was dramatically reduced within 2 hours of the shift. Cell number for *cdc37* temperature-sensitive mutants stabilised after 2 hours at 36 °C and remained fairly constant thereafter.

Figure 5.9: Analysis of cell number of *cdc37* temperature-sensitive mutant and *cdc37⁺* cells of ED1022 by Coulter electronic particle counter. Strains were cultured at 28 and 36 °C and cell number was determined for the *cdc37⁺* strain ED1022 and all four *cdc37* temperature-sensitive mutants.
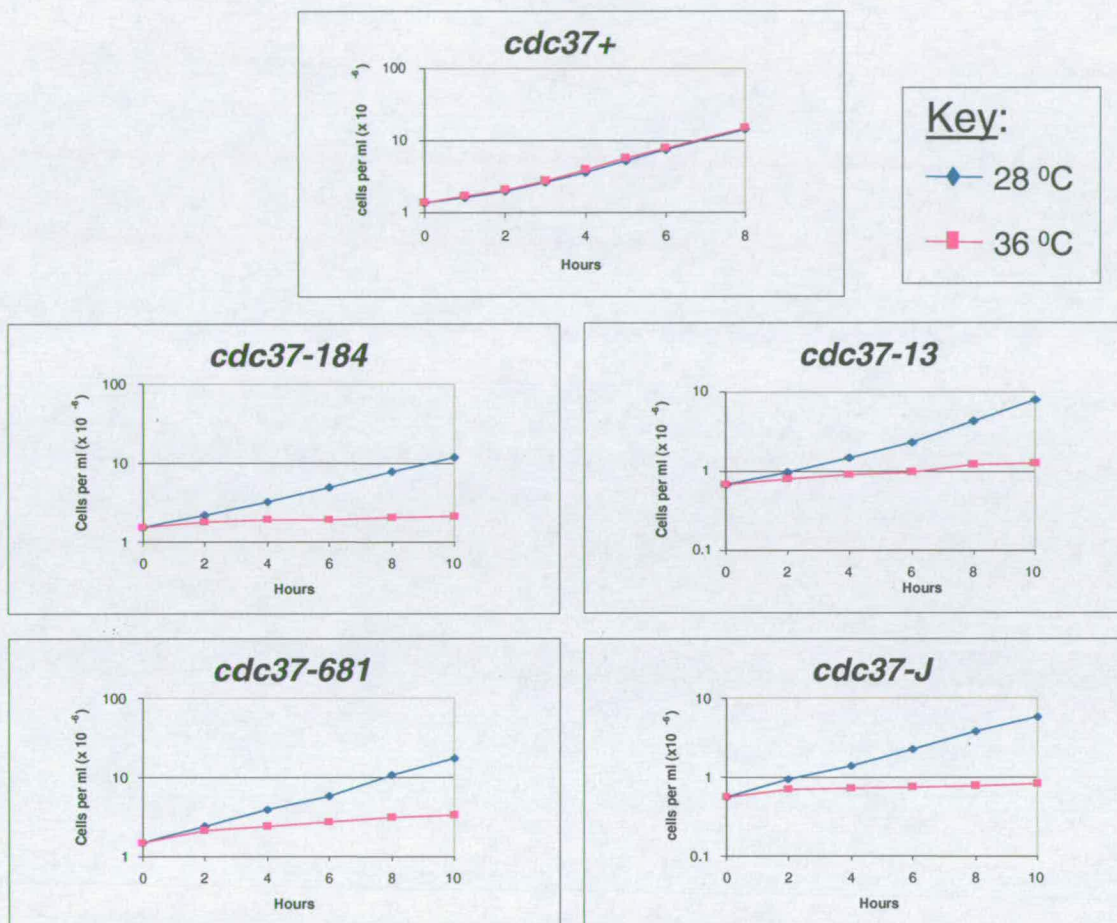
Figure 5.10: Comparison of Cdc37 protein levels in *cdc37* temperature-sensitive mutants and the *cdc37⁺* strain ED1022 after 8 hours at 28 and 36 °C. Western blot analysis were carried out on whole cell protein extracts using the anti-*S. pombe* Cdc37 and TAT1 antibodies as a loading control.

5.3.5 Cdc37 protein levels in *cdc37* temperature-sensitive mutants

The reason why *cdc37* temperature-sensitive mutant cells stop dividing after 2 hours at the restrictive temperature might be due to loss of Cdc37 function or reduced Cdc37 protein levels. To investigate the latter possibility, *cdc37* temperature-sensitive mutant strains and the *cdc37$^+$* strain ED1022 were cultured as described in Section 2.7.4.3 at 28 and 36 °C over an 8 hour time course. Denatured protein extracts were made and run by SDS PAGE for visualisation by western blot which with the anti-*S. pombe* Cdc37 antibody and TAT1 antibody as a loading control. Western blot analysis revealed that Cdc37 protein levels were not reduced in the *cdc37* temperature-sensitive mutants incubated at 36 °C (Figure 5.10). However, *cdc37-13* and *cdc37-J* mutant Cdc37 protein levels were slightly reduced compared to other strains but this was not temperature related. These data indicate that loss of cell viability in *cdc37* temperature-sensitive mutants is not due to lowered protein levels but presumably to reduced Cdc37 function.

5.3.6 Cell morphology of *cdc37* temperature-sensitive mutant cells

The cell morphologies of *cdc37* temperature-sensitive mutants and the *cdc37$^+$* strain ED1022 were examined after incubation for 24 hours on YE plates at a range of temperatures between 25 and 36 °C. At 25, 28 and 32 °C *cdc37$^+$* and *cdc37* temperature-sensitive mutant cells were morphologically wildtype in appearance. At 36 °C all *cdc37* temperature-sensitive mutant cells were elongated (Figure 5.11), characteristic of the cell division cycle (*cdc*) phenotype. The *cdc* phenotype indicates that cells are unable to progress through the cell cycle and divide, but growth is less compromised and the cells continue to elongate.

To quantitate this observed cell elongation, cell length was measured for *cdc37* temperature-sensitive mutant and *cdc37$^+$* strains, cultured as described in Section 2.7.4.3 over an 8 hours time course. Samples were taken from cultures at 0 hours and then at subsequent 2 hour intervals, fixed with formaldehyde and stained with

Figure 5.11: Cell morphology of *cdc37* temperature-sensitive mutants and the *cdc37*⁺ strain ED1022 on YE plates incubated at 25, 28, 32 and 36 °C for 24 hours. Bar is 10 µm.
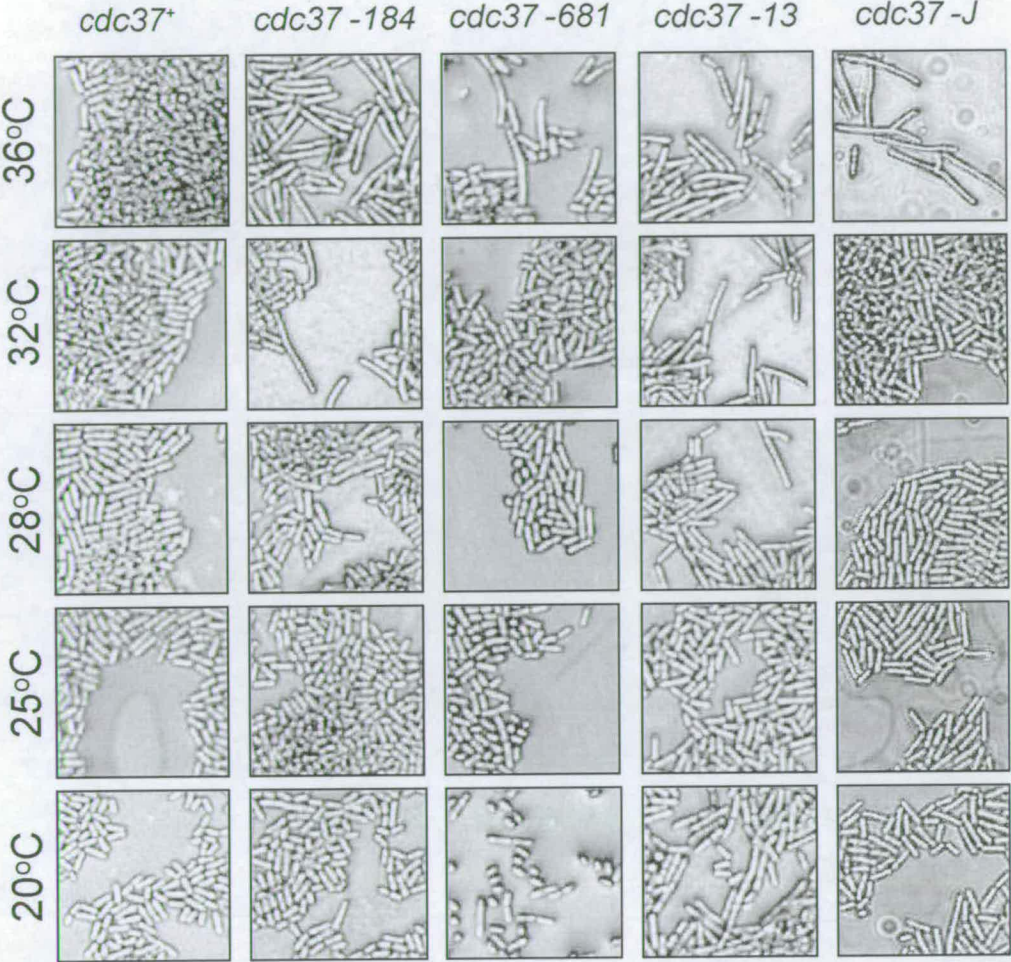
Calcofluor (Section 2.8.1) which is a fluorescent brightener that efficiently binds the yeast cell wall and septum (Streiblova et al., 1984). The lengths of 200 cells were measured for each strain at each time point (Section 2.8.5).

At 28 °C, the average cell length for $cdc37^+$ and $cdc37$ temperature-sensitive mutant cells did not differ dramatically (Figure 5.12). Cells of the $cdc37^+$, $cdc37-184$, $cdc37-681$ and $cdc37-13$ strains at 28 °C had an average cell length of 9.8 μm, 9.1 μm, 8.1 μm and 10 μm respectively. The average cell length for $cdc37-J$ at the permissive temperature was 12.3 μm, slightly longer than for $cdc37^+$ strain. At the restrictive temperature, the average cell length of $cdc37^+$ cells was equal to those observed at 28 °C and did not change over the 8 hour time course. In contrast, the average cell length for all $cdc37$ temperature-sensitive mutants increased with time at 36 °C (Figure 5.12). Table 5.2 shows the average cell lengths for $cdc37$ temperature-sensitive mutants at 28 °C and after 8 hours at 36 °C. $cdc37-J$ cells displayed the greatest elongation, increasing by approximately 3-fold in length over 8 hours at the restrictive temperature.

5.3.7 Identification of the cell cycle stage in which $cdc37$ temperature-sensitive mutant cells arrest

Previous data indicate that $cdc37$ temperature-sensitive mutant cells arrest the cell cycle, but continue to elongate producing a cdc phenotype. To identify the stage in the cell cycle at which the $cdc37$ temperature-sensitive mutants arrest, flow cytometry (FACS) (Section 2.8.7) analysis was carried out. This technique determines whether cells contain a 1C (unreplicated) or a 2C (replicated) DNA content by staining of the cellular DNA content with propidium iodide. The level of fluorescence from a 1C DNA content is half that of a 2C, and this allows cells with replicated and unreplicated DNA to be distinguished. In a wildtype *S. pombe* population very few cells contain a 1C DNA content as G1 is an extremely quick event. To identify the 1C DNA content peak by flow cytometry the *S. pombe* temperature-sensitive mutant

Table 5.2: Average cell lengths of *cdc37* temperature-sensitive mutant and *cdc37⁺* cells and the percentage of increase in cell length between 28 °C and after 8 hours at 36 °C. Strains were cultured in liquid YE at 28 and 36 °C and the lengths of 200 cells measured for each sample.

Strain	Average length (µm)		Length increase
	0 hours 28 °C	**8 hours 36 °C**	
Wildtype	10.4	9.7	-7%
Cdc37-184	9.1	15.6	71%
Cdc37-681	8.1	17.4	115%
Cdc37-13	10.0	21.7	117%
Cdc37-J	12.3	33.4	172%

Figure 5.12: Mean cell length of *cdc37* temperature-sensitive mutant and *cdc37⁺* cells (with standard deviation bars). Strains were cultured in liquid YE at 28 and 36 °C over an 8 hour time course and the lengths of 200 cells measured for each sample.
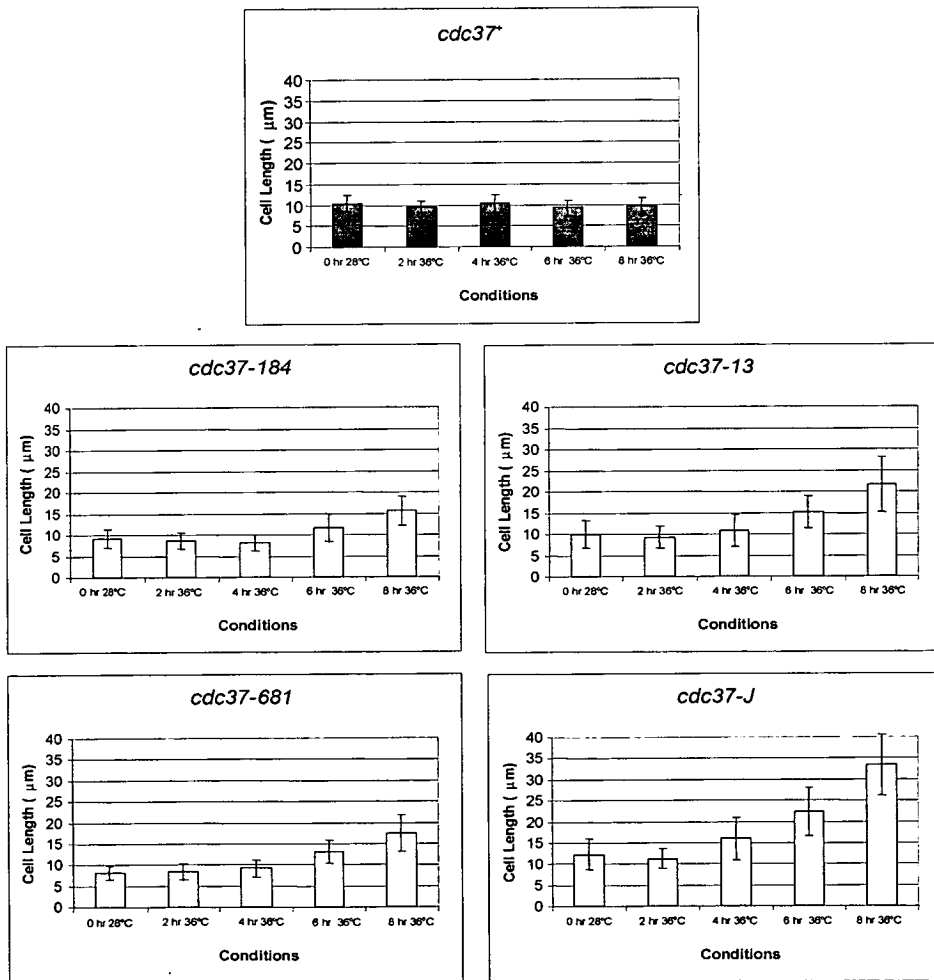
Figure 5.13: Flow cytometry of *cdc37* temperature-sensitive mutants, *cdc37*[+] ED1022 and *cdc10-129* was used to identify the DNA content of cells. Strains were cultured in YE at 28 and 36 °C over an 8 hour time course and samples of cells were taken every two hours. Cells were processed for flow cytometry, being stained with propidium iodide, and their DNA content was assayed in a FACScan (Becton Dickson).
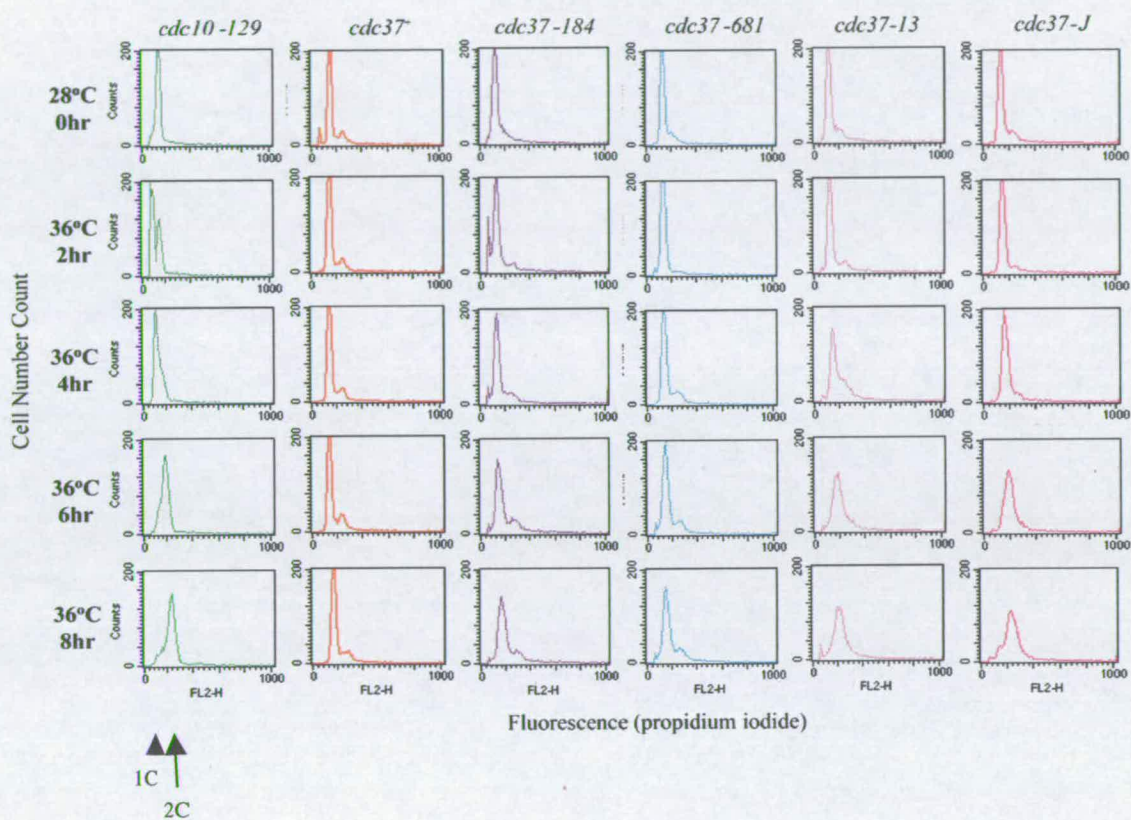
cdc10-129 was employed, which is defective in DNA synthesis at 36 °C (Nurse et al., 1976) and cells arrest unable to replicate their DNA producing a 1C DNA peak detectable by flow cytometry. The *cdc37* temperature-sensitive mutants, *cdc10-129* and the *cdc37⁺* strain ED1022 were cultured as described in Section 2.7.4.3. Samples were taken from each cell culture incubated at 28 and 36 °C every 2 hours over an 8 hour time course and were processed for flow cytometry (Section 2.8.7) by staining with propidium iodide.

At 28 °C the *cdc37⁺* strain ED1022, *cdc10-129*, *cdc37-681*, *cdc37-184*, *cdc37-J* and *cdc37-13* cell populations predominantly contained a 2C DNA content (Figure 5.13). At 36 °C the cell population of *cdc10-129* was split between a 1C and a 2C DNA content after 2 hours establishing the distribution for 1C and 2C DNA content peaks for other strains in this experiment. At 36 °C *cdc37⁺* and *cdc37* temperature-sensitive mutant cells predominantly contained a 2C DNA content (Figure 5.13). These data indicate that the *cdc37* temperature-sensitive mutants arrest with a replicated genome having passed through S phase. It is interesting to note that *cdc37-184* produces a distinct peak of 1C DNA content after 2 hours at 36 °C, which completely disappears by the 4 hour time point (Figure 5.13). This could suggest that cells undergo a delay in G1 at 36 °C, producing a 1C DNA content peak. For all strains, the position of the peaks determined by flow cytometry shifted to the right along the graph x-axis at the later time points. This is thought to be a result of an increase in the mitochondrial DNA and RNA cellular content following elongation (Sazer and Sherwood, 1990).

Flow cytometry data indicated that *cdc37* temperature-sensitive mutants arrest at the restrictive temperature with a 2C DNA content, suggesting a G2 or M phase (mitosis) arrest. Nuclear morphology was examined with the aim of gaining a better understanding of the stage in which *cdc37* temperature-sensitive mutant cells were arresting. The *cdc37* temperature-sensitive mutant and *cdc37⁺* strains were cultured as described in Section 2.7.4.3 and samples of cells were taken every 2 hours. Cells were fixed and stained with DAPI that binds double stranded DNA and RNA and produces a blue fluorescence which can be visualised by microscopy.

Figure 5.14: Frequency of phenotypes observed with DAPI staining of *cdc37* temperature-sensitive mutant and *cdc37+* cells. Strains were cultured in YE at 28 and 36 °C over an 8 hour time course and samples of cells were taken every two hours, fixed in formaldehyde and stained with DAPI. Bar is 10μm.
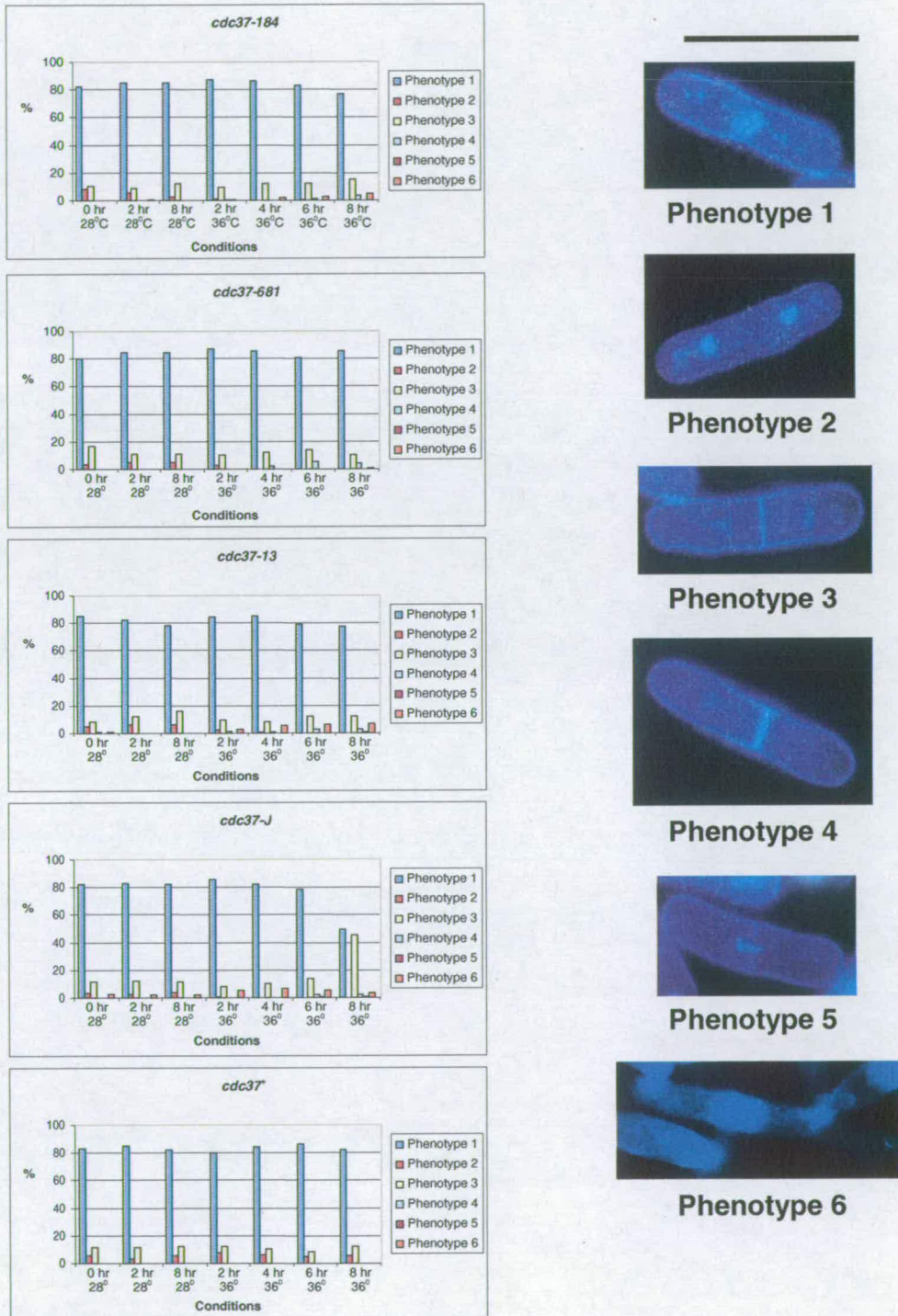
Three distinct nuclear morphologies were observed for all *cdc37* temperature-sensitive mutants and *cdc37*⁺ cells at the permissive temperature of 28 °C (Figure 5.14). Around 80% of the cell population were classified as phenotype 1, a single cell with one nucleus. Phenotype 2, consisting of a single cell with a divided nucleus, was observed for ~5% of the cell population and phenotype 3, cells which had 2 nuclei separated by a septum, made up ~15%.

With increasing time at the restrictive temperature, *cdc37* temperature-sensitive mutant cells were predominantly phenotype 1 with a small sub-population of phenotype 3 (~15%) (Figure 5.14). Phenotype 2 is absent from the cell population in all temperature-sensitive mutants after 2 hours at the restrictive temperature (Figure 5.14). These data indicate that most *cdc37* temperature-sensitive mutant cells arrest at the restrictive temperature with a single nucleus, most likely in G2 as chromatin was uncondensed and no individual chromosomes were observed. It is interesting to note that over the 8 hour time course at the restrictive temperature all *cdc37* temperature-sensitive mutants showed a slight increase in the number of cells with aberrant morphologies, categorised as phenotype 4 with a single nucleus and a single septum, phenotype 5 with a single nucleus with a septum running through it, and phenotype 6 containing multiple septa. However, these aberrant morphologies classified as phenotypes 4, 5 and 6 only represent a very small proportion of the total cell population. Interestingly, *cdc37-J* differs in phenotype after 6 to 8 hours at the restrictive temperature compared to the other *cdc37* temperature-sensitive mutants, as around half of the cells have leaked into mitosis, having divided their nuclei and formed septa.

Figure 5.15: Number of cells with and without septa. *cdc37* temperature-sensitive mutant and *cdc37*⁺ strains were cultured at 28 and 36 °C in YE over an 8 hour time course. Samples of cells were taken every two hours, fixed with formaldehyde and stained with Calcofluor to visualise the cell wall and septa.
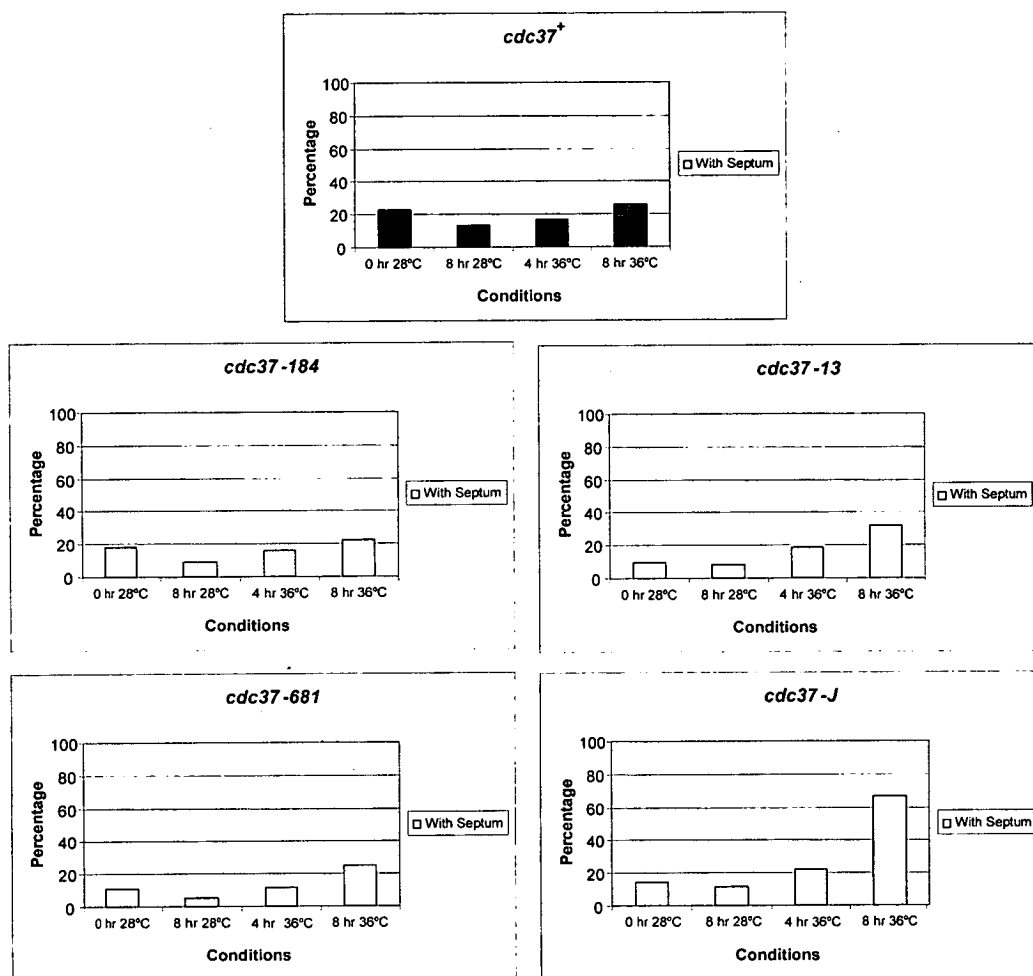
When cells were stained with Calcofluor to measure the lengths of *cdc37* temperature-sensitive mutant cells, a large proportion of *cdc37-J* cells were seen to be septated at the later time points at 36 °C. In wildtype cells, septa formed between two divided nuclei, in line with the actinomyosin contractile ring and are essential for cytokinesis, cleaving the mother cell to produce two daughter cells. To study further this observation, *cdc37* temperature-sensitive and *cdc37$^+$* strains were cultured in YE as described in Section 2.7.4.3, and samples of cells were taken every two hours, fixed and stained with Calcofluor for visualisation by microscopy. The proportion of cells with and without septa was determined for a minimum of 200 cells per sample. The proportion of cells containing septa did not dramatically change for the *cdc37$^+$* strain ED1022, *cdc37-184*, *cdc37-681* and *cdc37-13* at 28 and 36 °C (Figure 5.15). In contrast 67% of *cdc37-J* cells were septated after 8 hours at the restrictive temperature (Figure 5.15). Septa in *cdc37-J* cells were only observed at the later time points and may occur as a result of cells leaking through their G2 cell cycle arrest, entering mitosis and developing septa.

The septal morphology of *cdc37* temperature-sensitive mutant and *cdc37$^+$* ED1022 cells was then studied to look for any defects in cytokinesis. Septated cells of *cdc37* temperature-sensitive mutants at the permissive temperature contained only 1 septum per cell. At the non-permissive temperature, *cdc37-184*, *cdc37-681* and *cdc37-13* all exhibited a very slight increase in the proportion of cells with 2 or more septa per cell after 6 hours at the restrictive temperature. In contrast, the number of septa observed in *cdc37-J* cells increased dramatically over the 8 hour time course at 36 °C (Figure 5.16). After 8 hours at 36 °C only 52% of the cell population contained 1 septum, 38% contained 2 septa, 7.4% of cells contained 3 septa and 2.6% had multiple septa. These septa were often aberrant in morphology, curving and folding across the cell (Figure 5.16). *cdc37-J* cells may be leaking through their G2 arrest after 8 hours at 36 °C and arresting, unable to carry out cytokinesis and develop multiple, often aberrant septa in a large proportion of cells.

Figure 5.16: The number of septa per cell was quantitated for *cdc37* temperature-sensitive mutant strains cultured in YE at 28 and 36 °C. Samples of cells were taken every two hours, fixed and stained with Calcofluor to visualise septa by microscopy. The mean number of septa per cell in *cdc37* temperature sensitive mutants by staining with Calcofluor.
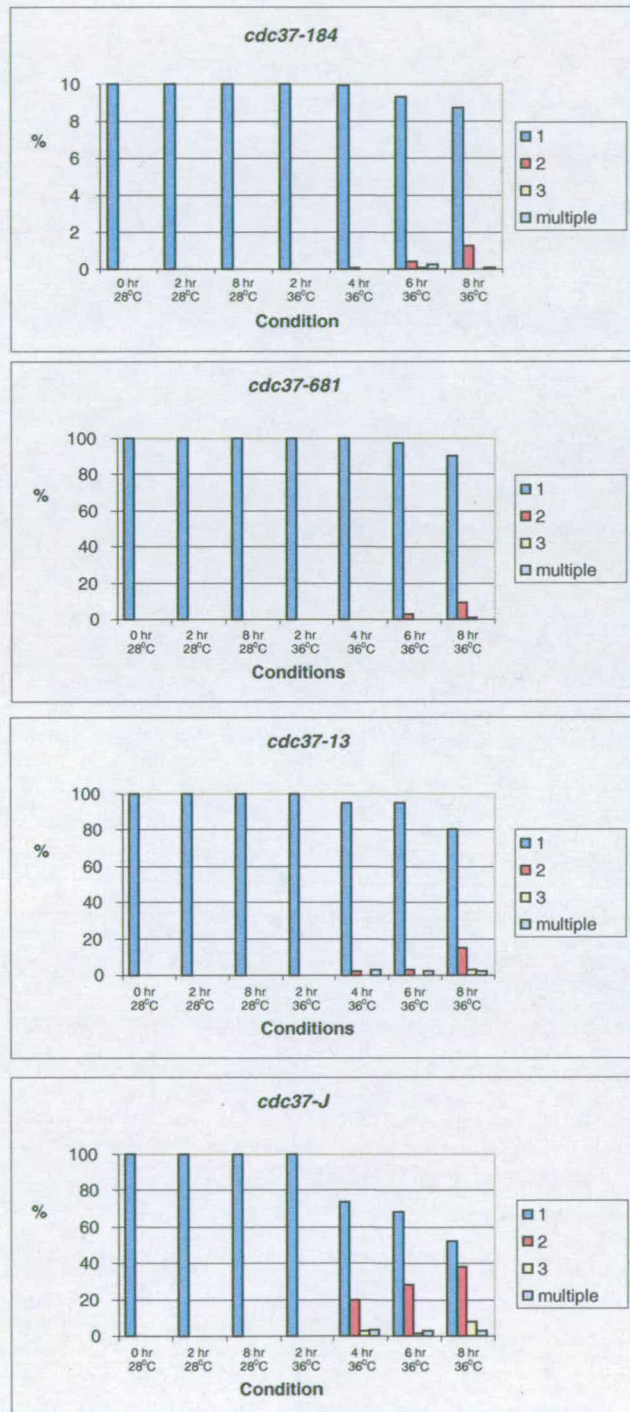
During normal cytokinesis the septum is positioned centrally along the length of the mother cell for division into two equally sized daughter cells. To better understand the aberrant septal morphologies observed in *cdc37-J* cells and to determine whether these mutants are defective in septum positioning within the cell, the position of septa was determined for *cdc37* temperature-sensitive and the *cdc37⁺* strains stained with Calcofluor. 200 cells from each sample were assayed, determining by eye whether septa were centrally positioned within the cell. Except for *cdc37-J*, *cdc37* temperature-sensitive mutant and *cdc37⁺* cells contained centrally positioned septa at 36 °C over the 8 hour time course (data not shown). 93% of septa in *cdc37-J* cells after 8 hours at the restrictive temperature were centrally positioned, indicating that *cdc37-J* cells with aberrant septal morphologies are not defective in septa positioning.

As nuclear division proceeds, an actinomyosin ring forms medially positioned within the cell, and the septum develops in alignment with this as the ring contracts during cytokinesis to produce two daughter cells (Krapp et al., 2004). To determine whether defects of the actinomyosin ring may contribute to aberrant septa morphology in *cdc37-J* cells, *cdc37* temperature-sensitive and *cdc37⁺* strains were cultured in YE as described in Section 2.7.4.3 and samples of cells were taken every two hours, fixed and stained with rhodamine-conjugated phalloidin (Chang et al., 1996) to visualise actin localisation by microscopy and DAPI to observe nuclear morphology. In ~80.8% of *cdc37⁺* cells at 28 and 36 °C the actin was localised at the cell tips (phenotype 1(the classification of phenotypes is given in Figure 5.18)), which occurs to permit cell growth in the form of elongation (Figure 5.17A). On average 6% of the cell population contained two nuclei and actin located at the cell tips (phenotype 2) and cells containing two nuclei separated by an actin contractile ring (phenotype 3) represented on average 13.1% of the cell population (Figure 5.17C). After 6 hours at 36 °C *cdc37* temperature-sensitive mutants showed a loss of actin localisation to cell tips and the actin contractile ring was absent (Figure 5.17). After 8 hours at 36 °C all *cdc37* temperature-sensitive mutants were seen to contain mislocalised abnormal actin patches in cells containing a single nucleus (phenotype 11) or divided nuclei separated by a division septa (phenotype 12) (Figure 5.17D). After 8 hours at the restrictive

Figure 5.17: The localisation of actin was examined in *cdc37* temperature-sensitive mutant and *cdc37⁺* strains cultured at 28 and 36 °C over an 8 hour time course. Samples were taken every two hours and stained with rhodamine-conjugated phalloidin and DAPI. **A** Phentoypes observed by staining with rhodamine-conjugated phalloidin and DAPI **B** pictures of phenotypes 1, 3 and 12.
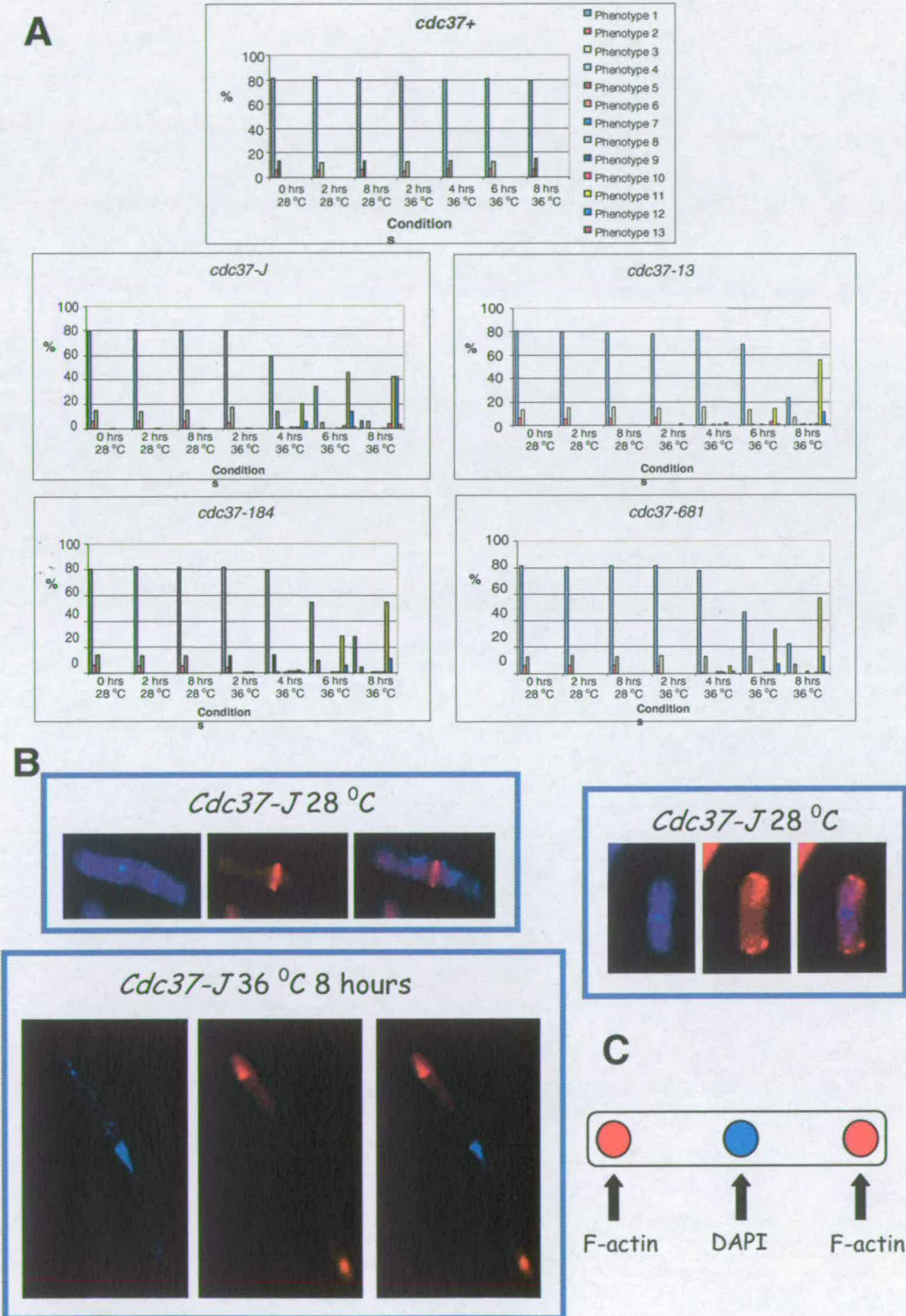
Figure 5.18: Classification of phenotype observed in *cdc37* temperature-sensitive mutant and *cdc37*[+] strains cultured at 28 and 36 °C over an 8 hour time course and stained with rhodamine-conjugated phalloidin and DAPI to investigate actin localisation.
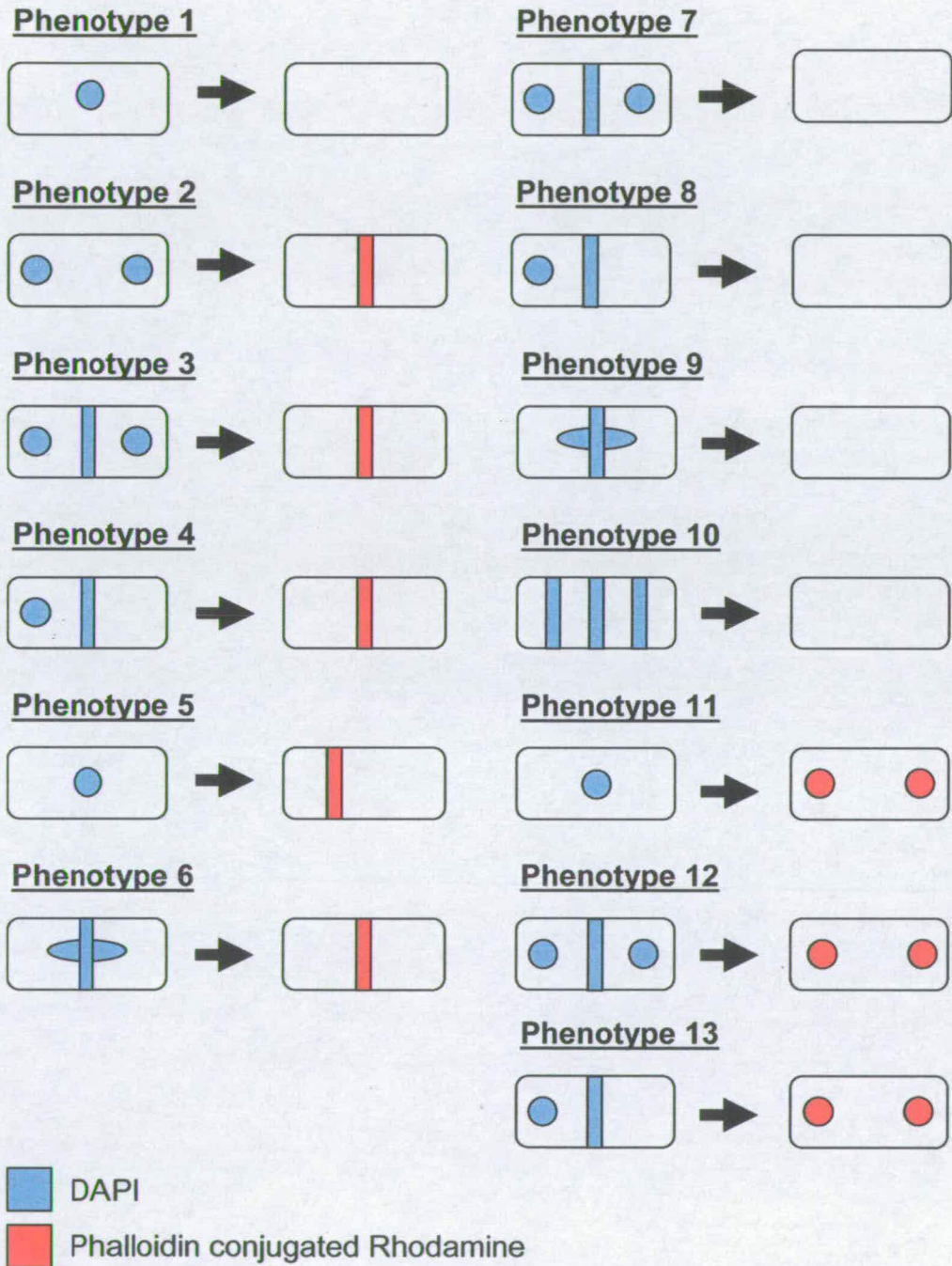
temperature 65.6%, 70%, 64.85% and 85.5% of cells from the strains *cdc37-184*, *cdc37-681*, *cdc37-13* and *cdc37-J* respectively displayed these abnormal actin patches (Phenotypes 11, 12 and 13) which were located at either end of the cell, but were separate from the cell tip wall as detailed schematically in Figure 5.17E. These aberrant actin patches may arise as a consequence of the cell cycle arrest in *cdc37* temperature-sensitive mutants or as an independent event due to loss of Cdc37 function in these mutants.

5.3.9 Morphology of microtubules in *cdc37* temperature-sensitive mutants

To identify cellular defects that may explain the cell cycle arrest and observed cytological abnormalities in the *cdc37* temperature-sensitive mutants at the restrictive temperature, immunofluorescence was carried out with the TAT1 antibody that allows microtubules to be visualised. The *cdc37⁺* strain ED1022 and *cdc37* temperature-sensitive mutants were cultured as described in Section 2.7.4.3 at 28 and 36 °C over an 8 hour time course. Samples were taken at 0, 4 and 8 hours and cells were fixed and treated for immunofluorescence with the TAT1 antibody and the anti-mouse IgG antibody Alexfluor[488] for visualisation by microscopy (Section 2.8.4). In *cdc37⁺* and *cdc37* temperature-sensitive mutant cells at both temperatures, microtubules appeared normal (Figure 5.19). The microtubules of *cdc37* temperature-sensitive mutant cells cultured at the restrictive temperature were slightly more diffuse in appearance, but this may be a visual artefact as the cells are elongated. No mitotic spindles were observed in *cdc37* temperature-sensitive mutant cells at the restrictive temperature, supporting the conclusion that cells arrest in G2.

5.3.10 Cdc37 localisation in *cdc37* temperature-sensitive mutants

The cellular distribution of the Cdc37 protein was analysed by immunofluorescence to determine its natural distribution pattern and whether its localisation changed in *cdc37* temperature-sensitive mutants at 36 °C. The *cdc37* temperature-sensitive

Figure 5.19: Microtubule morphology in *cdc37* temperature-sensitive mutants and the *cdc37⁺* strain ED1022. Strains were cultured at 28 and 36 °C over an 8 hour time course. Samples of cells were taken every two hours and processed for immunofluorescence with the TAT1 antibody against tubulin (kind gift from Keith Gull). Bar is 10 μm.
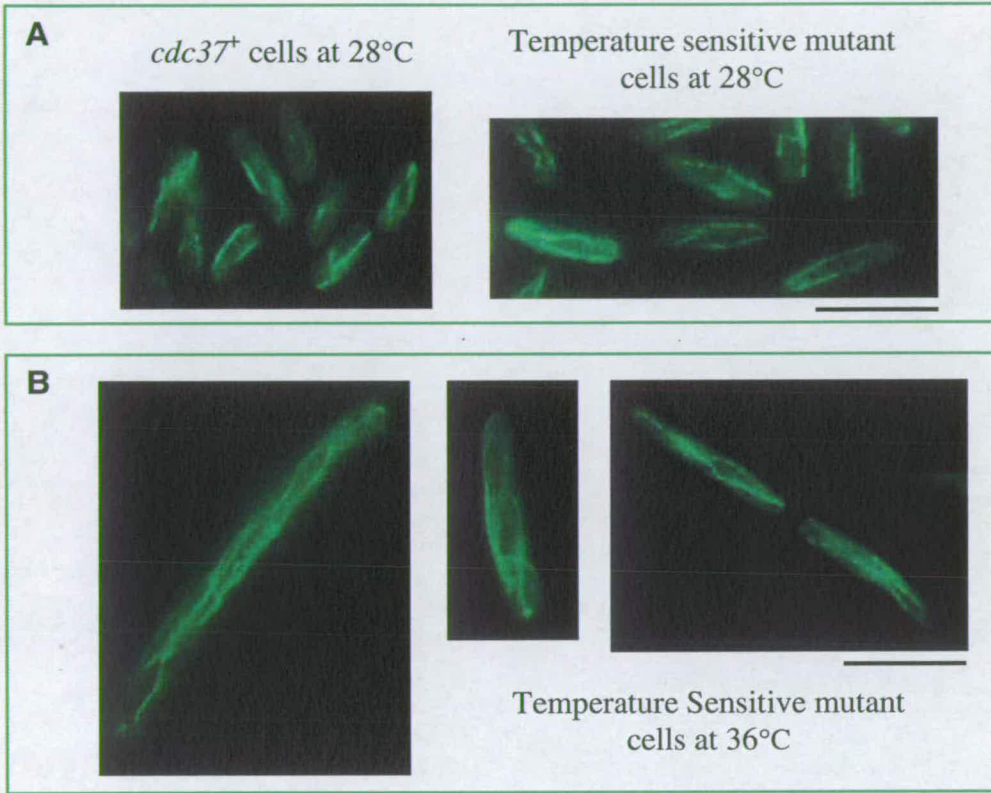
mutants and *cdc37*⁺ strain ED1022 were cultured at the 28 and 36 °C over an 8 hours time course as described in 2.7.4.3 and samples were taken every 2 hours and processed for immunofluorescence with the anti-*S. pombe* Cdc37 antibody (Section 2.8.4) and anti–rabbit IgG antibody Alexfluor[680] (Molecular Probes). In *cdc37* temperature-sensitive mutant and the *cdc37*⁺ strains, Cdc37 localisation was diffuse throughout the cell at both 28 and 36 °C over the entire 8 hour time course (Figure 5.20A). Cdc37 was seen to form punctate spots throughout the cells and does not display any specific localised pattern.

A number of conditions were tested for immunofluorescence with the anti-*S. pombe* Cdc37 antibody to ensure specific staining and to optimise the technique. Cells from *cdc37-184* and *cdc37*⁺ incubated at 28 and 36 °C for 8 hours were treated with 1:90, 1:60 and 1:30 dilutions of the *S. pombe* Cdc37 antibody, secondary antibody only and a 1:30 dilution using anti-*S. pombe* Cdc37 serum that had been depleted of antibodies. Depletion of the anti-*S. pombe* Cdc37 serum was carried out by incubating a sample of the serum with GST-Cdc37 bound to glutathione beads (Section 2.9.9). The depleted antibody serum was used in western blots against GST-Cdc37 and total *S. pombe* protein extracts to show loss of anti-*S. pombe* Cdc37 antibodies from serum (Figure 5.20B). Fluorescence of Cdc37 was clearly observed with 1:30 and 1:60 dilutions of the anti-*S. pombe* cdc37 antibody (Figure 5.20A). Staining cells with only secondary antibody or Cdc37 depleted serum did not produce any fluorescence (Figure 5.20A).

5.4   Discussion

In *S. pombe* four *cdc37* temperature-sensitive mutants have been identified and their characterisation is described in this chapter. Three temperature-sensitive mutants of *cdc37* were created in this work by random and directed mutagenesis. The temperature-sensitive mutant *cdc37-184* was generated by introducing the equivalent mutation to the *S. cerevisiae* mutant *cdc37-184* into the *S. pombe cdc37* gene. The mutants *cdc37-J* and *cdc37-13* were isolated from a total of 11,750 G418 resistant

Figure 5.20: Immunofluorescence of *cdc37* temperature-sensitive mutants and *cdc37⁺* cells with the anti-*S. pombe* Cdc37 antibody. **A** *cdc37⁺* and *cdc37-184* cells were stained with the anti-*S. pombe* Cdc37 antibody by immunofluorescence using a number of different conditions to verify specific staining. **B** Verification that the anti-*S. pombe* serum had been depleted of Cdc37 antibodies by incubation with GST-Cdc37 bound to glutathione beads. Bar is 10 μm.
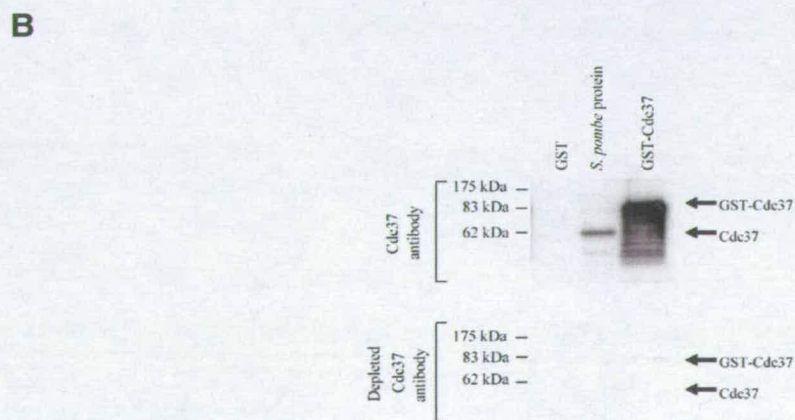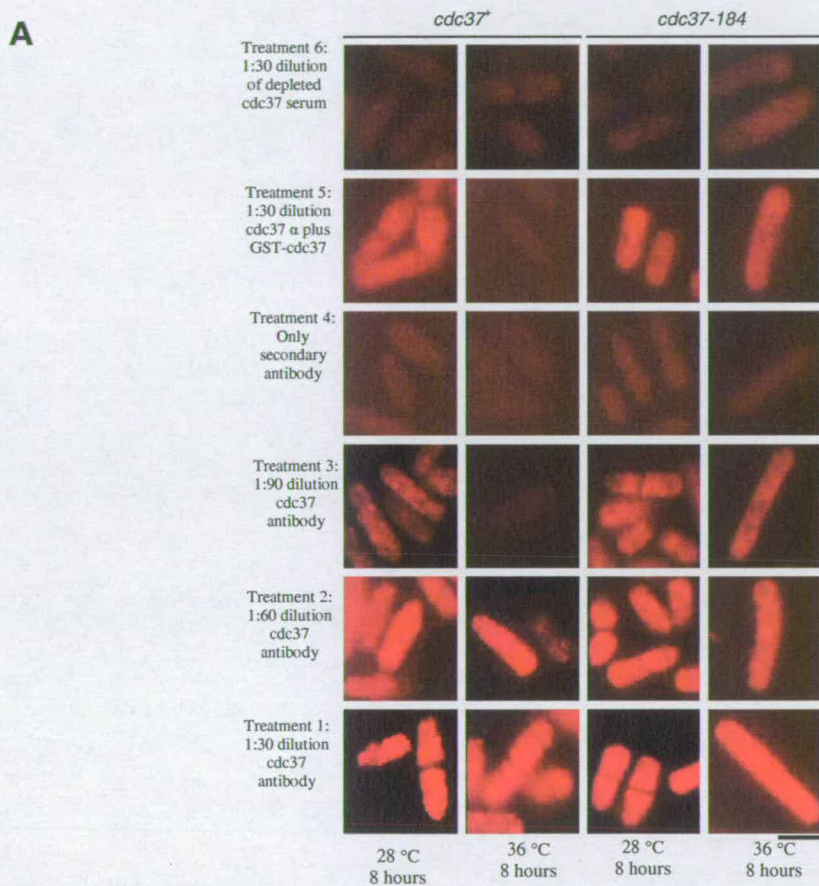
colonies, generated by random mutagenic PCR with the inclusion of $MnCl_2$ into PCR reactions. The low yield of *cdc37* temperature-sensitive mutants was not due to incorrect insertion of the PCR amplified fragments into the *S. pombe* genome, as random mutagenic PCR products were efficiently targeted to the *cdc37* locus with 92% efficiency. Therefore, mutagenic PCR conditions may have been the limiting factor in the generation of *cdc37* temperature-sensitive mutants. Equally, the number of possible temperature-sensitive mutants that can be generated for *S. pombe cdc37* may be low due to conformational and/or functional constraints. It is interesting to note that all the mutations identified in these *cdc37* temperature-sensitive mutants are located in very close proximity within the middle domain of the Cdc37 protein. Temperature-sensitive mutants of *S. pombe cdc37* may only occur by mutations within this central region, around the postulated Hsp90-binding and homodimerisation domains in the 6-helix bundle (Roe et al., 2004). Mutations within this region may inhibit Cdc37 dimerisation, Hsp90 interactions and/or negatively affect protein conformation, inhibiting proper function in a temperature dependent manner.

All four *cdc37* temperature-sensitive mutants were able to form single colonies at 25, 28 and 32 °C, but were unable to sustain colony formation at 36 °C. The ability to sustain growth was lost at 36 °C in *cdc37-681*, *cdc37-184*, *cdc37-13* and *cdc37-J* due to loss of Cdc37 function and not instability of the chaperone protein, as Cdc37 protein levels remained constant at the restrictive temperature. In the *S. cerevisiae* temperature-sensitive mutants *cdc37-34* (Fliss et al., 1997) and *cdc37-1* (Gerber et al., 1995) mutant Cdc37 protein levels were reduced at both the permissive and the restrictive temperature. Loss of Cdc37 function at the restrictive temperature in *S. pombe cdc37* temperature-sensitive mutants did not affect Cdc37 cellular localisation. The Cdc37 protein remained diffuse in its localisation throughout the cell at 28 and 36 °C over the 8 hour time course and no distinct alterations in the pattern of distribution were observed. When *S. pombe* Cdc37 was tagged with GFP, it was also found to localise throughout the cell, although distinct localisation was observed in the chromatin region of the nucleus (Tatebe and Shiozaki, 2003). This slight dissimilarity in localisation may reflect differences in experimental technique.

The loss of Cdc37 function in *S. pombe cdc37* temperature-sensitive mutants was rapid as cells stopped dividing within 2 hours at the restrictive temperature. The *cdc37* temperature-sensitive mutants arrest at the non-permissive temperature with elongated cells, displaying a *cdc* phenotype. The *cdc* phenotype is characteristic of cells that have undergone a cell cycle arrest but can continue to grow at the tips producing elongated cells. Further analysis showed that all four *S. pombe cdc37* temperature-sensitive mutants arrest with a replicated DNA content, having passed through S phase. Cytological analysis revealed that the majority of *cdc37* temperature-sensitive mutant cells at the restrictive temperature contained a single nucleus per cell with uncondensed chromatin, indicating a G2 arrest. In accordance with this, Drosophila cells treated with *cdc37* RNAi arrested cell division with a 4N DNA content, having passed through S-phase (Lange et al., 2002). This is distinct from *S. cerevisiae* where the majority of *CDC37* temperature-sensitive mutants arrest in Start (Reed, 1980; Reed, 1980; Valay et al., 1995). The exception is *cdc37-34* which arrests at the restrictive temperature with a phenotypically mixed cell population that can be grouped into three categories; single uninucleated cells in G1 arrest, budded uninucleated cells arrested in G2 and budded binucleate cells with a mitotic arrest (Dey et al., 1996). It is interesting to note that a proportion of *S. pombe cdc37-184* cells produced an unreplicated 1C DNA content peak after 2 hours at the restrictive temperature, and then all cells arrested in G2 after 4 hours. It is possible that at the restrictive temperature, *cdc37-184* cells underwent a delay in G1 prior to arresting in G2.

cdc37-J was the only temperature-sensitive mutant to leak beyond this G2 cell cycle arrest after 8 hours at the restrictive temperature. These cells had entered mitosis, dividing their nuclei and forming a division septum between them. These septa were often morphologically abnormal, occurring in multiple copies or being distorted in structure, which may contribute to the cells inability to accomplish cytokinesis. Interestingly, these aberrant septa were always positioned correctly within the cell, indicating that positioning of septa was not defective. Actin was not found to form a contractile ring after 6 hour at 36 °C in these mutant cells, which may have been a contributing factor to the failure to carry out cytokinesis and the aberrant septal

morphologies observed. Interestingly, Drosophila cells harbouring *cdc37* mutants have also been seen to be defective in cytokinesis (Lange et al., 2002).

In all four *cdc37* temperature-sensitive mutants the microtubules displayed no aberrant features at any time point at the restrictive temperature. However, after 6 hours at 36 °C all *cdc37* temperature-sensitive mutants contained mislocalised actin. The appearance of aberrant actin patches towards the cell tips has not been reported elsewhere in the literature. Actin mislocalising to produce numerous actin patches has previously been observed in the *S. pombe* Δ*Myo1* mutant which has defects of the actin cytoskeleton, forming numerous delocalised actin patches (Lee et al., 2000). In *S. pombe cdc37* temperature-sensitive mutants the mislocalisation of actin to form two distinct patches at the restrictive temperature may be a secondary effect of cell cycle arrest in these mutants, or independent of the cell cycle arrest.

The aim of generating temperature-sensitive mutants that show the effect of loss of Cdc37 function within a cell cycle at the restrictive temperature was achieved. All four *S. pombe cdc37* temperature-sensitive mutants arrest within 2 hours at the non-permissive temperature in G2 with a classic *cdc* phenotype. Interestingly, when Cdc2 was inactivated in *S. pombe,* cells arrested at the G2/mitosis boundary but continued to elongate with an undivided nucleus (Snell and Nurse, 1994). Cdc2 could be a client of Cdc37 in *S. pombe* that relies on this chaperone for function. When Cdc37 function is lost in *cdc37* temperature-sensitive mutants at the restrictive temperature, Cdc2 function could be abolished inducing a cell cycle arrest at the G2/mitosis boundary. The Cdc2 homologue in *S. cerevisiae*, Cdc28, was found to physically interact with Cdc37 when truncated and expressed in the yeast two hybrid assay (Mort-Bontemps-Soret et al., 2002). Investigations of Cdc2 as a postulated client of Cdc37 and the principal cause for the observed cell cycle arrest in Cdc37 temperature-sensitive mutants is described in Chapter 6.

Chapter 6:

S. pombe Cdc37 interactions with the co-chaperone Hsp90 and the putative client protein kinase Cdc2

6.0   Cdc37 interactions with co-chaperones and clients in *S. pombe*

6.1   Introduction

Cdc37 has been identified in a range of high molecular weight complexes (Boudeau et al., 2003; Mahony et al., 1998; Stepanova et al., 1996; Wang et al., 2002) indicating that this chaperone interacts with a wide variety of client proteins and co-chaperones. Cdc37 and the co-chaperone Hsp90 have been found by gel filtration in the same 450 kDa high molecular weight complex, associated with the client protein kinase Cdk4 in NIH-3T3 cells (Stepanova et al., 1996). It is widely believed that the molecular chaperone Cdc37 binds client proteins and delivers them to the co-chaperone Hsp90, forming a tertiary complex (MacLean and Picard, 2003). Although, no tertiary complex between Cdc37, Hsp90 and a client protein has been isolated to date; most likely due to the transient nature of this complex which makes detection of these interactions problematic and these associations are believed to be more unstable in yeast systems than higher eukaryotes (Farrell and Morgan, 2000).

A complex consisting of the interacting domains of yeast Hsp90 and human Cdc37 has been crystallised and its structure determined (Roe et al., 2004). Amino acids 164-170 and 204-208 of human Cdc37 were found to form a hydrophobic patch that interacts with the N-terminal region of yeast Hsp90 (Roe et al., 2004). Human Cdc37 binds both the N-terminal domains and the adjacent linker regions of the Hsp90 dimer (Zhang et al., 2004). Cdc37 binds to Hsp90 as a dimer (Siligardi et al., 2002) at a 1:1 molar ratio (Zhang et al., 2004), preferentially binding the non-ATP bound form of Hsp90 and suppressing ATP hydrolysis (Siligardi et al., 2002). It is widely believed that once Hsp90 has bound the client, Cdc37 is released from the tertiary complex. Hsp90 can then carry out ATP turnover which occurs as a two step process, promoting conformational changes of the Hsp90-client complex (McLaughlin et al., 2004).

The interaction between Cdc37 and Hsp90 in yeast has been less well studied as the tertiary complex is much less stable than in higher eukaryotes (Farrell and Morgan, 2000). In *S. cerevisiae* recombinant GST-Cdc37 was found to interact with Hsp90 in pull-down experiments (Donze and Picard, 1999). An interaction between these two co-chaperones has also been detected in *S. cerevisiae* by yeast two-hybrid assay using an Hsp90 containing mutations that inhibit ATP hydrolysis (Millson et al., 2004). The work described in this chapter investigates the association between Hsp90 and Cdc37 in the yeast *S. pombe*.

Client proteins rely on molecular chaperones such as Cdc37 and Hsp90 for activation, folding and maintenance of cellular protein levels. Client proteins of Cdc37 bind the N-terminal domain of Cdc37 (Shao et al., 2003) and are predominantly protein kinases such as Raf-1 (Grammatikakis et al., 1999) and the cyclin dependent kinase (Cdk) Cdk4 (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996). Cdk proteins are crucial for progression of the cell cycle; loss of Cdks from the cell results in a cell cycle arrest. In the previous chapter (Chapter 5) *cdc37* temperature-sensitive mutants in *S. pombe* were found to arrest the cell cycle at the restrictive temperature in G2. In *S. pombe*, Cdc2 is the Cdk that is crucial for the transitions between G2/mitosis and G1/S phase. Cdc2 protein levels remain constant throughout the cell cycle (Alfa et al., 1989). Regulation of cell cycle progression by Cdc2 is controlled by modulating its activity at different stages by associating with specific cyclin partners and both negative and positive phosphorylation. Prior to the G2/mitosis transition, Cdc2 is phosphorylated on threonine 167 (Gould et al., 1991) to promote cyclin association and on tyrosine 15 (Gould and Nurse, 1989) to keep the Cdk inactive during interphase. Phosphorylation on threonine 167 is carried out by the Cdk-activating kinases (CAKs) (Lee et al., 1999) while tyrosine 15 is phosphorylated primarily by Wee1 (Gould and Nurse, 1989). Phosphorylation on threonine 167 allows Cdc2 to stably associate with a 482 amino acid B-type cyclin, Cdc13 (Booher and Beach, 1988; Booher et al., 1989; Hagan et al., 1988). The Cdc13 protein shows periodic changes in abundance (Alfa et al., 1989), accumulating through interphase and then being actively degraded at the metaphase-anaphase transition. To initiate mitosis, Cdc2 bound to Cdc13 is activated by dephosphorylation on tyrosine 15 by the

phosphatase Cdc25 (Nurse, 1997). Active Cdc2 is then localised to the nucleus by cyclin Cdc13 and the active complex promotes mitosis (Alfa et al., 1989). In light of the findings with *S. pombe cdc37* temperature-sensitive mutants, interactions between Cdc2 and Cdc37 were investigated in *S. pombe* and are described in this chapter.

Little is known about the interactions between Cdc37 and the co-chaperone Hsp90 and client protein kinases in *S. pombe*. These interactions in yeast systems are though to be extremely unstable (Farrell and Morgan, 2000) making biochemical studies problematic. In addition, the non-specific binding affinity of both Cdc37 and Hsp90 further complicates these investigations. Often mutational analysis or *in vitro* experiments have had to be employed to identify Cdc37 interactions, such as using truncation mutants of both *cdc37* and *cdc28* (the *S. cerevisiae* homologue of Cdc2) to detect an interaction by yeast two-hybrid assay (Mort-Bontemps-Soret et al., 2002). In *S. pombe* the only client protein identified to date is the kinase Spc1, which was found to co-immunoprecipitate with Cdc37 (Tatebe and Shiozaki, 2003). In this work the interactions between the co-chaperone Hsp90 and the putative client protein kinase Cdc2 with Cdc37 in *S. pombe* have been investigated using both genetic and biochemical techniques.

6.2 Hsp90

6.2.1 Genetic interactions between Hsp90 and Cdc37 in *S. pombe*

To investigate whether *cdc37* and *swo1* (the gene in *S. pombe* that codes for Hsp90) interact at the genetic level, *cdc37* temperature-sensitive mutants *cdc37-681*, *cdc37-184*, *cdc37-13* and *cdc37-J* were crossed with the Hsp90 temperature-sensitive mutant *swo1-26* (Aligue et al., 1994). (The cross between *cdc37-681* and *swo1-26* was carried out by Ina Martin). Resulting asci were dissected and the progeny were incubated at 28 °C and then replicated to 28 and 36 °C to determine the genotype of germinated spores. Table 6.1 shows the phenotypes of progeny from dissected asci for each cross. Progeny that contained both mutant alleles were identified by determining the phenotypes of each viable spore in each tetrad, assuming that segregation is 2:2.

Spores containing both mutant alleles were found to be synthetically lethal at temperatures permissive for the individual mutations indicating that *cdc37* and *swo1* interact at the genetic level in *S. pombe*.

To investigate further the genetic interaction between Hsp90 and Cdc37 in *S. pombe*, *swo1* was expressed from pREP81 and pREP1 in all four *cdc37* temperature-sensitive mutants to determine whether it could compensate for reduced Cdc37 function at the restrictive temperature. Strains were plated as serial dilutions and incubated at 28 and 36 °C for 4 days. The increased expression of *swo1* from high and low copy plasmids (pREP1 and pREP81 respectively) was able to partially rescue one of the four *cdc37* temperature-sensitive mutants at the restrictive temperature, *cdc37-13* (Figure 6.1A). Increased expression of *swo1* was able to support a low level of growth at the restrictive temperature in *cdc37-13*, although the cells displayed distinctly aberrant phenotypes, being either elongated or small and round (Figure 6.1B). These data indicate that Hsp90 can partially compensate for reduced Cdc37 function, in certain circumstances. It should be noted that, *cdc37-J* was able to support colony formation at the restrictive temperature with expression of all four plasmids, including pREP vector only. However, at 36 °C cells were elongated in appearance indicating that the phenotype of this mutant cannot be rescued by increased expression of *swo1*.

6.2.2 Biochemical interactions between Cdc37 and Hsp90 in *S. pombe*

6.2.2.1 Yeast two-hybrid assay

To study the interaction between the *S. pombe* co-chaperones Hsp90 and Cdc37 the yeast two-hybrid system was employed for detecting protein-protein interactions. A schematic diagram of the yeast two-hybrid system in Figure 2.6 shows how this system works. The DNA activating domain of Gal4 was fused to one candidate protein and the DNA binding element of Gal4 was fused to the other candidate protein. If the two candidate proteins physically interact then the DNA binding and

Table 6.1: Tetrad types and progeny phenotypes from crosses between *cdc37* temperature-sensitive mutants *cdc37-681* (carried out by Ina Martin), *cdc37-184*, *cdc37-13* and *cdc37-J* with the Hsp90 temperature-sensitive mutant *swo1-26* (Aligue et al., 1994).

Tetrad types	Genotypes of progeny	*cdc37-681*	*cdc37-184*	*cdc37-13*	*cdc37-J*
Parental ditype	**2** *cdc37* **and 2** *swo1* **temperature-sensitive mutants spores**	4	6	9	9
Tetrad type	**1 wildtype, 1 dead, 1** *cdc37* **and 1** *swo1* **temperature-sensitive mutant spores**	2	6	4	2
Non-parental ditype	**2 wildtype and 2 dead spores**	1	2	7	2

Figure 6.1: Expression of *swo1* from pREP81 and pREP1 in *cdc37* temperature-sensitive mutants, *cdc37-681*, *cdc37-184*, *cdc37-13* and *cdc37-J*, at 28 and 36 °C. **A** Growth on plates of serial dilutions of *S. pombe* strains at 28 and 36 °C **B** cell morphology of *cdc37-13* with overexpressed *swo1* at 36 °C.
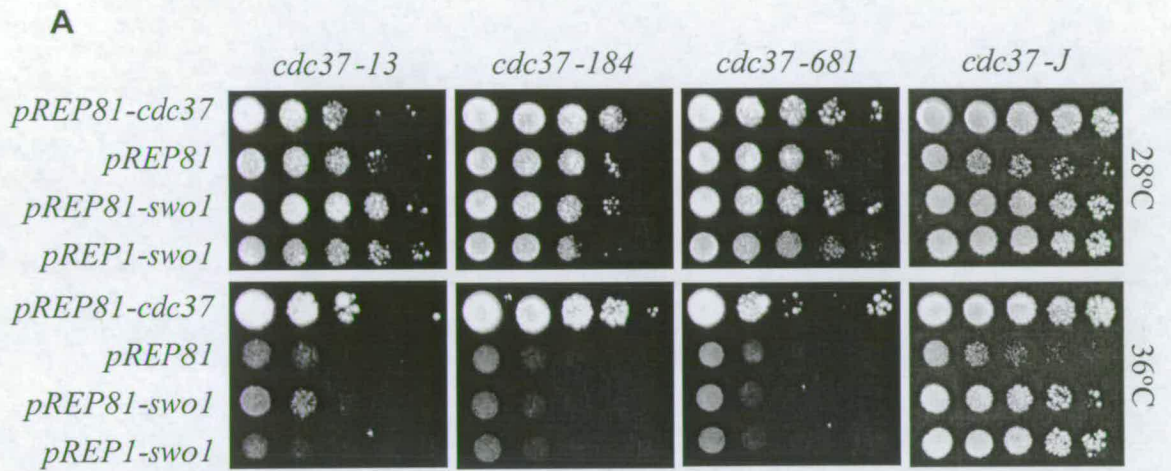
DNA activating domains of Gal4 come together and enable transcriptional activation of the reporter gene β-galactosidase. The level of β-galactosidase can be assayed by its enzymatic ability to hydrolyse the substrate X-gal to produce a blue color.

The vectors pADC (DNA activating domain) and pBDC (DNA binding domain) (Millson et al., 2003) were used to enable N- and C- terminal tagging of Cdc37 and Hsp90. *S. pombe swo1* was cloned into both vectors to allow C-terminal tagging as the N-terminal domain of this chaperone is known to bind Cdc37 (Roe et al., 2004). *S. pombe cdc37* was cloned into both vectors for both N- and C- terminal tagging. Human *cdc37* (p50) was also used, as an interaction with Hsp90 has been determined using various techniques, and was cloned into both vectors to enable N- and C- terminal tagging. The *swo1, S. pombe* and human *cdc37* genes were PCR amplified with mutagenised oligonucleotides to introduce *SmaI* sites at the 5' and 3' ends, and to remove stop codons where appropriate. Fragments were then cloned into the appropriate vectors. Plasmid constructs were transformed into *S. cerevisiae* strain AH109 using the lithium acetate method (Section 2.10.3.1) in combination (Table 6.2) and individually, to ensure that they did not auto-activate. Positive and negative control plasmids pGADT7-T and pGBKT7-53 were also used to determine whether the system was working. The colony lift filter assay (Section 2.10.5.1) was carried out, identifying positive interactions by their ability to turn the yeast cells blue. The colonies tested did not turn blue except for the positive control, indicating that an interaction between Hsp90 and *S. pombe* or human Cdc37 was not detected by yeast two-hybrid (Figure 6.2).

6.2.2.2 GST-Cdc37 pull-down to detect an interaction between recombinant Cdc37 and Hsp90

The GST pull-down technique was used with the aim of detecting an interaction between recombinant *S. pombe* or human Cdc37 and native *S. pombe* Hsp90. *S. pombe* and human *cdc37* were cloned into the vector pGEX-1 (N-terminal GST tag) for high level expression in BL21 *E. coli*. (*S. pombe cdc37* was cloned into the pGEX-

Table 6.2: Combination of plasmids transformed into AH109 *S. cerevisiae* strain for yeast two-hybrid analysis.

Plasmid 1	Plasmid 2
pADC-Cdc37	Hsp90-pBDC
pBDC-Cdc37	Hsp90-pADC
Cdc37-pADC	Hsp90-pBDC
Cdc37-pBDC	Hsp90-pADC
pADC-p50	Hsp90-pBDC
pBDC-p50	Hsp90-pADC
P50-pADC	Hsp90-pBDC
P50-pBDC	Hsp90-pADC
pGADT7-T	pGBKT753

Figure 6.2: Yeast two-hybrid colony lift filter assay using the *Gal4* system to identify an interaction between *S. pombe* or human Cdc37 and Hsp90. Colonies were incubated with X-gal to observe their ability to turn blue to identify an interaction between candidate proteins.

<u>Controls</u>

Positive control
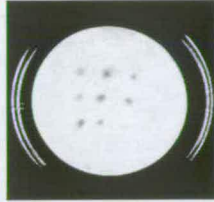
Negative control

pGADT7-T &
pGBKT7-53
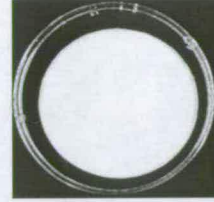
pADC & *cdc37*-pBDC

<u>*S. Pombe* Cdc37 and Hsp90</u>
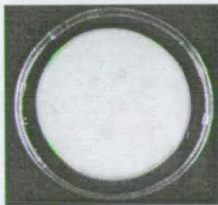
cdc37-pADC &
swo1-pBDC
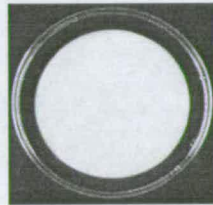
pADC-*cdc37* &
swo1-pBDC
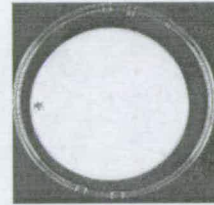
cdc37-pBDC &
swo1-pADC
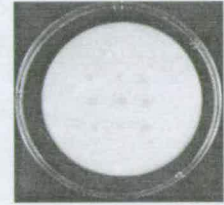
pBDC-*cdc37* &
swo1-pADC

<u>Human Cdc37 (p50) and Hsp90</u>
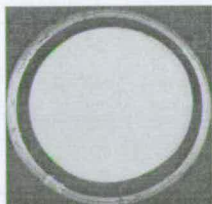
cdc37-pADC &
swo1-pBDC
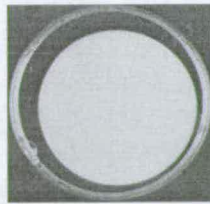
pADC-*cdc37* &
swo1-pBDC
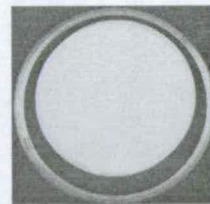
cdc37-pBDC &
swo1-pADC
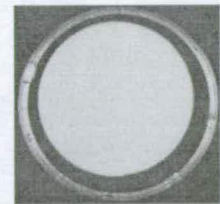
pBDC-*cdc37* &
swo1-pADC

1 vector by Ina Martin). GST and GST fusion proteins were purified from bacterial lysates by affinity chromatography using immobilised glutathione. GST, GST-*S. pombe* Cdc37 and GST-human Cdc37 were then bound to glutathione beads and incubated with whole cell protein extracts of the *S. pombe* strain ED1537 in which the chromosomal *swo1* gene is tagged with the HA epitope (Section 2.9.9). Samples were washed in appropriate buffer and treated with PreScission™ Protease to cleave the GST-tag from fusion proteins, as GST-*S. pombe* Cdc37 has the same mobility by SDS PAGE as Hsp90-HA. The supernatant, containing the recombinant Cdc37 proteins, and the glutathione beads were separated by centrifugation and analysed by SDS PAGE and western blot. Probing western blots with the anti-HA antibody revealed that Hsp90-HA was present in both the beads matrix and supernatant fractions, presumably due to non-specific binding of GST, GST fusion proteins and glutathione bead matrix (Figure 6.3). To try to reduce the non-specific binding of Hsp90-HA a range of buffers was used with this technique: PBS, TEG, RIPA and R Buffer (See Materials and Methods). However, in all cases Hsp90-HA was found in both the bead matrix and supernatant fractions, presumably interacting non-specifically with GST, GST-fusion proteins and the bead matrix.

An interaction between Hsp90 and Cdc37 could not be detected by GST pull-down experiments. The non-specific binding affinity of Hsp90-HA makes detection of an interaction between Cdc37 and Hsp90 problematic, especially if the association between these co-chaperones is weak and unstable in *S. pombe*. Furthermore, Cdc37 is known to be phosphorylated *in vivo* in *S. cerevisiae* (Bandhakavi et al., 2003), rat (Miyata and Nishida, 2004) and human (Shao et al., 2003) cells and mutation of the phosphorylatable residues in *S. cerevisiae* Cdc37 greatly reduced its binding affinity towards the co-chaperone Hsp90 by yeast two-hybrid (Millson et al., 2004). Recombinant Cdc37 from *E. coli* is not phosphorylated and may reduce the binding affinity of Cdc37 towards Hsp90 in this *in vitro* GST pull-down assay. However, mutations of human Cdc37 serine 13 to alanine or glutamic acid only reduced Cdc37 binding affinity towards Hsp90 by 40 and 10% respectively (Shao et al., 2003), although the interaction between these two co-chaperones is thought to be tighter in mammalian systems than in yeast (Farrell and Morgan, 2000).

Figure 6.3: GST pull-down assay using recombinant *S. pombe* and human Cdc37 to detect an interaction with Hsp90-HA from native *S. pombe* protein extracts. These experiments were treated with PreScission^{TM} Protease as GST-*S. pombe* Cdc37 has the same mobility as Hsp90-HA by SDS PAGE. Hsp90-HA was identified by western blot analysis with anti-HA antibodies and all other proteins were identified by Coomassie staining of SDS polyacrylamide gels.
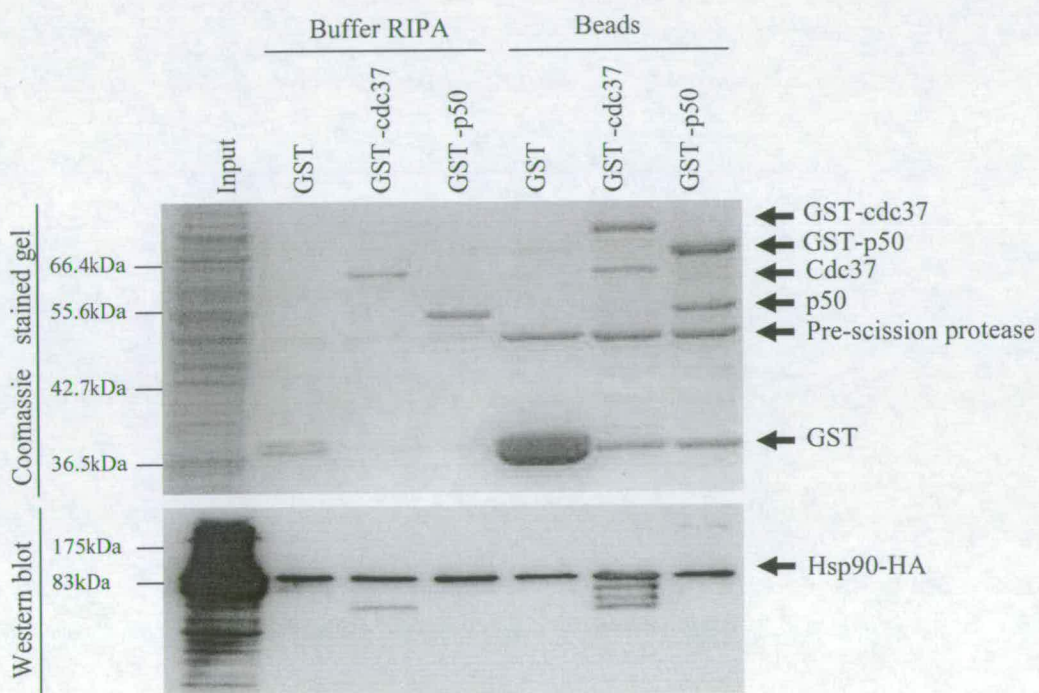
6.2.2.3 Immunoprecipitation (IP)

An alternative approach was taken to detect an interaction between Cdc37 and Hsp90 using co-immunoprecipitation from whole cell protein extracts, so that phosphorylation or other modifications would not be a limiting factor. Whole cell protein extracts from the *S. pombe* strain ED1537 containing a chromosomally tagged Hsp90-HA were made and co-immunoprecipitation (IP) experiments were carried out with both the anti-HA antibody and anti-*S. pombe* Cdc37 antibody, and no antibody or anti-rat IgG antibody in control IPs (Section 2.9.5). The immunoprecipitates were then analysed by western blot with anti-HA and anti-*S. pombe* Cdc37 antibodies. IPs with the anti-HA and anti-*S. pombe* Cdc37 antibodies precipitated Cdc37 and Hsp90-HA respectively. However, control immunoprecipitates also precipitated Hsp90-HA and/or Cdc37 (Figure 6.4A), indicating that these chaperone proteins display non-specific binding in these experiments. Therefore, numerous IP conditions were changed, as laid out in Table 6.3 in order to optimise the experimental technique. IP conditions were optimised so that non-specific binding by either co-chaperone did not occur (the technique is described in Section 2.9.5), but no interaction between Hsp90-HA and Cdc37 was detected by IP (Figure 6.4B). Detecting an interaction between Cdc37 and Hsp90-HA may be problematic as the association may be extremely unstable, although it is possible that these two proteins do not interact in *S. pombe*.

6.2.2.4 Size exclusion chromatography

Previous experiments had been unable to identify an interaction between Hsp90 and Cdc37. A new approach was taken using size exclusion chromatography, to determine whether Cdc37 was present in a high molecular weight complex in *S. pombe* which might also include the co-chaperone Hsp90. Molybdate was included throughout these experiments as it is thought to stabilise Cdc37 interactions (Hartson et al., 2000; Hutchison et al., 1992). First, recombinant Cdc37 was studied using a Superose 6 column to establish the elution pattern of the chaperone alone. The majority of recombinant Cdc37 was found to elute at around 200 kDa (Figure 6.5A), which was surprising given the predicted molecular weight of 64kDa. However, human Cdc37 is

Table 6.3: Conditions changed in co-immunoprecipitation experiments to optimise the technique.

Condition changed	Details of variables	
Buffer	RIPA	150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer 1	150 mM NaCl, 0.5% NP-40, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer 2	50 mM NaCl, 1% NP-40, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer 3	100 mM NaCl, 1% NP-40, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer A	10 mM NaCl, 0.01% Triton-X, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer B	50 mM NaCl, 0.1% Triton-X, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer C	10 mM NaCl, 0.35% Triton-X, 50 mM Tris [pH7.5], 10% glycerol & protease inhibitors
	Buffer TS	As described in Tatebe & Shiozaki 2004
Number of Washes	IPs were washed 3, 4 and 5 times for comparison	
Amount of buffer used in washes	500 µl and 1 ml of buffer used to wash IPs	
Antibody	Incubation of antibody with the Sepharose beads prior to the addition of *S. pombe* whole cell protein extracts.	
	Incubation of antibody with whole cell protein extracts from *S. pombe* prior to addition of Sepharose beads.	
Amount of antibody in IPs	2, 4 or 6 µl of antibody in IPs	
Method of making *S. pombe* whole cell protein extracts	Harvesting cells by centrifugation and making protein in a Ribolyser.	
	Harvesting cells by centrifugation and making protein by vortexing tubes containing 500 µl or 1 ml of acid washed glass beads.	
	Harvesting cells by filtration and making protein by vortexing tubes containing 500 µl or 1 ml of acid washed glass beads.	
Protein concentration	125 µg, 250 µg, 500 µg, & 1 mg of whole cell protein extracts	
Amount of Cdc37	Protein was made from strains with endogenous Cdc37	
	Increasing the level of Cdc37 by expressing *cdc37* from high and low copy plasmids pREP1 or pREP81	
Amount of Sepharose beads	5, 10, 20, 30 and 50 µl of beads in an equal volume of buffer	
Type of Sepharose beads	Protein A Sepharose beads	
	Protein G Sepharose beads	
BSA	Incubation of Sepharose beads with BSA prior to IPs to reduce non-specific binding	
Eppindorf	Transfer to a new tube after washes 1 and 4	
Molybdate	IPs were carried out with and without 20 mM molybdate	

Figure 6.4: Immunoprecipitation (IP) experiments to detect an interaction between Hsp90-HA and Cdc37 from native *S. pombe* protein extracts. Western blots were carried out with anti-HA and anti *S. pombe* Cdc37 antibodies. **A** IP experiments show that Cdc37 non-specifically binds in control IPs. **B** IP experiments after the technique was optimised eliminating non-specific Cdc37 and Hsp90-HA binding, but no interaction was detected.
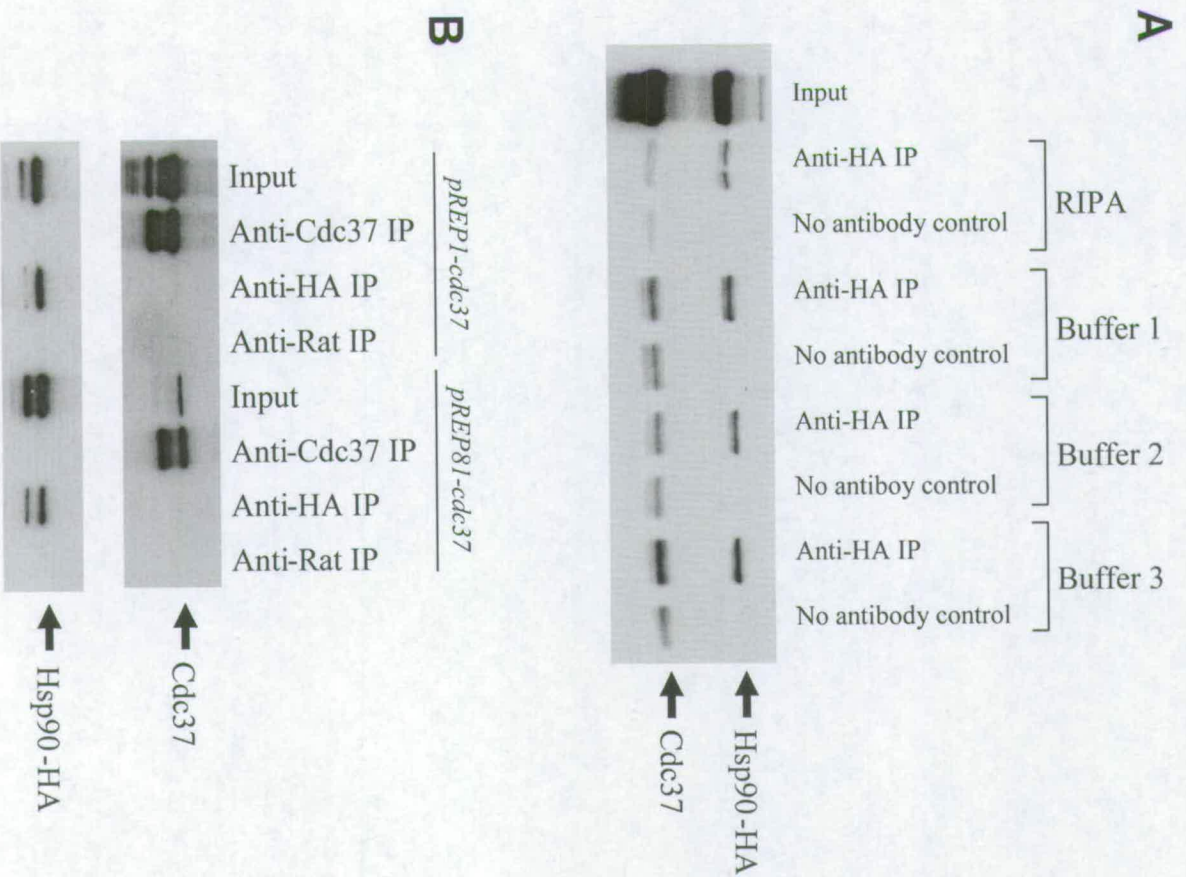
Figure 6.5: **A** Small scale size exclusion chromatography of recombinant Cdc37 and native *S. pombe* protein extracts using a Superose 6 column. Western blot analysis with anti-*S. pombe* Cdc37 antibody and anti-HA antibody to determine the elution pattern of Cdc37 and Hsp90-HA. **B** Large scale size exclusion chromatography using a Sephacryl S-300 HR 26/60 column and western blot with anti-*S. pombe* Cdc37 antibody and anti-HA antibody to determine the elution pattern of Cdc37 and Hsp90. Immunoprecipitation experiments on fractions containing the Cdc37 high molecular weight complex were carried out to identify an interaction between Cdc37 and Hsp90 and western blots were probed with anti-*S. pombe* Cdc37 antibody and anti-HA antibody.
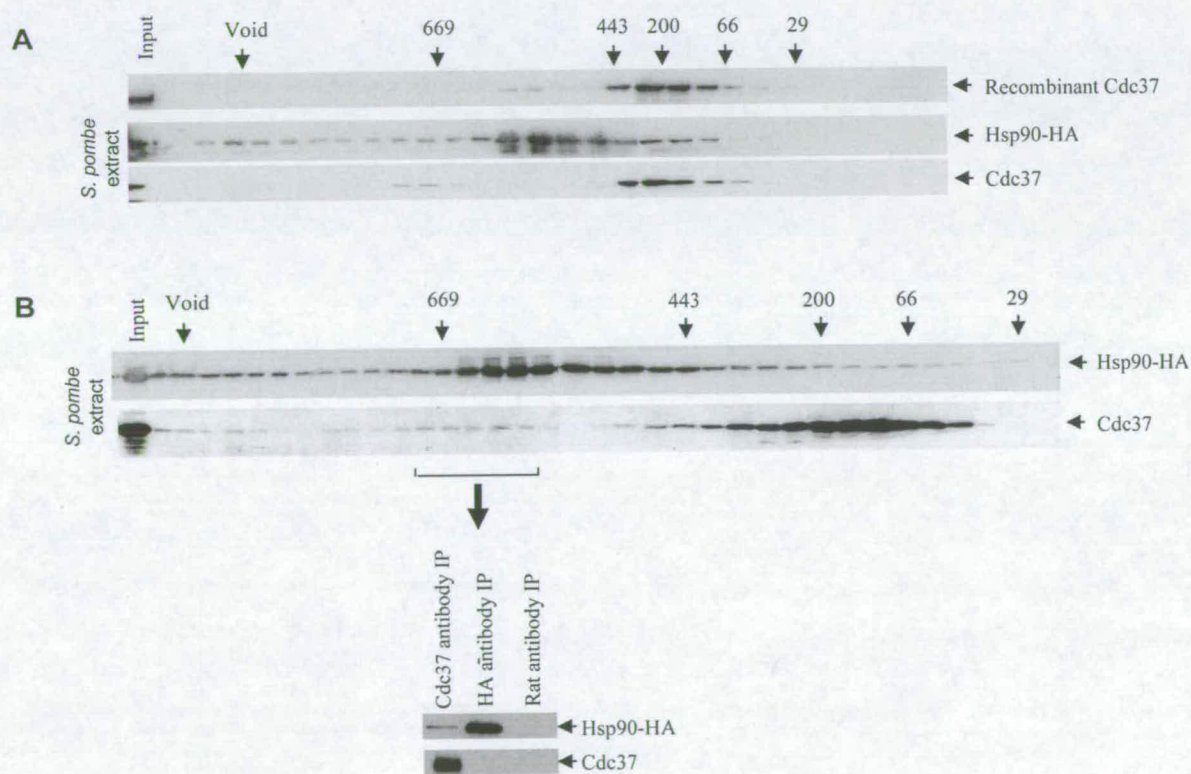
a ~50 kDa protein that exists as a dimer in its native state (Roe et al., 2004) and is structurally elongated which might affect its apparent size by size exclusion chromatography and explain the elution profile of *S. pombe* Cdc37. Size exclusion chromatography of *S. pombe* whole cell protein extracts showed the majority of Cdc37 also eluted at around 200 kDa (Figure 6.5B). Interestingly a small proportion of total Cdc37 protein eluted as a high molecular weight complex(es) at approximately 669 kDa, but no recombinant Cdc37 was found to elute at this position. These data suggest that *in vivo*, a small fraction of Cdc37 interacts stably with other proteins to form a high molecular weight complex. It is interesting to note that the elution pattern of the Cdc37 high molecular weight complex overlaps with the elution pattern of Hsp90-HA in these experiments.

To analyse further the high molecular weight complex of Cdc37, a larger size exclusion chromatography experiment was carried out. The aim was to generate adequate material to carry out IPs on fractions containing the Cdc37 high molecular weight complex to determine whether it also contains Hsp90-HA. Whole cell protein extracts from *S. pombe* ED1537 were made and treated by size exclusion chromatography using a Sephacryl S-300 HR 26/60 column. Fractions were run on SDS PAGE and western blot analysis was carried out using anti-*S. pombe* Cdc37 and anti-HA antibodies. As previously observed the majority of *S. pombe* Cdc37 eluted at around 200 kDa (Figure 6.5B). Only a small fraction of total Cdc37 protein eluted as a high molecular weight complex of approximately 669 kDa which overlapped with the elution pattern of Hsp90-HA (Figure 6.5A). Fractions containing Cdc37 in a high molecular weight complex were pooled and used as a source for IPs (Section 2.9.5). IPs using the anti-*S. pombe* Cdc37 antibody co-precipitated Hsp90-HA, indicating that these two co-chaperones co-IP from the high molecular weight complex (Figure 6.5B). However, the reverse IP using the HA antibody did not yield Cdc37. This may be due to a small relative amount of Hsp90 in the Cdc37 complex or an inability of the HA-antibody to access the epitope tag within the complex.

6.3 Cdc2

6.3.1 Genetic interactions between Cdc37 and Cdc2

To investigate the genetic interaction between Cdc2 and Cdc37, the temperature-sensitive mutant *cdc37-681* was crossed with *cdc2-L7* and *cdc2-33* and the progeny phenotypes studied. (These experiments were carried out by Ina Martin). Tetrad dissection was carried out and spores were allowed to germinate at 28 °C. Progeny were then replicated to the permissive and restrictive temperature to determine their genotype. Double mutants of the *cdc2* and *cdc37* temperature-sensitive mutants were synthetically lethal at temperatures permissive for each individual mutant indicating that *cdc2* and *cdc37* interact genetically in *S. pombe*.

To investigate further the genetic interaction between *cdc37* and *cdc2*, the level of *cdc2* expression was increased in all four *cdc37* temperature-sensitive mutants to see whether the G2 cell cycle arrest could be overcome at the restrictive temperature. This was carried out by the introduction of a multicopy plasmid, *pIRT2-cdc2* (a gift from Stuart MacNeill Lab), in *cdc37* temperature-sensitive mutants that were plated as serial dilutions and incubated for 4 days at the 28 and 36 °C. At the restrictive temperature (36 °C) increased expression of Cdc2 fully rescued the temperature-sensitive lethality of *cdc37-J* and *cdc37-13* (Figure 6.6A), producing cells that were phenotypically wildtype in appearance (Figure 6.6B). The increased expression of *cdc2* in *cdc37-184* and *cdc37-681* was able to partially rescue these temperature-sensitive mutants at the restrictive temperature. A low level of growth was observed at 36 °C (Figure 6.6A) and cells were either wildtype or slightly elongated in appearance (Figure 6.6B). These data indicate that loss of fully active Cdc2 in *cdc37* temperature-sensitive mutants could be the cause of G2 cell cycle arrest, as increasing the cellular level of Cdc2 alleviates the arrest, indicating that Cdc2 may be a client of Cdc37 in *S. pombe*.

Figure 6.6: Increased expression of Cdc2 in *cdc37* temperature-sensitive mutant strain, plated as serial dilution and incubated at 28 and 36 °C. **A** Growth of strains at the different temperatures **B** cell morphology of strains expressing different constructs at the restrictive temperature of 36 °C.
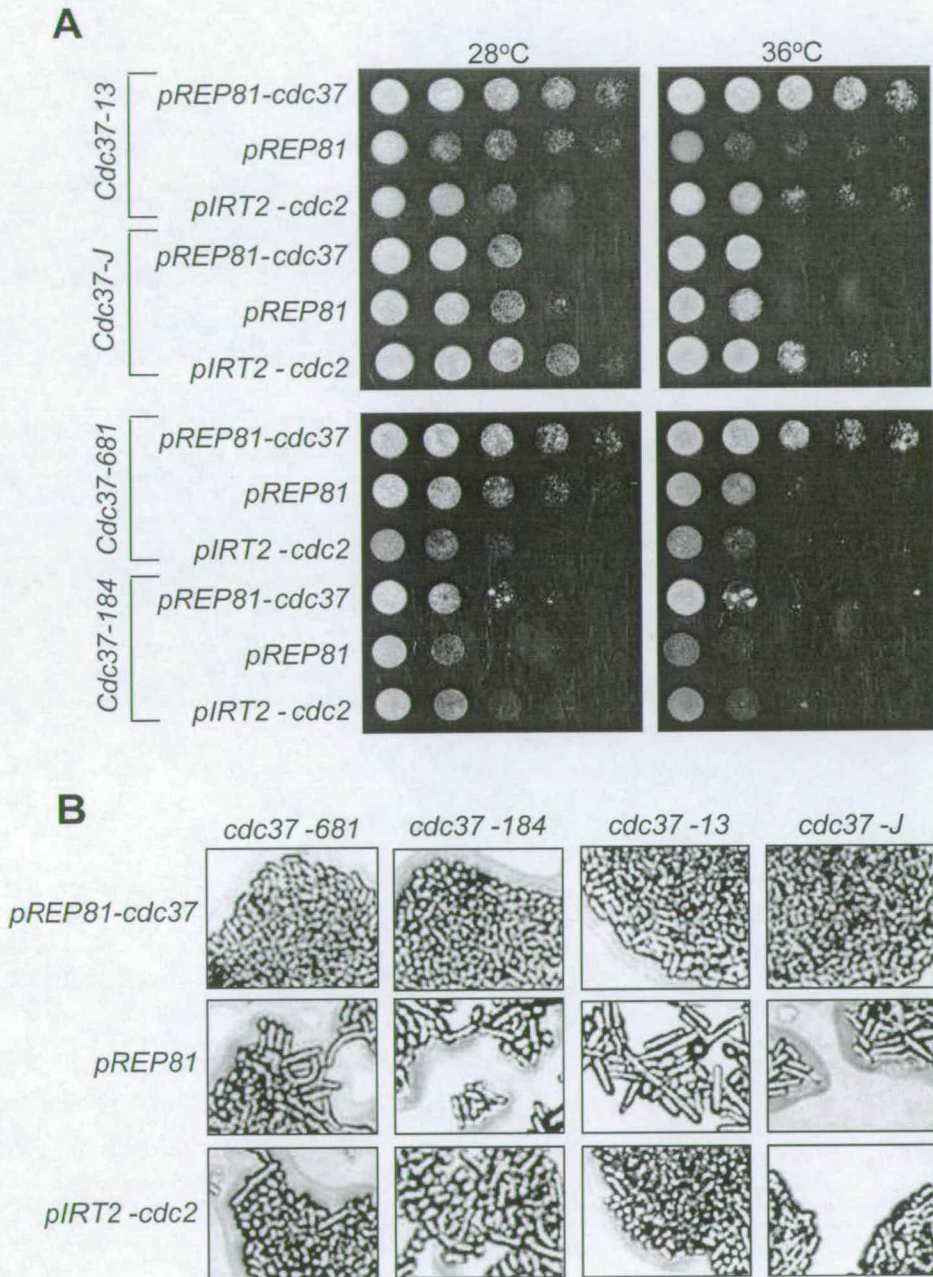
6.3.2 Studies of Cdc2 in *cdc37* temperature-sensitive mutants

To investigate the role of Cdc2 in the G2 cell cycle arrest in *cdc37* temperature-sensitive mutants, the kinase activity of Cdc2 in these mutants was assayed over a time course at the restrictive temperature. Cdc2 kinase activity was also investigated in the Hsp90 temperature-sensitive mutant *swo1-26*, as these co-chaperones were previously found to co-IP from a high molecular weight complex in *S. pombe* (Section 6.2.4). Cdc2 kinase activity can be assayed by its ability to phosphorylate histone H1 *in vitro*. Temperature-sensitive mutants and the *cdc37⁺* strain ED1022 for comparison were cultured in liquid at 28 °C, then split and incubated at 28 and shifted to 36 °C. Samples of cells were taken at 0 hours and then at hourly intervals over a 3 hour time course. Native protein extracts were made, Cdc2 was affinity-precipitated by p13^{Suc1} beads and its activity assayed as described in Section 2.9.13. Cdc2 from wildtype cells showed a high level of histone H1 phosphorylation at all time points at both temperatures. In *cdc37* and *swo1* temperature-sensitive mutants the level of histone H1 phosphorylation was dramatically reduced when cells were shifted to the restrictive temperature (Figure 6.7) indicating that at 36 °C Cdc2 activity is rapidly reduced in these mutants. This data strongly points towards a role for Cdc2 in the cell cycle arrest after 2 hours at the restrictive temperature in *cdc37* temperature-sensitive mutants, due to its reduced activity and therefore inability to promote mitosis.

Cdc2 protein levels were also assayed to determine whether reduced Cdc2 activity was due to lower cellular Cdc2 protein in *cdc37* and *swo1* temperature-sensitive mutants, as Cdc28 protein levels in the *S. cerevisiae* temperature-sensitive mutant *cdc37-1* were reduced (Gerber et al., 1995). *swo1* and *cdc37* temperature-sensitive mutants, and ED1022 were cultured as previously described, and whole cell protein extracts were made for samples taken hourly at the permissive and restrictive temperature. Western blot analysis with anti-PSTAIR antibody that recognises *S. pombe* Cdc2 and TAT1 antibodies as a loading control, showed that Cdc2 protein levels did not decrease over time at 36 °C in the *swo1* (Figure 6.7) or *cdc37* (Figure 6.8) temperature-sensitive mutants. These data indicate that Cdc2 protein levels are

Figure 6.7: Cdc2 kinase activity and protein levels were assayed in **A** *swo1* and **B** *cdc37* temperature-sensitive mutants at the permissive and restrictive temperatures. Temperature-sensitive mutants were cultured at 28 and 36 °C over a 3 hour time course. Samples of cells were taken hourly and native protein extracts were made. Cdc2 was affinity-precipitated on p13^{Suc1} beads and its activity determined by its ability to phosphorylate histone H1. Cdc2 protein levels were determined by western blot with the anti-PSTAIR antibody. * indicates p31 that is also recognised by the anti-PSTAIR antibody.
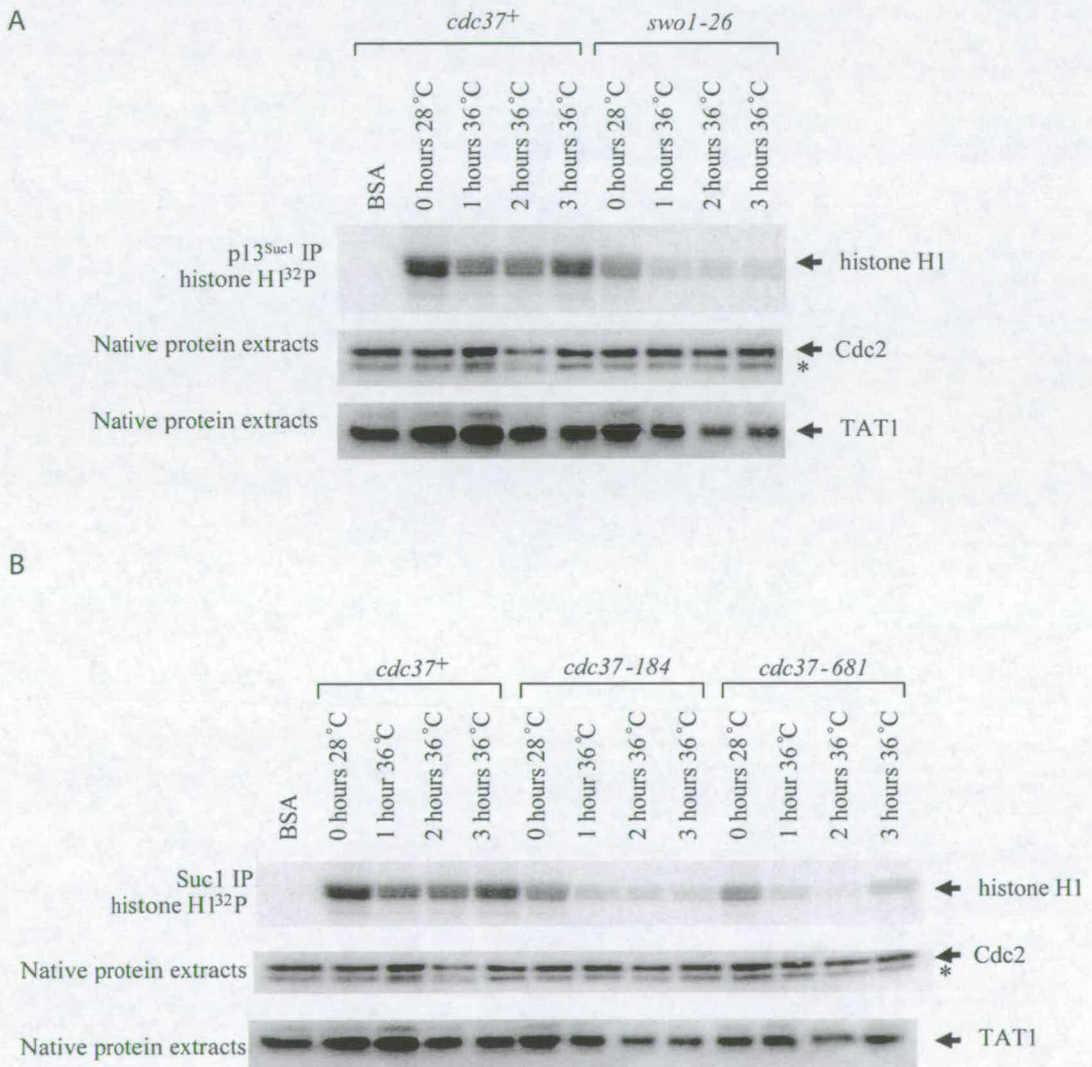
Figure 6.8: Cdc2 protein levels were assayed by western blot with anti-Cdc2 Y63.2 antibody and TAT1 antibodies as a loading control. Whole cell protein extracts were made from *cdc37* temperature-sensitive mutants at the permissive and restrictive temperature at hourly time points over a 5 hour time course.
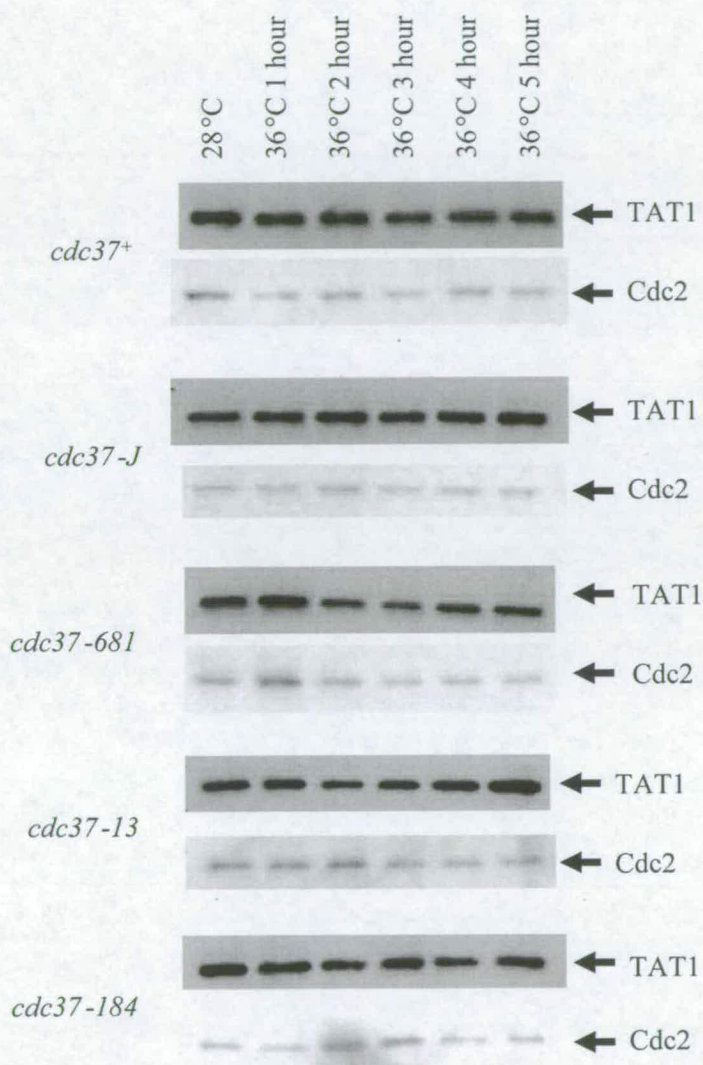
not the cause for reduced Cdc2 kinase activity in *swo1* and *cdc37* temperature-sensitive mutants.

Cdc2 activity might also be compromised by the aggregation of the Cdk into insoluble complexes in *cdc37* temperature-sensitive mutants at 36 °C, as found in the *S. cerevisiae* mutant *cdc37-1* (Farrell and Morgan, 2000). This would probably not affect overall cellular Cdc2 protein levels. To test this, whole cell protein extracts were made from *cdc37* temperature-sensitive mutant and the *cdc37*⁺ strains incubated at 28 and 36 °C over a three hour time course. The soluble and insoluble fractions of each protein preparation were separated by centrifugation at 11,000 xg for 5 minutes at 4 °C. The level of Cdc2 protein in soluble and insoluble fractions was analysed by SDS PAGE and western blot with anti-PSTAIR antibodies against Cdc2 (that also recognises the Cdk p31) and TAT1 antibodies as a loading control. Cdc2 was found in both the soluble and insoluble fraction of all strains, the proportion of which did not change over the time course at 36 °C (Figure 6.9). Therefore, the kinase activity of Cdc2 is not reduced in·*cdc37* temperature-sensitive mutants as a result of forming insoluble aggregates at the restrictive temperature.

Cdc2 can also be inactivated, preventing entry into mitosis, by phosphorylation at tyrosine 15 by Wee1 (Lee et al., 1994), and this can be detected using the specific anti-phospho tyrosine 15 antibody. *cdc37* temperature-sensitive mutants and ED1022 were cultured as previously described at 28 and 36 °C. Whole cell protein extracts were made and run on SDS PAGE. Western blot analysis was carried out using TBS buffer, blocking with BSA, and the anti-phospho tyrosine 15 Cdc2 antibody with TAT1 antibody as a loading control. For all temperature-sensitive mutants and the strain ED1022 the level of phosphorylation at tyrosine 15 was not seen to change over the time course at the restrictive temperature (Figure 6.10). These data show that inactivation of Cdc2 in temperature-sensitive mutants was not caused by increased phosphorylation at tyrosine 15 by Wee1.

Figure 6.9: The level Cdc2 protein in soluble and insoluble fractions in *cdc37* temperature-sensitive mutants was analysed at 28 and 36 °C. Strains were cultured in liquid at 28 °C, then split and incubated over a 3 hour time course at 28 and 36 °C. Whole cell protein extracts were made and the soluble and insoluble fractions separated by centrifugation at 11,000 xg for 5 minute at 4 °C. Samples were run on SDS PAGE and analysed by western blot with anti-PSTAIR antibody against Cdc2 and TAT1 antibody as a loading control. * indicates p31 that is also recognised by the anti-PSTAIR antibody.
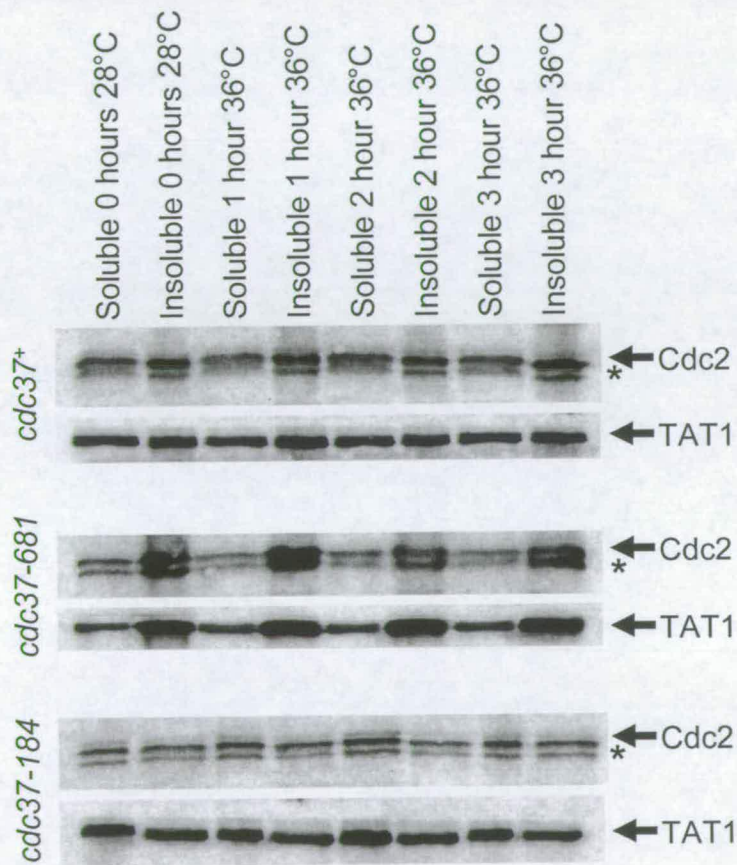
Figure 6.10: Level of phosphorylation on tyrosine 15 of Cdc2 in native *S. pombe* protein extracts from *cdc37* temperature-sensitive mutants incubated at 28 and 36 °C over a 3 hour time course. Samples of cells were taken hourly, and native *S. pombe* protein extracts were made and western blot analysis was carried out with anti-phospho tyrosine 15 antibody for Cdc2.
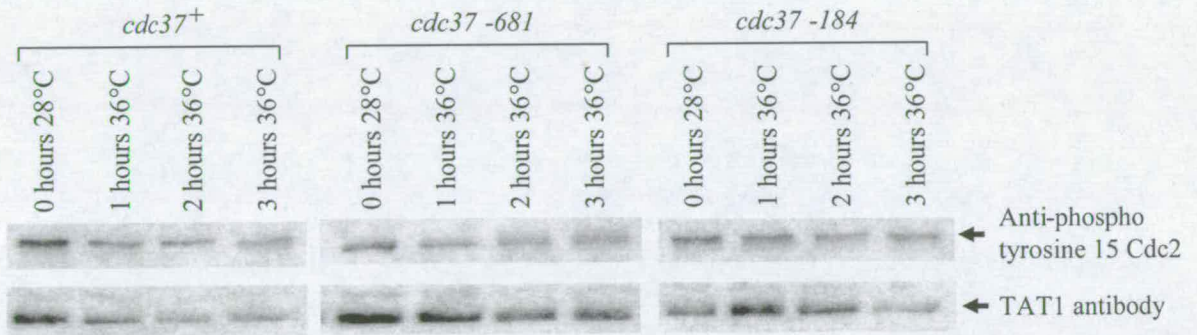
To investigate further the reduction of Cdc2 activity in *cdc37* temperature-sensitive mutants, the level of association between Cdc2 and its cyclin partner Cdc13 was assayed. Cdc2 associates with Cdc13 to promote the onset of mitosis. *cdc37* temperature-sensitive mutants and ED1022 were cultured as previously described over a 4 hour time course at 28 and 36 °C. Native protein extracts were made from samples taken hourly and IPs with the anti-Cdc13 6F 10/11 antibody (kind gift from Paul Nurse Lab) were carried out to examine the level of Cdc2 association over the time course at the restrictive temperature. The level of Cdc2 that precipitated with Cdc13 from the strain ED1022 did not change over the time course at the restrictive temperature. The level of Cdc2 associated with Cdc13 in *cdc37* temperature-sensitive mutants was seen to decrease over the time course at the restrictive temperature (Figure 6.11). Cdc2 activity may be compromised at 36 °C in *cdc37* temperature-sensitive mutants by the inability of Cdc2 and Cdc13 to sustain a stable complex, resulting in a G2 cell cycle arrest.

6.3.3   Biochemical interaction between Cdc37 and Cdc2

To investigate further the possibility that Cdc2 is a client of Cdc37, immunoprecipitation experiments were carried out to determine whether Cdc2 and Cdc37 interact biochemically in *S. pombe*. IPs with anti-*S. pombe* Cdc37 antibody were carried out on native protein extracts made from the strain ED1022 and showed that Cdc37 precipitated Cdc2 (Figure 6.12A). Interestingly, the anti-PSTAIR antibody also recognises the Cdk p31 in *S. pombe*, which also precipitated with Cdc37 in these experiments (Figure 6.12A). The reverse IPs were also carried out using anti-HA antibodies with native protein extracts from the strain ED1576 (gift from Paul Russell Lab), which contains a chromosomally tagged Cdc2-HA. IPs with the anti-HA antibody for Cdc2-HA were unable to precipitate Cdc37 (Figure 6.12B), this may be due to the inability of the HA-antibody to access the epitope tag within the complex.

Figure 6.11: The interaction between Cdc2 and Cdc13 in *cdc37* temperature-sensitive mutants at was analysed. Strains were cultured at 28 °C then split and incubated at 28 and 36 °C over a 4 hour time course. Whole cell protein extracts were made and immunoprecipitation (IP) experiments were carried out with the anti-Cdc13 6F 10/11 antibody (from Paul Nurse lab) to determine whether this cyclin was associated with Cdc2 with increasing time at the restrictive temperature.
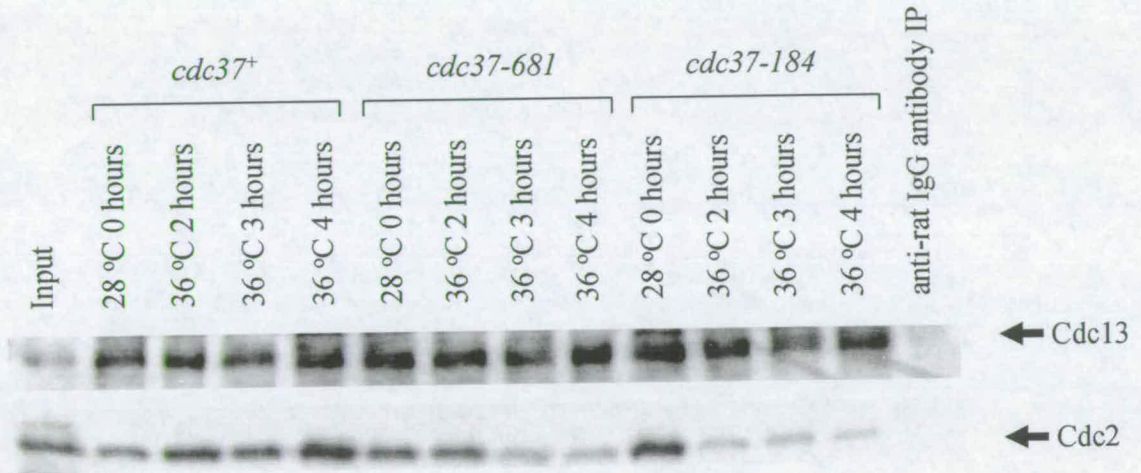
Figure 6.12: Immunoprecipitation experiments to detect an interaction biochemically between Cdc2 and Cdc37 in native *S. pombe* protein extracts. **A** Immunoprecipitations with the anti-*S. pombe* Cdc37 antibody to determine whether Cdc2 precipitates with Cdc37 from native protein extracts from the *S. pombe* strain ED1022. **B** Immunoprecipitations with the anti-HA antibody to determine whether Cdc37 precipitates with Cdc2-HA from native protein extracts from the *S. pombe* strain ED1576.

6.4 Discussion
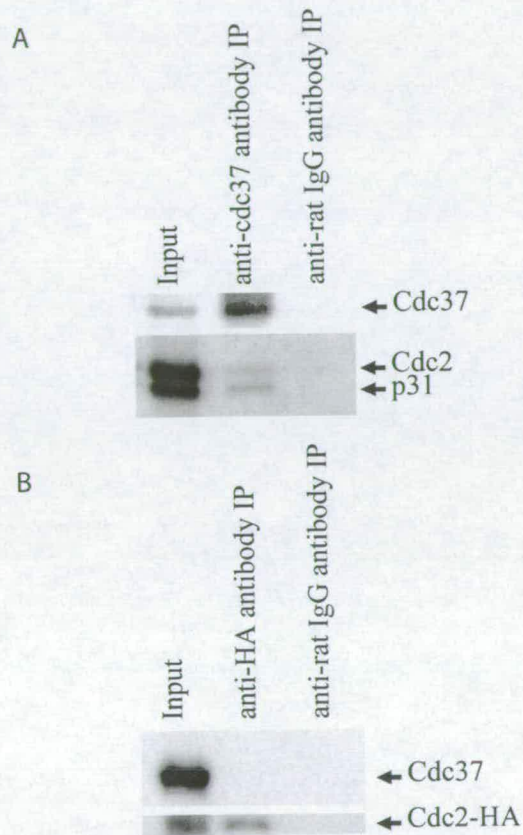
A small proportion of total cellular Cdc37 protein forms a high molecular weight complex, which was also found to contain the co-chaperone Hsp90. A biochemical interaction between the two co-chaperones, Hsp90 and Cdc37, was verified by immunoprecipitation from fractions containing the Cdc37 high molecular weight complex. The inclusion of molybdate in these experiments may have stabilised the interaction between Hsp90 and Cdc37, making it easier to detect, as molybdate inhibits the ATPase driven reaction cycle of Hsp90 thus freezing Cdc37 into association (Shao et al., 2003). These data indicate that Cdc37 and Hsp90 may associate in a high molecular weight complex. The high molecular weight complex is ~669 kDa which is greater than the predicted size for the combined dimers of Cdc37 and Hsp90, suggesting that other proteins, possibly co-chaperones or clients proteins are present.

An interaction between Cdc37 and Hsp90 has also been detected genetically, similar to data from *S. cerevisiae* that also showed that *CDC37* and *HSP90* temperature-sensitive mutants are synthetically lethal at temperatures permissive for each mutant (Kimura et al., 1997). Double mutants in *S. pombe* of *swo1* and *cdc37* temperature-sensitive genes in *S. pombe* may be unable to sustain cellular viability at the permissive temperature because Cdc37 and Hsp90 carry out important functions together, such as forming a tertiary complex with protein kinase clients. Alternatively, Hsp90 and Cdc37 may carry out the same or similar essential roles, being able to compensate for one another in some instances, shown by the ability of Hsp90 to partially rescue the temperature-sensitive mutant *cdc37-13*.

However, identification of an interaction between Hsp90 and Cdc37 in *S. pombe* has been problematic. Yeast two-hybrid assay, GST pull-down experiments with recombinant Cdc37 and immunoprecipitations from native *S. pombe* protein extracts all failed to detect any interaction between these two co-chaperones. Yeast two-hybrid experiments may have been unsuccessful in identifying an interaction between Hsp90 and Cdc37 because the N- and C- terminal tags may have interfered with the function

of Cdc37 and/or Hsp90 preventing any form of interaction between the co-chaperones. GST pull-down experiments were complicated by the non-specific binding affinity of Hsp90-HA. Furthermore, this technique used recombinant Cdc37 which is in a non-phosphorylated form, and unphosphorylated *S. cerevisiae* Cdc37 has be seen to have reduced binding affinity towards Hsp90 (Millson et al., 2004). Although phosphorylation mutants of rat and human Cdc37 were still able to interact with Hsp90 (Miyata and Nishida, 2004; Shao et al., 2003), possibly because this interaction is more stable in animal systems than yeast. Immunoprecipitation experiments using native protein were not limited by post-translational modifications required by Cdc37, but were problematic due to the non-specific binding of both Cdc37 and Hsp90-HA. An interaction between Hsp90 and Cdc37 could not be detected using total native protein extracts from *S. pombe* by IP. However, immunoprecipitation with the anti-*S. pombe* Cdc37 antibody from the Cdc37 high molecular weight complex precipitated Hsp90-HA and was the only technique that could repeatedly detect an interaction biochemically between Hsp90 and Cdc37.

In the second part of this work, Cdc2 has been identified as a possible client of Cdc37 in *S. pombe* as an interaction was biochemically identified between Cdc37 and Cdc2 by immunoprecipitation. Further evidence comes from studies with *S. pombe cdc37* temperature-sensitive mutants. Consistent with findings in the *S. cerevisiae* temperature-sensitive mutant *cdc37-1* (Gerber et al., 1995), phosphorylation of histone H1 by Cdc2 was dramatically reduced in *S. pombe cdc37* temperature-sensitive mutants at the restrictive temperature. These data indicate that the G2 cell cycle arrest observed in *S. pombe cdc37* temperature-sensitive mutants may have resulted from reduced Cdc2 activity.

Investigations were then carried out to determine the cause for reduced Cdc2 activity in *S. pombe cdc37* temperature-sensitive mutants at 36 °C. In the *S. cerevisiae* temperature-sensitive mutant *cdc37-1*, Cdc28 protein levels were reduced and the protein was seen to aggregate (Farrell and Morgan, 2000; Gerber et al., 1995). In contrast in *S. pombe*, Cdc2 protein levels remained constant and aggregation of the Cdc2 protein did not increase in *cdc37* temperature-sensitive mutants at 36 °C.

Furthermore, this work has established that Cdc2 was not inactivated at the restrictive temperature in *S. pombe cdc37* temperature-sensitive mutants by increased phosphorylation at tyrosine 15 by Wee1. The results presented here indicate that reduced Cdc2 activity in *cdc37* temperature-sensitive mutants at 36 °C resulted from an inability of Cdc2 and Cdc13 to maintain a stable complex and loss of this complex would promote a G2 cell cycle arrest. This has also been observed in the *S. cerevisiae* temperature-sensitive mutant *cdc37-1*, where mutant Cdc37 showed reduced binding of the G1 cyclin Cln2 and the mitotic cyclin Clb2 at the restrictive temperature (Gerber et al., 1995). Further analysis of this mutant revealed that Cdc28 was largely unphosphorylated at the restrictive temperature (Farrell and Morgan, 2000), specifically on threonine 169 (Gerber et al., 1995) which would inhibit cyclin association.

Increased expression of Cdc2 rescued *cdc37-13* and *cdc37-J* and partially rescued *cdc37-681* and *cdc37-184* from lethality at the restrictive temperature. These data indicate that when Cdc37 is compromised in *cdc37* temperature-sensitive mutants at 36 °C, increasing the level of Cdc2 protein, raising its activity above a threshold level can overcome the G2 cell cycle arrest. Among *S. cerevisiae* temperature-sensitive mutants that arrest at Start, *cdc37-184* was rescued by overexpression of *CDC28* (Mort-Bontemps-Soret et al., 2002), but *cdc37-1* was not (Gerber et al., 1995). It is interesting to note that overexpression of Cdc37 did not rescue the *S. cerevisiae* temperature-sensitive mutants *cdc28-1* and *cdc28-4* temperature-sensitive mutants (Mort-Bontemps-Soret et al., 2002).

In *S. pombe cdc37* temperature-sensitive mutants, cells arrest at the non-permissive temperature in G2 which correlates with reduced Cdc2 activity. The decrease in Cdc2 activity is most likely due to an inability to stably complex with the cyclin Cdc13. Cdc37 may be important for promoting the interaction between Cdc2 and Cdc13 as this has been observed with Cdk4 and cyclin D in mammalian systems, where Cdc37 does not activate the complex but aids its assembly (Lamphere et al., 1997; Stepanova et al., 1996). Cdc2 activity was also rapidly reduced in the *S. pombe swo1* temperature-sensitive mutant at the restrictive temperature. Previous data show that

Cdc37 and Hsp90 interact in a high molecular weight complex, indicating that Cdc37 and Hsp90 may act on Cdc2 as molecular chaperones forming a tertiary complex.

Chapter 7:

General discussion and conclusions

7.0   General discussion and conclusions

The aim of this work was to generate *cdc37* mutants and to analyse them using various experimental techniques to gain a better understanding of the molecular chaperone Cdc37 in *S. pombe*. In order to generate a range of *cdc37* mutants, several techniques were employed, including site directed mutagenesis, random mutagenic PCR and *in vitro* pentapeptide insertion mutagenesis. A variety of *cdc37* mutants were created using these techniques: truncation mutants, in-frame insertions of five amino acids mutants, point mutations and temperature-sensitive mutants. By generating a range of *cdc37* mutants, a variety of different investigations could be undertaken to study the cellular role of Cdc37, protein-protein interactions and to identify essential domains and residues of the Cdc37 protein.

Truncation mutants were created by introducing stop codons throughout the *cdc37* gene by random and directed mutagenesis. These mutants were expressed from pREP81 in a *cdc37Δ* strain by plasmid shuffle assay with the aim of identifying the essential domains of Cdc37. The predicted domains for *S. pombe* Cdc37 were determined by alignment and mapping of the domains identified in the human Cdc37 protein by limited proteolysis and peptide analysis (Shao et al., 2003) as shown in Chapter 3. Expression of the *cdc37* truncation mutants from pREP81 revealed that the C-terminal domain is completely dispensable for function, consistent with findings for Cdc37 in *S. cerevisiae* (Lee et al., 2002). In *S. cerevisiae,* mutants lacking most of the middle and all of the C-terminal domain were only able to support growth in a temperature dependent manner when overexpressed in a *cdc37Δ* (Lee et al., 2002). The equivalent *cdc37* truncation mutants in *S. pombe* were able to support a low level of growth at all temperatures with expression from pREP81, and successfully sustained growth and produced cells that were wildtype in appearance with overexpression from pREP1. The protein levels of these truncation mutants expressed from pREP81 were reduced compared to endogenous Cdc37, but with overexpression from pREP1, protein levels were dramatically greater than endogenous Cdc37, as determined by western blot with the anti-*S. pombe* Cdc37 antibody. Therefore,

expression of the N-terminal domain of Cdc37 is sufficient to sustain cell viability providing the mutant protein levels are above a critical threshold level.

It is interesting to note that the middle domain of Cdc37 is dispensable, as this region contains the predicted Hsp90-binding and homodimerisation domains as identified in human Cdc37 (Roe et al., 2004). These data suggest that Hsp90-binding and homodimerisation are not required for Cdc37 to carry out its essential functions in *S. pombe*. It is possible that dimerisation of Cdc37 is not crucial for interactions with client proteins, that bind the N-terminal domain of Cdc37 (Shao et al., 2003), and that a single monomer of the Cdc37 N-terminal domain is sufficient to bind the client protein and carry out the required chaperone activity. Therefore, one could speculate that Cdc37 does not play an active role in folding or activation of the client protein, but instead the N-terminal domain holds the client in an activation/folding competent state for other ATPase chaperones to act on. Evidence for Cdc37 holding clients in an activation/folding competent state for other ATPase chaperones has been shown for Hdj1 and Hsp70 (Kimura et al., 1997) and Cdc37 also holds and stabilises clients such as Cdk4 to promote binding of their cyclin partner (Lamphere et al., 1997; Stepanova et al., 1996). Non-ATPase chaperones holding and transferring client proteins to ATPase chaperones has been observed with other pairs of chaperones such the Hsp40s and the ATPase Hsp70 chaperones in *S. cerevisiae* (Lu and Cyr, 1998).

Although the Hsp90-binding domain is not essential for Cdc37 to sustain cell viability, this work has shown that Cdc37 is present in a high molecular weight complex in *S. pombe* which also contains the chaperone Hsp90. Combined with the genetic evidence presented here, this strongly suggests that Cdc37 and Hsp90 interact directly or indirectly in *S. pombe*. Cdc37 is believed to deliver client proteins to the ATPase chaperone Hsp90 (MacLean and Picard, 2003), which then actively folds and/or activates the client. Although an interaction between Cdc37 and Hsp90 occurs in *S. pombe*, this association does not appear to be essential. Alternatively, when *cdc37* C-terminally truncated constructs are overexpressed, their high abundance means that they may encounter chaperones such as Hsp90 by chance and do not require the binding domain for the interaction. However, Cdc37 interactions are

thought to be unstable, especially in yeast systems (Farrell and Morgan, 2000) and interactions between Cdc37 and Hsp90 where Cdc37 lacks the Hsp90-binding domain may be extremely unstable and inefficient at transfer of the client protein.

Alternatively, the essential function of the N-terminal domain of Cdc37 may involve interactions with chaperones other than Hsp90. Cdc37 also interacts with other chaperones such as Hop/Sti1 (Abbas-Terki et al., 2002), Hsp70 and Hdj1 (Kimura et al., 1997), and associations with these chaperones may be able to compensate for the inability of Cdc37 truncation mutants lacking the Hsp90-binding domain to interact with Hsp90. The binding domains of Cdc37 co-chaperones such as Sti1/Hop and Hsp70 have not been determined and it is possible that their binding regions are located within the N-terminal domain of Cdc37. The relationship between Cdc37 and chaperones other than Hsp90 is an interesting area for future investigations.

The essential N-terminal domain of Cdc37 in *S. pombe* is the region that interacts with client proteins (Shao et al., 2003), and these associations with rat Cdc37 are dependent on phosphorylation on serine 13 by casein kinase II (CKII) (Miyata and Nishida, 2004). Serine 14 and 17 in *S. cerevisiae* (Bandhakavi et al., 2003), and serine 13 in rat (Miyata and Nishida, 2004) and human (Shao et al., 2003), which are equivalent to *S. cerevisiae* serine 14, are phosphorylated by CKII. Mutational analysis of serine 14 in *S. pombe* Cdc37, equivalent to serine 13 in rat and human and serine 14 in *S. cerevisiae*, identified this amino acid as important for Cdc37 function. Mutation of *S. pombe* Cdc37 serine 14 to glutamic acid (*cdc37-S14E*) to mimic a phosphoserine was able to support growth in the plasmid shuffle assay when expressed from pREP81, whereas *cdc37-S14A* and *cdc37-S14L* were not. Although glutamic acid was not a complete substitute for a phosphoserine, as cells expressing this mutant allele were morphologically aberrant, even when overexpressed from pREP1.

Interestingly, overexpression of *cdc37-S14L* produced a temperature-sensitive phenotype and overexpression of *cdc37-S14A* supported growth at all temperatures and cells were wildtype in appearance. Therefore, mutations of serine 14 to alanine

and leucine do not completely abolish Cdc37 function, but appear to reduce it below a critical threshold as protein levels were not affected. Increasing the level of mutant Cdc37 protein by expression from pREP1 increases the overall level of Cdc37 activity in the cell above a critical threshold that can sustain cell viability. These mutations may negatively affect Cdc37 function by altering the conformation and proper folding of the molecular chaperone as the substituted amino acids are of a different size and structure to the serine residue. Alternatively, it is possible that phosphorylation does occur at serine 14 but that it is not essential, though important, for Cdc37 activation in *S. pombe*. Unfortunately development of a system by which to determine the phosphorylation status of Cdc37 was not successful and other avenues of investigation should be considered for the future. For example the phosphorylation status of Cdc37 could be assayed using mass spectrometry or 2-D gels. Furthermore, it would be interesting to investigate further the role of phosphorylation at serine 14 in *S. pombe* for interactions with client proteins and co-chaperones.

In order to analyse the cellular role of Cdc37 in *S. pombe*, *cdc37* temperature-sensitive mutants were generated by both random and directed mutagenesis. Three *cdc37* temperature-sensitive mutants were generated in this work and a fourth *cdc37-681* (Tatebe and Shiozaki, 2003) was analysed in parallel. It is interesting to note that all the mutations identified in these temperature-sensitive mutants were located in close proximity. By alignment with the human Cdc37 protein these mutations are found around the Hsp90-binding and homodimerisation domain in the middle domain, in the large 6-helix bundle (Roe et al., 1999). It is possible that temperature-sensitive mutants of *cdc37* in *S. pombe* may only be generated by mutations in this region. These mutations may produce temperature-sensitive mutants by negatively affecting proper folding and conformation of the 6-helix bundle of Cdc37 which dramatically reduces the essential activity of this molecular chaperone in a temperature dependent manner.

At the restrictive temperature the majority of cells for all four *cdc37* temperature-sensitive mutants displayed an elongated phenotype, characteristic of the *cdc* phenotype. All four *cdc37* temperature-sensitive mutants were found to arrest the cell

cycle in G2 with a single nucleus. Interestingly, a proportion of *cdc37-184* cells were seen to contain an unreplicated DNA content after 2 hours at the restrictive temperature, but all arrested with a replicated DNA content after 4 hours. These cells may undergo a delay in G1 due to defects caused by loss of Cdc37 function, and then finally arrest the cell cycle in G2, consistent with the other three *cdc37* temperature-sensitive mutants.

All four *cdc37* temperature-sensitive mutants arrest with a phenotype that has also been observed with loss of Cdc2 activity which resulted in elongated *S. pombe* cells that arrest at the G2/M boundary with an undivided nucleus (Snell and Nurse, 1994). Cdc2 is the cyclin-dependent kinase (Cdk) in *S. pombe* that regulates the cell cycle transitions between G1/S phase and G2/mitosis (Nurse, 1997). For cells to enter mitosis, Cdc2 must be associated with the cyclin Cdc13 (Booher et al., 1989) and phosphorylated on threonine 167 (Gould et al., 1991) by Cdk-activating kinases (CAKs) (Lee et al., 1999). Cdc2 is then activated by dephosphorylation on tyrosine 15 by Cdc25 (Nurse, 1997). In this work, Cdc2 activity was dramatically reduced at the restrictive temperature in *cdc37* temperature-sensitive mutants. Increased expression of Cdc2 in these temperature-sensitive mutants fully rescued *cdc37-13* and *cdc37-J*, and partially rescued *cdc37-184* and *cdc37-681*. These data indicate that that loss of Cdc2 activity may be the principal cause for the cell cycle arrest and suggest that Cdc2 could be a client of Cdc37 in *S. pombe*. One explanation is that Cdc37 function may be reduced in *cdc37* temperature-sensitive mutants below a critical threshold level, but not abolished, so that elevating the cellular level of Cdc2 increases the chances of Cdc37 carrying out its required chaperone activity on Cdc2. Differences in the ability of Cdc2 to rescue the *cdc37* temperature-sensitive mutants may arise from the different types of mutations that affect Cdc37 at varying levels of severity.

Investigations into the cause of reduced Cdc2 activity in *cdc37* temperature-sensitive mutants determined that interactions between Cdc2 and Cdc13 were dramatically reduced at the restrictive temperature. This may be an indirect consequence of loss of Cdc37 function in these temperature-sensitive mutants or Cdc2 may be a client of Cdc37 that relies on this molecular chaperone to promote its activation by aiding in

the complex assembly with the Cdc13 cyclin. Cdc37 has been seen to promote the assembly of Cdk-cyclin complexes in other systems, such as Cdk4 and its cyclin partners (Lamphere et al., 1997; Stepanova et al., 1996). Similar observations have been observed in the *S. cerevisiae* temperature-sensitive mutant *cdc37-1*, where Cdc28, the *S. cerevisiae* homologue of Cdc2, failed to bind the cyclin partner at the restrictive temperature and was found to be largely unphosphorylated on Threonine 169 (Farrell and Morgan, 2000; Gerber et al., 1995). However, in contrast to the findings in *S. pombe*, the Cdc28 protein in *S. cerevisiae cdc37-1* was present at reduced levels and was found to aggregate into insoluble complexes (Farrell and Morgan, 2000; Gerber et al., 1995). Further investigations should be carried out to gain a better understanding of the inability of Cdc13 and Cdc2 to interact in these mutants and to examine phosphorylation on threonine 167, equivalent to *S. cerevisiae* threonine 169, which can be analysed by 2D gel (Gould et al., 1991). In the *S. cerevisiae* temperature-sensitive mutant *cdc37-1* at the restrictive temperature, the CAK Cak1 that phosphorylates threonine 169 was present at reduced protein levels and displayed decreased activity (Farrell and Morgan, 2000). This may be a direct or indirect consequence of reduced Cdc37 function in *S. cerevisiae* temperature-sensitive mutants, but may be an interesting avenue for investigation in the *S. pombe cdc37* mutants generated in this work.

Evidence supporting the idea of Cdc2 as a client of Cdc37 comes from the identification of both biochemical and genetical interactions between them. Cdc2 co-immunoprecipitates with Cdc37 and mutants containing temperature-sensitive mutant alleles for both Cdc2 and Cdc37 are synthetically lethal. Cdc2 may rely on Cdc37 for folding, activating or promoting its association with Cdc13, independently of Cdc2 interactions with Hsp90, or Cdc37 may deliver the Cdk to a co-chaperone such as Hsp90 for these activities to be carried out. Interactions between Hsp90 and Cdc2 have also been identified both biochemically by co-immunoprecipitation (A. Santino, personal communication) and genetically in *S. pombe* (Munoz and Jimenez, 1999). Furthermore, this work has shown that Cdc2 activity is dramatically reduced in the *S. pombe* Hsp90 temperature-sensitive mutant *swo1-26* at the restrictive temperature, indicating that Hsp90 may play a role in maintaining the activity of Cdc2. However,

Cdc2 activity was not affected in the *S. pombe* mutant *cdc2-33* in combination with *swo1*[+] or the mutant *swo1-w1*, but samples were only examined at the permissive temperature (Munoz and Jimenez, 1999). A future direction for further experiments would be to investigate further the relationships between Hsp90, Cdc37 and Cdc2.

In conclusion the N-terminal domain of Cdc37 in *S. pombe* is essential for Cdc37 function to sustain cell viability. These mutants lack the postulated Hsp90-binding and homodimerisation domains indicating that these interactions are not essential for Cdc37 function. However, a small proportion of Cdc37 in *S. pombe* is present in high molecular weight complexes that were also found to contain Hsp90, indicating that these two co-chaperones do interact, though possibly in a non-essential manner. This work proposes that Cdc37 holds client proteins in an activation/folding competent state for other ATPase co-chaperones to act on or to enable clients to interact with partner proteins. A likely client of Cdc37 in *S. pombe* that has been identified in this work is Cdc2, which is an exciting finding as the only other client identified in *S. pombe* to date is the kinase Spc1 (Tatebe and Shiozaki, 2003). In *cdc37* temperature-sensitive mutants at the restrictive temperature, Cdc2 activity was dramatically reduced possibly because the complex between Cdc13 and Cdc2 breaks down. The reduction of this Cdk-cyclin complex from *cdc37* temperature-sensitive mutants at the restrictive is a possible cause for the G2 cell cycle arrest observed in all four mutants. These data indicate that Cdc37 or its association with a co-chaperone such as Hsp90 may act on Cdc2 by holding the Cdk so that it can associate with the cyclin Cdc13.

Chapter 8:

References

8.0   References

Abbas-Terki, T., Briand, P. A., Donze, O., and Picard, D. (2002). The Hsp90 co-chaperones Cdc37 and Sti1 interact physically and genetically. Biol Chem *383*, 1335-1342.

Abbas-Terki, T., Donze, O., and Picard, D. (2000). The molecular chaperone Cdc37 is required for Ste11 function and pheromone-induced cell cycle arrest. FEBS Lett *467*, 111-116.

Alberti, S., Bohse, K., Arndt, V., Schmitz, A., and Hohfeld, J. (2004). The cochaperone HspBP1 inhibits the CHIP ubiquitin ligase and stimulates the maturation of the cystic fibrosis transmembrane conductance regulator. Mol Biol Cell *15*, 4003-4010.

Alberts, B., Johnson, A., Lewis, J. R., M, Roberts, K., and Walter, P. (2002). **Molecular Biology of the Cell**. *4th Edition*.

Alfa, C. E., Booher, R., Beach, D., and Hyams, J. S. (1989). Fission yeast cyclin: subcellular localisation and cell cycle regulation. J Cell Sci Suppl *12*, 9-19.

Alfa, C. E., Gallagher, I. M., and Hyams, J. S. (1993). Antigen localization in fission yeast. Methods Cell Biol *37*, 201-222.

Aligue, R., Akhavan-Niak, H., and Russell, P. (1994). A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. Embo J *13*, 6099-6106.

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943-951.

Bandhakavi, S., McCann, R. O., Hanna, D. E., and Glover, C. V. (2003). Genetic interactions among ZDS1,2, CDC37, and protein kinase CK2 in Saccharomyces cerevisiae. FEBS Lett *554*, 295-300.

Bandhakavi, S., McCann, R. O., Hanna, D. E., and Glover, C. V. (2003). A positive feedback loop between protein kinase CKII and Cdc37 promotes the activity of multiple protein kinases. J Biol Chem *278*, 2829-2836.

Barral, J. M., Broadley, S. A., Schaffar, G., and Hartl, F. U. (2004). Roles of molecular chaperones in protein misfolding diseases. Semin Cell Dev Biol *15*, 17-29.

Basi, G., Schmid, E., and Maundrell, K. (1993). TATA box mutations in the Schizosaccharomyces pombe nmt1 promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene *123*, 131-136.

Bonatti, S., Simili, M., and Abbondandolo, A. (1972). Isolation of temperature-sensitive mutants of Schizosaccharomyces pombe. J Bacteriol *109*, 484-491.

Booher, R., and Beach, D. (1988). Involvement of cdc13+ in mitotic control in Schizosaccharomyces pombe: possible interaction of the gene product with microtubules. Embo J *7*, 2321-2327.

Booher, R. N., Alfa, C. E., Hyams, J. S., and Beach, D. H. (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell *58*, 485-497.

Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulien, J., and Lindquist, S. (1989). hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol Cell Biol 9, 3919-3930.

Boudeau, J., Deak, M., Lawlor, M. A., Morrice, N. A., and Alessi, D. R. (2003). Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability. Biochem J 370, 849-857.

Cadwell, R. C., and Joyce, G. F. (1992). Randomization of genes by PCR mutagenesis. PCR Methods Appl 2, 28-33.

Carrello, A., Allan, R. K., Morgan, S. L., Owen, B. A., Mok, D., Ward, B. K., Minchin, R. F., Toft, D. O., and Ratajczak, T. (2004). Interaction of the Hsp90 cochaperone cyclophilin 40 with Hsc70. Cell Stress Chaperones 9, 167-181.

Casas, S., Ollila, J., Aventin, A., Vihinen, M., Sierra, J., and Knuutila, S. (2003). Changes in apoptosis-related pathways in acute myelocytic leukemia. Cancer Genet Cytogenet 146, 89-101.

Chang, F., Woollard, A., and Nurse, P. (1996). Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. J Cell Sci 109 (Pt 1), 131-142.

Chen, G., Cao, P., and Goeddel, D. V. (2002). TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. Mol Cell 9, 401-410.

Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996). Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. Mol Endocrinol 10, 682-693.

Chernoff, Y. O., Lindquist, S. L., Ono, B., Inge-Vechtomov, S. G., and Liebman, S. W. (1995). Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science 268, 880-884.

Cooper, G. M. (2000). **The Cell - A Molecular Approach**. *2nd Edition*.

Cyr, D. M. (1995). Cooperation of the molecular chaperone Ydj1 with specific Hsp70 homologs to suppress protein aggregation. FEBS Lett 359, 129-132.

Dai, K., Kobayashi, R., and Beach, D. (1996). Physical interaction of mammalian CDC37 with CDK4. J Biol Chem 271, 22030-22034.

Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol 11, 1569-1577.

Dey, B., Lightbody, J. J., and Boschelli, F. (1996). CDC37 is required for p60v-src activity in yeast. Mol Biol Cell 7, 1405-1417.

Dobson, C. M. (2004). Principles of protein folding, misfolding and aggregation. Semin Cell Dev Biol 15, 3-16.

Donze, O., and Picard, D. (1999). Hsp90 binds and regulates Gcn2, the ligand-inducible kinase of the alpha subunit of eukaryotic translation initiation factor 2 [corrected]. Mol Cell Biol 19, 8422-8432.

Fan, C. Y., Lee, S., Ren, H. Y., and Cyr, D. M. (2004). Exchangeable chaperone modules contribute to specification of type I and type II Hsp40 cellular function. Mol Biol Cell 15, 761-773.

Farrell, A., and Morgan, D. O. (2000). Cdc37 promotes the stability of protein kinases Cdc28 and Cak1. Mol Cell Biol 20, 749-754.

Finkelstein, D. B., and Strausberg, S. (1983). Identification and expression of a cloned yeast heat shock gene. J Biol Chem 258, 1908-1913.

Finkelstein, D. B., Strausberg, S., and McAlister, L. (1982). Alterations of transcription during heat shock of Saccharomyces cerevisiae. J Biol Chem 257, 8405-8411.

Fliss, A. E., Fang, Y., Boschelli, F., and Caplan, A. J. (1997). Differential in vivo regulation of steroid hormone receptor activation by Cdc37p. Mol Biol Cell 8, 2501-2509.

Forsburg, S. L. (1994). Codon usage table for Schizosaccharomyces pombe. Yeast 10, 1045-1047.

Fromant, M., Blanquet, S., and Plateau, P. (1995). Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. Anal Biochem 224, 347-353.

Gao, Y., Thomas, J. O., Chow, R. L., Lee, G. H., and Cowan, N. J. (1992). A cytoplasmic chaperonin that catalyzes beta-actin folding. Cell 69, 1043-1050.

Gerber, M. R., Farrell, A., Deshaies, R. J., Herskowitz, I., and Morgan, D. O. (1995). Cdc37 is required for association of the protein kinase Cdc28 with G1 and mitotic cyclins. Proc Natl Acad Sci U S A 92, 4651-4655.

Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20, 1425.

Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991). Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. Embo J 10, 3297-3309.

Gould, K. L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature 342, 39-45.

Grammatikakis, N., Grammatikakis, A., Yoneda, M., Yu, Q., Banerjee, S. D., and Toole, B. P. (1995). A novel glycosaminoglycan-binding protein is the vertebrate homologue of the cell cycle control protein, Cdc37. J Biol Chem 270, 16198-16205.

Grammatikakis, N., Lin, J. H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999). p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. Mol Cell Biol 19, 1661-1672.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988). Genetic engineering of Schizosaccharomyces pombe: a system for gene disruption and replacement using the ura4 gene as a selectable marker. Mol Gen Genet 215, 81-86.

Hagan, I., Hayles, J., and Nurse, P. (1988). Cloning and sequencing of the cyclin-related cdc13+ gene and a cytological study of its role in fission yeast mitosis. J Cell Sci 91 (Pt 4), 587-595.

Hartson, S. D., Irwin, A. D., Shao, J., Scroggins, B. T., Volk, L., Huang, W., and Matts, R. L. (2000). p50(cdc37) is a nonexclusive Hsp90 cohort which participates intimately in Hsp90-mediated folding of immature kinase molecules. Biochemistry 39, 7631-7644.

Hartwell, L. H. (1974). Saccharomyces cerevisiae cell cycle. Bacteriol Rev 38, 164-198.

Heikkila, J. J. (2004). Regulation and function of small heat shock protein genes during amphibian development. J Cell Biochem 93, 672-680.

Hesterkamp, T., Hauser, S., Lutcke, H., and Bukau, B. (1996). Escherichia coli trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. Proc Natl Acad Sci U S A 93, 4437-4441.

Horowitz, N. H., and Leupold, U. (1951). Some recent studies bearing on the one geneone enzyme hypothesis. Cold Spring Harb Symp Quant Biol *16*, 65-74.

Huang, L., Grammatikakis, N., and Toole, B. P. (1998). Organization of the chick CDC37 gene. J Biol Chem *273*, 3598-3603.

Hunter, T., and Sefton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci U S A *77*, 1311-1315.

Hutchison, K. A., Brott, B. K., De Leon, J. H., Perdew, G. H., Jove, R., and Pratt, W. B. (1992). Reconstitution of the multiprotein complex of pp60src, hsp90, and p50 in a cell-free system. J Biol Chem *267*, 2902-2908.

Hutchison, K. A., Stancato, L. F., Jove, R., and Pratt, W. B. (1992). The protein-protein complex between pp60v-src and hsp90 is stabilized by molybdate, vanadate, tungstate, and an endogenous cytosolic metal. J Biol Chem *267*, 13952-13957.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene *96*, 23-28.

Ish-Horowicz, D., Pinchin, S. M., Gausz, J., Gyurkovics, H., Bencze, G., Goldschmidt-Clermont, M., and Holden, J. J. (1979). Deletion mapping of two D. melanogaster loci that code for the 70,000 dalton heat-induced protein. Cell *17*, 565-571.

Jakob, U., Lilie, H., Meyer, I., and Buchner, J. (1995). Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo. J Biol Chem *270*, 7288-7294.

Jana, N. R., Dikshit, P., Goswami, A., Kotliarova, S., Murata, S., Tanaka, K., and Nukina, N. (2005). Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. J Biol Chem.

Javerzat, J. P., Cranston, G., and Allshire, R. C. (1996). Fission yeast genes which disrupt mitotic chromosome segregation when overexpressed. Nucleic Acids Res *24*, 4676-4683.

Kimura, Y., Matsumoto, S., and Yahara, I. (1994). Temperature-sensitive mutants of hsp82 of the budding yeast Saccharomyces cerevisiae. Mol Gen Genet *242*, 517-527.

Kimura, Y., Rutherford, S. L., Miyata, Y., Yahara, I., Freeman, B. C., Yue, L., Morimoto, R. I., and Lindquist, S. (1997). Cdc37 is a molecular chaperone with specific functions in signal transduction. Genes Dev *11*, 1775-1785.

Kleizen, B., and Braakman, I. (2004). Protein folding and quality control in the endoplasmic reticulum. Curr Opin Cell Biol *16*, 343-349.

Krapp, A., Gulli, M. P., and Simanis, V. (2004). SIN and the art of splitting the fission yeast cell. Curr Biol *14*, R722-730.

Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., and Krebs, E. G. (1987). Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. J Biol Chem *262*, 9136-9140.

Kumar, R., Grammatikakis, N., and Chinkers, M. (2001). Regulation of the atrial natriuretic peptide receptor by heat shock protein 90 complexes. J Biol Chem *276*, 11371-11375.

Lamphere, L., Fiore, F., Xu, X., Brizuela, L., Keezer, S., Sardet, C., Draetta, G. F., and Gyuris, J. (1997). Interaction between Cdc37 and Cdk4 in human cells. Oncogene *14*, 1999-2004.

Lange, B. M., Rebollo, E., Herold, A., and Gonzalez, C. (2002). Cdc37 is essential for chromosome segregation and cytokinesis in higher eukaryotes. Embo J *21*, 5364-5374.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. Nature *356*, 683-689.

Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P. J., Jr., Kumamoto, C. A., and Wickner, W. (1989). Three pure chaperone proteins of Escherichia coli--SecB, trigger factor and GroEL--form soluble complexes with precursor proteins in vitro. Embo J *8*, 2703-2709.

Lecker, S. H., Driessen, A. J., and Wickner, W. (1990). ProOmpA contains secondary and tertiary structure prior to translocation and is shielded from aggregation by association with SecB protein. Embo J *9*, 2309-2314.

Lee, K. M., Saiz, J. E., Barton, W. A., and Fisher, R. P. (1999). Cdc2 activation in fission yeast depends on Mcs6 and Csk1, two partially redundant Cdk-activating kinases (CAKs). Curr Biol *9*, 441-444.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature *327*, 31-35.

Lee, M. S., Enoch, T., and Piwnica-Worms, H. (1994). mik1+ encodes a tyrosine kinase that phosphorylates p34cdc2 on tyrosine 15. J Biol Chem *269*, 30530-30537.

Lee, P., Rao, J., Fliss, A., Yang, E., Garrett, S., and Caplan, A. J. (2002). The Cdc37 protein kinase-binding domain is sufficient for protein kinase activity and cell viability. J Cell Biol *159*, 1051-1059.

Lee, P., Shabbir, A., Cardozo, C., and Caplan, A. J. (2004). Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. Mol Biol Cell *15*, 1785-1792.

Lee, W. L., Bezanilla, M., and Pollard, T. D. (2000). Fission yeast myosin-I, Myo1p, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. J Cell Biol *151*, 789-800.

Lin-Goerke, J. L., Robbins, D. J., and Burczak, J. D. (1997). PCR-based random mutagenesis using manganese and reduced dNTP concentration. Biotechniques *23*, 409-412.

Litchfield, D. W., Luscher, B., Lozeman, F. J., Eisenman, R. N., and Krebs, E. G. (1992). Phosphorylation of casein kinase II by p34cdc2 in vitro and at mitosis. J Biol Chem *267*, 13943-13951.

Lopez, N., Aron, R., and Craig, E. A. (2003). Specificity of class II Hsp40 Sis1 in maintenance of yeast prion [RNQ+]. Mol Biol Cell *14*, 1172-1181.

Lu, Z., and Cyr, D. M. (1998). Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J Biol Chem *273*, 27824-27830.

MacLean, M., and Picard, D. (2003). Cdc37 goes beyond Hsp90 and kinases. Cell Stress Chaperones *8*, 114-119.

MacNeill, S. A., and Fantes, P. A. (1993). Controlling entry into Mitosis in Fission yeast. Frontiers in Molecular Biology: The Cell Cycle IRL Press.

Mahony, D., Parry, D. A., and Lees, E. (1998). Active cdk6 complexes are predominantly nuclear and represent only a minority of the cdk6 in T cells. Oncogene *16*, 603-611.

Maier, R., Scholz, C., and Schmid, F. X. (2001). Dynamic association of trigger factor with protein substrates. J Mol Biol *314*, 1181-1190.

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991). Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. Nature *352*, 36-42.

Martin, J., Mayhew, M., Langer, T., and Hartl, F. U. (1993). The reaction cycle of GroEL and GroES in chaperonin-assisted protein folding. Nature *366*, 228-233.

Matsuda, S., Suzuki-Fujimoto, T., Minowa, A., Ueno, H., Katamura, K., and Koyasu, S. (1999). Temperature-sensitive ZAP70 mutants degrading through a proteasome-independent pathway. Restoration of a kinase domain mutant by Cdc37. J Biol Chem *274*, 34515-34518.

Maundrell, K. (1990). nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J Biol Chem *265*, 10857-10864.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene *123*, 127-130.

McLaughlin, S. H., Ventouras, L. A., Lobbezoo, B., and Jackson, S. E. (2004). Independent ATPase activity of Hsp90 subunits creates a flexible assembly platform. J Mol Biol *344*, 813-826.

Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat Cell Biol *3*, 100-105.

Mendenhall, M. D., and Hodge, A. E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev *62*, 1191-1243.

Millson, S. H., Truman, A. W., and Piper, P. W. (2003). Vectors for N- or C-terminal positioning of the yeast Gal4p DNA binding or activator domains. Biotechniques *35*, 60-64.

Millson, S. H., Truman, A. W., Wolfram, F., King, V., Panaretou, B., Prodromou, C., Pearl, L. H., and Piper, P. W. (2004). Investigating the protein-protein interactions of the yeast Hsp90 chaperone system by two-hybrid analysis: potential uses and limitations of this approach. Cell Stress Chaperones *9*, 359-368.

Mitchison, J. M. (1957). The growth of single cells. I. Schizosaccharomyces pombe. Exp Cell Res *13*, 244-262.

Mitchison, J. M. (1990). The fission yeast, Schizosaccharomyces pombe. Bioessays *12*, 189-191.

Miyata, Y., and Nishida, E. (2004). CK2 controls multiple protein kinases by phosphorylating a kinase-targeting molecular chaperone, Cdc37. Mol Cell Biol *24*, 4065-4074.

Mort-Bontemps-Soret, M., Facca, C., and Faye, G. (2002). Physical interaction of Cdc28 with Cdc37 in Saccharomyces cerevisiae. Mol Genet Genomics *267*, 447-458.

Moser, B. A., and Russell, P. (2000). Cell cycle regulation in Schizosaccharomyces pombe. Curr Opin Microbiol *3*, 631-636.

Munoz, M. J., and Jimenez, J. (1999). Genetic interactions between Hsp90 and the Cdc2 mitotic machinery in the fission yeast Schizosaccharomyces pombe. Mol Gen Genet *261*, 242-250.

Nguyen, T. H., Law, D. T., and Williams, D. B. (1991). Binding protein BiP is required for translocation of secretory proteins into the endoplasmic reticulum in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *88*, 1565-1569.

Ni, J., Gao, Y., Liu, H., and Chen, J. (2004). Candida albicans Cdc37 interacts with the Crk1 kinase and is required for Crk1 production. FEBS Lett *561*, 223-230.

Nurse, P. (1997). Regulation of the eukaryotic cell cycle. Eur J Cancer *33*, 1002-1004.

Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Mol Gen Genet *146*, 167-178.

O'Keeffe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000). Requirement for a kinase-specific chaperone pathway in the production of a Cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated tat stimulation of HIV-1 transcription. J Biol Chem *275*, 279-287.

Obermann, W. M., Sondermann, H., Russo, A. A., Pavletich, N. P., and Hartl, F. U. (1998). In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. J Cell Biol *143*, 901-910.

Pain, R. (2000). Mechanisms of Protein Folding. Oxford University Press.

Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. Embo J *17*, 4829-4836.

Panaretou, B., Siligardi, G., Meyer, P., Maloney, A., Sullivan, J. K., Singh, S., Millson, S. H., Clarke, P. A., Naaby-Hansen, S., Stein, R., *et al.* (2002). Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. Mol Cell *10*, 1307-1318.

Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci U S A *71*, 1286-1290.

Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994). Protein disaggregation mediated by heat-shock protein Hsp104. Nature *372*, 475-478.

Peng, H. M., Morishima, Y., Jenkins, G. J., Dunbar, A. Y., Lau, M., Patterson, C., Pratt, W. B., and Osawa, Y. (2004). Ubiquitylation of neuronal nitric-oxide synthase by CHIP, a chaperone-dependent E3 ligase. J Biol Chem *279*, 52970-52977.

Perdew, G. H., Wiegand, H., Vanden Heuvel, J. P., Mitchell, C., and Singh, S. S. (1997). A 50 kilodalton protein associated with raf and pp60(v-src) protein kinases is a mammalian homolog of the cell cycle control protein cdc37. Biochemistry *36*, 3600-3607.

Picard, D. (2002). Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci *59*, 1640-1648.

Pollard, T., and Earnshaw, W. (2002). Cell Biology. Saunders Publishing, Elsevier Science.

Prince, T., and Matts, R. L. (2004). Definition of protein kinase sequence motifs that trigger high affinity binding of Hsp90 and Cdc37. J Biol Chem *279*, 39975-39981.

Rao, J., Lee, P., Benzeno, S., Cardozo, C., Albertus, J., Robins, D. M., and Caplan, A. J. (2001). Functional interaction of human Cdc37 with the androgen receptor but not with the glucocorticoid receptor. J Biol Chem *276*, 5814-5820.

Reed, S. I. (1980). The selection of amber mutations in genes required for completion of start, the controlling event of the cell division cycle of S. cerevisiae. Genetics *95*, 579-588.

Reed, S. I. (1980). The selection of S. cerevisiae mutants defective in the start event of cell division. Genetics *95*, 561-577.

Roe, S. M., Ali, M. M., Meyer, P., Vaughan, C. K., Panaretou, B., Piper, P. W., Prodromou, C., and Pearl, L. H. (2004). The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37). Cell *116*, 87-98.

Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. J Med Chem *42*, 260-266.

Rustici, G., Mata, J., Kivinen, K., Lio, P., Penkett, C. J., Burns, G., Hayles, J., Brazma, A., Nurse, P., and Bahler, J. (2004). Periodic gene expression program of the fission yeast cell cycle. Nat Genet *36*, 809-817.

Samejima, I., Mackie, S., and Fantes, P. A. (1997). Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. Embo J *16*, 6162-6170.

Sazer, S., and Sherwood, S. W. (1990). Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast. J Cell Sci *97 (Pt 3)*, 509-516.

Scheibel, T., Bloom, J., and Lindquist, S. L. (2004). The elongation of yeast prion fibers involves separable steps of association and conversion. Proc Natl Acad Sci U S A *101*, 2287-2292.

Scholz, G., Hartson, S. D., Cartledge, K., Hall, N., Shao, J., Dunn, A. R., and Matts, R. L. (2000). p50(Cdc37) can buffer the temperature-sensitive properties of a mutant of Hck. Mol Cell Biol *20*, 6984-6995.

Scholz, G. M., Cartledge, K., and Hall, N. E. (2001). Identification and characterization of Harc, a novel Hsp90-associating relative of Cdc37. J Biol Chem *276*, 30971-30979.

Schutz, A. R., Giddings, T. H., Jr., Steiner, E., and Winey, M. (1997). The yeast CDC37 gene interacts with MPS1 and is required for proper execution of spindle pole body duplication. J Cell Biol *136*, 969-982.

Schwarze, S. R., Fu, V. X., and Jarrard, D. F. (2003). Cdc37 enhances proliferation and is necessary for normal human prostate epithelial cell survival. Cancer Res *63*, 4614-4619.

Scroggins, B. T., Prince, T., Shao, J., Uma, S., Huang, W., Guo, Y., Yun, B. G., Hedman, K., Matts, R. L., and Hartson, S. D. (2003). High affinity binding of Hsp90 is triggered by multiple discrete segments of its kinase clients. Biochemistry *42*, 12550-12561.

Shao, J., Grammatikakis, N., Scroggins, B. T., Uma, S., Huang, W., Chen, J. J., Hartson, S. D., and Matts, R. L. (2001). Hsp90 regulates p50(cdc37) function during the biogenesis of the activeconformation of the heme-regulated eIF2 alpha kinase. J Biol Chem *276*, 206-214.

Shao, J., Irwin, A., Hartson, S. D., and Matts, R. L. (2003). Functional dissection of cdc37: characterization of domain structure and amino acid residues critical for protein kinase binding. Biochemistry *42*, 12577-12588.

Shao, J., Prince, T., Hartson, S. D., and Matts, R. L. (2003). Phosphorylation of serine 13 is required for the proper function of the Hsp90 co-chaperone, Cdc37. J Biol Chem *278*, 38117-38120.

Siligardi, G., Panaretou, B., Meyer, P., Singh, S., Woolfson, D. N., Piper, P. W., Pearl, L. H., and Prodromou, C. (2002). Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. J Biol Chem *277*, 20151-20159.

Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M., and Pratt, W. B. (1998). p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. J Biol Chem *273*, 20090-20095.

Snell, V., and Nurse, P. (1994). Genetic analysis of cell morphogenesis in fission yeast--a role for casein kinase II in the establishment of polarized growth. Embo J *13*, 2066-2074.

Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., *et al.* (1996). A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. Mol Cell Biol *16*, 6486-6493.

Sorger, P. K., and Pelham, H. R. (1987). The glucose-regulated protein grp94 is related to heat shock protein hsp90. J Mol Biol *194*, 341-344.

Stepanova, L., Finegold, M., DeMayo, F., Schmidt, E. V., and Harper, J. W. (2000). The oncoprotein kinase chaperone CDC37 functions as an oncogene in mice and collaborates with both c-myc and cyclin D1 in transformation of multiple tissues. Mol Cell Biol *20*, 4462-4473.

Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996). Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. Genes Dev *10*, 1491-1502.

Stepanova, L., Yang, G., DeMayo, F., Wheeler, T. M., Finegold, M., Thompson, T. C., and Harper, J. W. (2000). Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to promote prostatic hyperplasia. Oncogene *19*, 2186-2193.

Streiblova, E., Hasek, J., and Jelke, E. (1984). Septum pattern in ts mutants of Schizosaccharomyces pombe defective in genes cdc3, cdc4, cdc8 and cdc12. J Cell Sci *69*, 47-65.

Tatebe, H., and Shiozaki, K. (2003). Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. Mol Cell Biol *23*, 5132-5142.

Tateishi, Y., Kawabe, Y., Chiba, T., Murata, S., Ichikawa, K., Murayama, A., Tanaka, K., Baba, T., Kato, S., and Yanagisawa, J. (2004). Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. Embo J *23*, 4813-4823.

Valay, J. G., Simon, M., Dubois, M. F., Bensaude, O., Facca, C., and Faye, G. (1995). The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. J Mol Biol *249*, 535-544.

Vogel, J. P., Misra, L. M., and Rose, M. D. (1990). Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J Cell Biol *110*, 1885-1895.

Wang, H., Goode, T., Iakova, P., Albrecht, J. H., and Timchenko, N. A. (2002). C/EBPalpha triggers proteasome-dependent degradation of cdk4 during growth arrest. Embo J *21*, 930-941.

Wang, X., Grammatikakis, N., and Hu, J. (2002). Role of p50/CDC37 in hepadnavirus assembly and replication. J Biol Chem *277*, 24361-24367.

Wartmann, M., and Davis, R. J. (1994). The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. J Biol Chem *269*, 6695-6701.

Watson, H. A., Cope, M. J., Groen, A. C., Drubin, D. G., and Wendland, B. (2001). In vivo role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. Mol Biol Cell *12*, 3668-3679.

Wawrzynow, A., and Zylicz, M. (1995). Divergent effects of ATP on the binding of the DnaK and DnaJ chaperones to each other, or to their various native and denatured protein substrates. J Biol Chem *270*, 19300-19306.

Wegele, H., Muller, L., and Buchner, J. (2004). Hsp70 and Hsp90--a relay team for protein folding. Rev Physiol Biochem Pharmacol *151*, 1-44.

Westwood, P. K., Martin, I. V., and Fantes, P. A. (2004). Fission yeast Cdc37 is required for multiple cell cycle functions. Mol Genet Genomics *271*, 82-90.

Xu, Y., Singer, M. A., and Lindquist, S. (1999). Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. Proc Natl Acad Sci U S A *96*, 109-114.

Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol *5*, 781-791.

Zhang, W., Hirshberg, M., McLaughlin, S. H., Lazar, G. A., Grossmann, J. G., Nielsen, P. R., Sobott, F., Robinson, C. V., Jackson, S. E., and Laue, E. D. (2004). Biochemical and structural studies of the interaction of Cdc37 with Hsp90. J Mol Biol *340*, 891-907.

Zhao, Q., Boschelli, F., Caplan, A. J., and Arndt, K. T. (2004). Identification of a conserved sequence motif that promotes Cdc37 and cyclin D1 binding to Cdk4. J Biol Chem *279*, 12560-12564.

Ziemienowicz, A., Skowyra, D., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C., and Zylicz, M. (1993). Both the Escherichia coli chaperone systems, GroEL/GroES and DnaK/DnaJ/GrpE, can reactivate heat-treated RNA polymerase. Different mechanisms for the same activity. J Biol Chem *268*, 25425-25431.