Cytoplasmic polyadenylation in *S. pombe*

A dissertation presented for the degree of D. Phil.

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Abstract

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Cid1 is a cytoplasmic member of a novel class of regulatory poly(A) polymerases discovered recently in yeast, worms and vertebrates. Previous genetic studies in the fission yeast, *Schizosaccharomyces pombe*, suggested a role for Cid1 in the checkpoint response to replication stress, but it was not known how a poly(A) polymerase might contribute to this response. Further investigations into the mode of action of Cid1 were therefore undertaken in this study. Cid1 is likely to target specific RNAs for polyadenylation; potential RNA substrates were identified using the complementary methods of microarray hybridisation and whole proteome analysis using two-dimensional liquid chromatography. These experiments revealed that Cid1 does not affect RNAs during normal, unperturbed growth but instead alters the expression of specific subsets of genes during replication stress. Many RNAs affected by Cid1 in these circumstances were cell-cycle dependent and telomeric transcripts, including those encoding histones and a novel RecQ helicase, Rqh2. As Cid1 lacks an RNA recognition motif, it is unlikely to bind selectively to RNA targets on its own. Cid1-interacting proteins were identified using yeast two-hybrid and tandem affinity purification methods. From these studies, novel members of a Cid1 complex have been discovered including: a previously uncharacterised metallo-beta-lactamase, RNA-binding proteins, ribosomal proteins and a telomere-binding protein. Together, these approaches are leading to a model for the role of cytoplasmic polyadenylation by Cid1 in checkpoint control.

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Abbreviations

A	amps
aa	amino acids
APS	ammonium persulphate
β-ME	beta-mercaptoethanol
β-gal	beta-galactosidase
BLAST	basic local alignment search tool
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
cpm	counts per minute
CRUK	Cancer Research UK
D. melanogaster	*Drosophila melanogaster*
Da	dalton(s)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanidine triphosphate
dH_20	distilled water
DMF	N, N-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-NNN',N'-tetra-acetic acid
eIF	eukaryotic initiation factor
ESI-qTOF	electro-spray ionisation quadrupole time-of-flight
g	gram(s)
g	relative centrifugal force
G418	geneticin sulphate
GFP	green fluorescent protein
HA	haemagglutin
hnRNP	heterogeneous nuclear ribonucleoprotein
hr(s)	hour(s)
HRP	horseradish peroxidase
H. sapiens	*Homo sapiens*
IP	immunoprecipitation
k	kilo
l	litre(s)
LB	Luria-Bertani

M	molar (moles per litre)
MBC	methyl benzimidazole-2-yl carbamate (carbenazim)
min(s)	minute(s)
MOPS	3-[N-morpholino] propanesulphonic acid
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
nt(s)	nucleotide(s)
OD	optical density
ORF	open reading frame
PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
PAP	poly(A) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
Pol	polymerase
RNA	ribonucleic acid
rpm	revolutions per minute
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	reverse transcription
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulphate
sec(s)	second(s)
ss	single-stranded
TBE	Tris/Borate/EDTA
TBS	tris-buffered saline
TCA	trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phosphine
TE	Tris/EDTA buffer
TEMED	N, N, N′,N′-tetramethylethylenediamine
TEV	Tobacco Etch Virus
Tris	tris[hydroxymethyl] aminomethane
tRNA	transfer RNA
ts	temperature sensitive
UV	ultra-violet
UTR	untranslated region
W	watts
XC	xylene cyanol
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X. laevis	*Xenopus laevis*

Chapter One

Introduction

1.1 The DNA integrity checkpoints of *S. pombe*

To ensure every daughter cell receives an accurate copy of the genome, each chromosome must be replicated faithfully and completely during S-phase and segregated accurately at M-phase. A cell can encounter a multitude of problems during the cell cycle that can lead to DNA damage or incompletely replicated chromosomes. If left unchecked, these insults would allow for the inheritance of damaged or incomplete copies of the genome; such genomic instability is a hallmark of cancer. To prevent this, cells have evolved checkpoint mechanisms to detect such insults and mount an appropriate response that ultimately arrests the cell cycle until the problems are resolved (Hartwell and Weinert 1989). In addition to halting progression of the cell cycle, checkpoints activate repair mechanisms, up-regulate transcriptional programmes and, in multi-cellular organisms, trigger apoptosis if the damage is irreparable.

The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for the study of cell cycle control and checkpoint mechanisms. This single-celled eukaryote has a four-phase cell cycle similar to that of higher eukaryotes, but like the budding yeast, *S. cerevisiae*, it is genetically tractable and can exist as a haploid, facilitating the identification of recessive mutants. Pioneering work in both budding and fission yeasts led to the discovery of cyclin-dependent kinases (CDKs), the enzymes that control the onset of each stage of the

cell cycle in all eukaryotes (Hartwell et al. 1974; Nurse 1975; Nurse and Thuriaux 1980). In *S. pombe*, a single CDK (Cdc2) is regulated by the association of different cyclins (Cdc13 and Cig2) to control cell cycle progression, whereas vertebrates have multiple CDK and cyclin homologues, which may have redundant functions (reviewed in Moser and Russell 2000; Kaldis and Aleem 2005). Sequencing and analysis of the complete fission yeast genome was published in 2002, which revealed that *S. pombe* is more similar to humans than *S. cerevisiae* in many respects, with more genes containing introns and larger centromeres (Wood et al. 2002).

Different checkpoint mechanisms are activated in response to different forms of damage. The replication checkpoint is activated during S-phase by agents that cause replication fork stalling, such as the replication inhibitor hydroxyurea (HU) or UV irradiation (reviewed in Osborn et al. 2002; Lambert and Carr 2005). The DNA damage checkpoint is activated throughout the cell cycle in response to agents that cause double-strand breaks (DSBs), such as ionising radiation or radio-mimetic drugs (reviewed in Zhou and Elledge 2000). Although the pathways remain distinct, the DNA damage and replication checkpoints share many components and have overlapping functions; the key components of DNA damage and replication checkpoints of *S. pombe* are illustrated in Fig. 1.1. DNA damage or replication block is detected by the "checkpoint rad" proteins, including the phosphatidylinositol-kinase-like-kinase (PIKK), Rad3, which becomes activated and phosphorylates the downstream effector kinases, Cds1 (upon replication block) or Chk1 (after DNA damage) via their associated mediator proteins, Mrc1 and Crb2. This allows for the phosphorylation of downstream targets, which ultimately leads to the inhibition of mitotic entry and subsequent cell cycle arrest. Checkpoint proteins have been identified throughout eukaryotes that are structurally and/or functionally similar to those

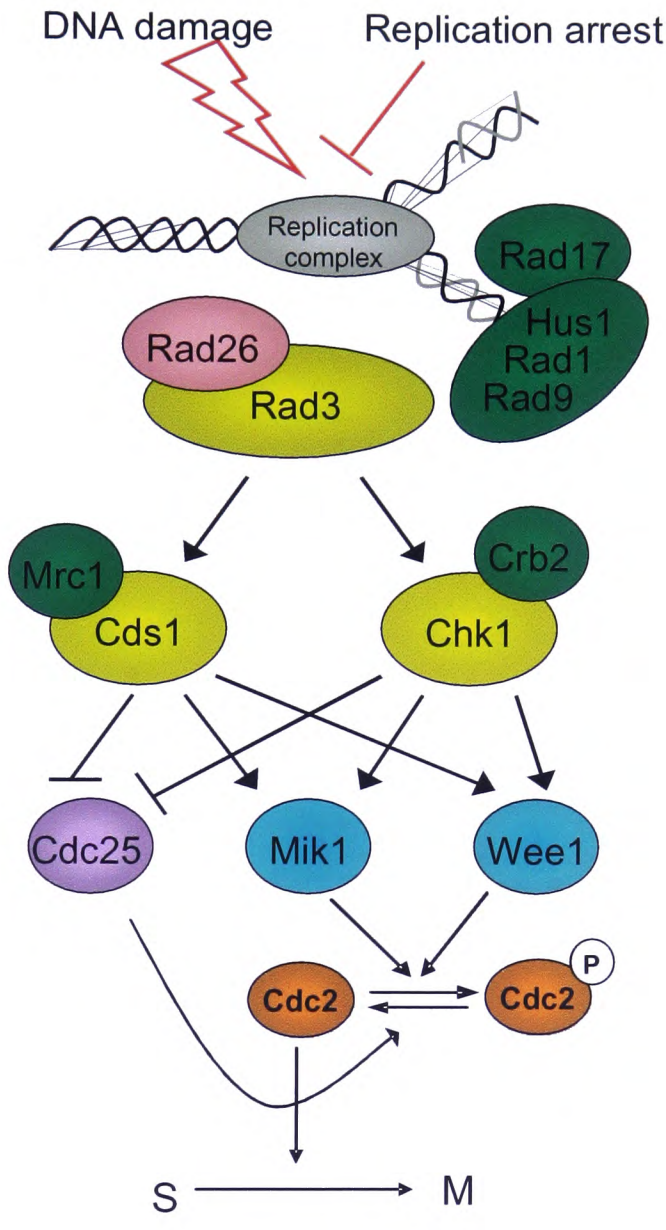


Figure 1.1. The DNA integrity checkpoints of *S. pombe*. DNA damage or replication block are sensed by the checkpoint rad proteins (Rad3, Rad26, Rad17, Rad1, Rad9, Hus1), which transduce the signal to the downstream effector kinases, Cds1 or Chk1, via their respective adaptor proteins, Mrc1 and Crb2. Cds1 and Chk1 phosphorylate various downstream effectors, which results in cell cycle arrrest.

in *S. pombe* (see Table 1.1). The focus of this section will be the checkpoint pathway in fission yeast, therefore all proteins described are those of *S. pombe*, unless indicated otherwise.

Table 1.1. Checkpoint components across eukaryotes.

S. pombe	*S. cerevisiae*	*H. sapiens*	*Description*
Rad3	Mec1	ATR	PIKK
Tel1	Tel1	ATM	PIKK
Rad26	Ddc2	ATRIP	Binds to Rad3 as a regulatory subunit
Mre11	Mre11	MRE11	MRN complex
Rad32	Rad50	RAD50	MRN complex
Nbs1	Xrs2	NBS1	MRN complex
Rad17	Rad24	RAD17	RF-C like complex (associated with Rfc 2-5)
Rad1	Rad17	RAD1	PCNA-like complex (associates with Rad9 and Hus1)
Rad9	Ddc1	RAD9	PCNA-like complex (associates with Rad1 and Hus1)
Hus1	Mec3	HUS1	PCNA-like complex (associates with Rad1 and Rad9)
Cds1	Rad53	CHK2	Ser/Thr kinase
Chk1	Chk1	CHK1	Ser/Thr kinase
Crb2/Rhp9	Rad9	BRCA1, 53BP1	BRCT domain, mediator protein for Chk1 activation
Mrc1	Mrc1	Claspin	Binds to Cds1, mediator required for replication checkpoint
Swi1	Tof1	?	Required for replication checkpoint
Rad4/Cut5	Dbp11	TOPBP1	BRCT domain protein required for replication, but also activation of Chk1 and Cds1

1.1.1 The PIKKs

Activation of a PIKK is an essential step in the initiation of a DNA integrity checkpoint signal in many eukaryotes. In fission yeast, Rad3 is required for activation of both the replication and DNA damage checkpoints (Bentley et al. 1996), whereas Tel1 is only thought to play a minor role, since *tel1Δ* mutants are not sensitive to HU or MMS (Matsuura et al. 1999). However, in vertebrates, both PIKK homologues are important for checkpoint activation; ATM (ataxia telangiectasia mutated) is essential for the response to DSBs and ATR (ATM and Rad3-related) is required for the response to replication stress. Rad3

associates with and phosphorylates its regulatory subunit, Rad26, upon checkpoint activation (Edwards et al. 1999). In vertebrates, the orthologue of Rad26 is ATRIP; the ATR-ATRIP complex is activated by ssDNA bound by replication protein A (RPA) (Zou and Elledge 2003). ATM is thought to be activated via its association with the heterotrimeric MRN complex (Mre11-Rad50-Nbs1), which binds to DSBs (Uziel et al. 2003; Falck et al. 2005). The PIKKs are large serine-threonine kinases that phosphorylate numerous downstream effectors to propagate the checkpoint signal and activate repair and replication mechanisms, however these targets vary depending on the type of damage and between organisms (reviewed in McGowan and Russell 2004).

1.1.2 The Checkpoint Rad proteins

In addition to PIKKs, other checkpoint Rad proteins are involved in sensing genomic perturbations and are required to generate a checkpoint response. The remaining four Rad proteins, Rad17, Hus1, Rad9 and Rad1, are structurally similar to components of the DNA replication machinery. Rad17 is an RFC (replication factor C) -like protein which can form a complex with RFC2-5 (Griffiths et al. 1995; Shimada et al. 1999). Hus1, Rad1 and Rad9 interact *in vivo* to form the heterotrimeric 9-1-1 complex, which is structurally similar to the homotrimeric PCNA (proliferating cell nuclear antigen) complex (Caspari et al. 2000). During replication, RFC loads PCNA onto chromatin, which encircles the DNA and tethers the polymerases to the template (reviewed in Johnson and O'Donnell 2005). By analogy with this model, the Rad17-containing RFC complex and the 9-1-1 complex are thought to act as a checkpoint-specific sliding clamp and loader. Studies in budding yeast and human cells suggest that Rad17 loads the 9-1-1 complex onto chromatin in response to DNA damage (Zou et al. 2002; Majka and Burgers 2003). These experiments also determined that

9-1-1 loading was independent of Rad3 and that this loading is a requirement for Rad3 phosphorylation of its downstream targets, which include Rad17 and Hus1. Together, these results suggest that PIKKs and Rad17/9-1-1 complexes bind independently to sites of damage and only when damage has been recognised by both can the checkpoint signal be propagated to the downstream effector kinases, Cds1 and Chk1 (Melo et al. 2001).

1.1.3 The Effector Kinases

In fission yeast, Chk1 is required for the DNA damage checkpoint (Walworth et al. 1993), whereas Cds1 is the effector of the replication checkpoint (Murakami and Okayama 1995). Cds1 and Chk1 are also serine/threonine kinases; once activated by PIKK-mediated phosphorylation, they in turn phosphorylate numerous downstream targets that activate repair and recombination mechanisms and effect cell cycle arrest. The effector kinases provide the flexibility in checkpoint signalling, allowing the cell to distinguish between various types of genomic damage and to respond in the most appropriate way to repair the lesion (reviewed in Rhind and Russell 2000).

In response to DNA damage or incomplete replication, Rad3 kinase phosphorylates Cds1 or Chk1 via different mediator proteins. Which effector kinases is activated by Rad3 also depends on the stage of the cell cycle; during S-phase, Cds1 is activated, whereas in late S or G2, Chk1 is activated (Martinho et al. 1998). In response to replication block, Rad3 phosphorylates the forkhead domain-containing mediator protein Mrc1, which allows for the activation of its interacting partner, Cds1 (Tanaka and Russell 2001). In G2 phase, DNA damage triggers Rad3 phosphorylation of Crb2, the BRCT-domain mediator protein required for Chk1 activation (Mochida et al. 2004). The activation and/or expression of mediator proteins are cell-cycle regulated, restricting their availability to the

Rad3 kinase. Exactly how this cell-cycle regulation is achieved is unclear, but Crb2 has been reported to be phosphorylated by Cdc2 and Mrc1 expression is restricted to S-phase (Tanaka and Russell 2001; Nakamura et al. 2005).

1.1.4 Effecting cell cycle arrest

In addition to activating damage-specific repair and recombination mechanisms, both the replication and the DNA damage checkpoints must ensure that the cell does not enter mitosis before any problems have been resolved. Both Cds1 and Chk1 activate mechanisms to inhibit the cell cycle machinery at the G2-M transition, by targeting the phosphorylation state of Cdc2 (Enoch and Nurse 1990; Rhind et al. 1997). Cdc2 is regulated by inhibitory phosphorylation of a tyrosine residue at position 15 (Y15) (Gould and Nurse 1989). This inactivates the CDK-cyclin complex, preventing entry into mitosis. After passing Start, Y15 is phosphorylated by the Wee1 and Mik1 tyrosine kinases (Lundgren et al. 1991; Hayles and Nurse 1995). Y15 remains phosphorylated until the onset of mitosis, when the phosphate is removed by Cdc25 and Pyp3 phosphatases (Millar et al. 1992; Millar and Russell 1992). Therefore, checkpoints could act by up-regulating the kinases or inhibiting the phosphatases that target Y15. Although the mechanisms are still unclear, there is evidence that Cds1 and Chk1 both activate Mik1 and Wee1 and inhibit Cdc25 (reviewed in Rhind and Russell 2000).

Both Cds1 and Chk1 can phosphorylate and inhibit the catalytic activity of Cdc25 (Furnari et al. 1999). In addition, phosphorylation of Cdc25 promotes binding of the 14-3-3 protein Rad24, which excludes Cdc25 from the nucleus, preventing activation of Cdc2 (Zeng and Piwnica-Worms 1999). Although *mik1Δ* mutants are only partially checkpoint-defective, Mik1 transcript and protein levels accumulate after DNA damage and HU treatment in a Cds1/Chk1 dependent manner, suggesting that it is also an important target

(Christensen et al. 2000). It is unclear whether Wee1 is targeted to effect cell cycle arrest; genetic experiments suggest Wee1 is not necessary for checkpoint function, yet Cds1 and Chk1 can phosphorylate Wee1 *in vitro* (Barbet and Carr 1993; O'Connell et al. 1997; Boddy et al. 1998). Together, these results suggest that the checkpoint kinases are likely to target more than one of these proteins to effect cell cycle arrest.

1.1.5 The replication checkpoint

The replication checkpoint is activated during S-phase when replication is perturbed. Replication forks can become blocked at endogenous pause sites or upon exposure to exogenous agents, such as replication inhibitors or DNA-damaging agents. For example, replication forks stall when exposed to the replication inhibitor hydroxyurea (HU), which down-regulates dNTP pools by inhibiting ribonucleotide reductase (RNR). This results in the exposure of ssDNA at forks, which become coated with RPA, triggering checkpoint activation (Sogo et al. 2002). The replication checkpoint has numerous functions in the response to such stresses; in addition to preventing progression into mitosis (the S-M checkpoint), Cds1-dependent mechanisms must be activated to stabilise stalled forks and to prevent firing of late origins (the intra-S-phase checkpoint) (Kai and Wang 2003; Lambert and Carr 2005). However, there is evidence of overlap with the DNA damage checkpoint; if stalled forks cannot be stabilised, they collapse to create lesions leading to subsequent activation of the DNA damage checkpoint. In this way, Chk1 is activated in HU-treated *cds1Δ* mutants, since the stalled forks cannot be stabilised effectively (Boddy et al. 1998; Lindsay et al. 1998). Chk1-dependent mechanisms are required to re-start collapsed forks by activating recombination mechanisms (Furuya et al. 2004). In addition, DNA polymerase

mutants, although defective in proteins essential for replication, also activate the DNA damage checkpoint (Francesconi et al. 1997; Mochida et al. 2004).

As well as the non-essential components of the checkpoint pathway, several proteins essential for DNA replication are required to generate a replication checkpoint signal, including DNA pol α, Cdc18, Orp1/Cdc30 and Cut5/Rad4 (which is also required for the DNA damage checkpoint). The corresponding mutants cannot initiate replication, but also fail to prevent entry into mitosis (Kelly et al. 1993; Saka and Yanagida 1993; D'Urso et al. 1995; Grallert and Nurse 1996). Genes required later in S-phase, such as DNA pol δ are required for replication, but not for activation of the replication checkpoint (D'Urso and Nurse 1997).

1.2 Cid1 is a cytoplasmic poly(A) polymerase involved in the replication checkpoint

Many checkpoint mutants abolish the dependence of mitosis on the completion of replication; in addition, several drugs are capable of overriding the replication (S-M) checkpoint. The methylxanthine caffeine can override the S-M checkpoint in fission yeast and mammalian cells (Schlegel and Pardee 1986; Wang et al. 1999). Caffeine inhibits Rad3, preventing the activation of Cds1 and Chk1 kinases (Moser et al. 2000). Therefore, when cells are treated with a combination of HU and caffeine, DNA replication is blocked without subsequent activation of the S-M checkpoint, leading to mitotic catastrophe and cell death. Over-expression of Cds1 and Chk1 can suppress the lethality of HU and caffeine (Wang et al. 1999). Based on these facts, an over-expression screen for novel components of the

replication checkpoint was performed in fission yeast, which identified an uncharacterised gene, *cid1*$^+$ (for caffeine induced death suppressor) (Wang et al. 1999).

A genetic study provided further evidence that Cid1 was involved in the replication checkpoint (Wang et al. 2000a). Deleting *cid1* caused sensitivity to a combination of HU and caffeine and *cid1*$^+$ over-expression partially suppressed the HU-sensitivity of checkpoint rad mutants, including *rad3Δ*, *rad9Δ* and *rad17Δ*. Also, DNA pol δ and ε mutants are checkpoint-defective in the absence of Cid1; *cdc27-P11* (pol δ mutant) and *cdc20-P7* (pol ε mutant) have intact S-M checkpoints, which are partially disrupted in *cid1Δ*. This study also determined that Cid1 is likely to act through Chk1, since *cds1Δcid1Δ* cells have an additive phenotype, similar to that of *cds1Δchk1Δ* cells upon HU treatment. Both HU-treated *cds1Δ* and DNA polymerase mutants depend on Chk1 to prevent mitotic entry (Mochida et al. 2004). Therefore, Cid1 may contribute to a Chk1-dependent sub-pathway of the S-M checkpoint in *S. pombe*.

Amino acid sequence analysis predicted Cid1 to be a member of the Pol β superfamily of nucleotidyltransferases, which includes DNA polymerase β and poly(A) polymerase (Aravind and Koonin 1999). A biochemical investigation determined that Cid1 was not a DNA polymerase, but a poly(A) polymerase *in vitro* (Read et al. 2002). The canonical poly(A) polymerase in yeast (PAP) is an essential nuclear enzyme, required for the general polyadenylation of mRNAs (to be discussed in the following section). However, Read et al. determined Cid1 to be exclusively cytoplasmic *in vivo* and to target a subset of RNAs *in vitro*. Also, amino acid sequence analysis indicated that Cid1 is a member of a sub-family of polymerases distinct from that of canonical PAP. Therefore, it was proposed that Cid1 is a novel type of cytoplasmic poly(A) polymerase important for cell cycle regulation (Read and Norbury 2002). It was hypothesised that Cid1 up-regulates the expression of

specific genes important for checkpoint control by targeting their mRNAs for polyadenylation in the cytoplasm, increasing their stability and/or translatability. Cytoplasmic polyadenylation is a well-known mechanism of gene regulation in metazoans (discussed below), but had not been described previously in yeast. The discovery of Cid1 in *S. pombe* suggested that cytoplasmic polyadenylation is an important mechanism for the control of gene expression in a wide range of eukaryotes.

1.3 Polyadenylation

Polyadenylation is an essential processing step for almost all eukaryotic mRNAs, with the notable exception of metazoan bulk histone mRNAs. The 3′ end of a pre-mRNA is polyadenylated in a two-step reaction, where endonucleolytic cleavage is followed by poly(A) addition. Like many RNA processing reactions, polyadenylation occurs co-transcriptionally, while the mRNA is being transcribed by RNA pol II. Several excellent reviews exist on the subject (Zhao et al. 1999; Proudfoot and O'Sullivan 2002), so only a brief description is outlined below.

Polyadenylation is a complex reaction requiring many multi-subunit protein complexes and several *cis*-acting sequences, which were first described in mammalian cells, but functional equivalents also exist in yeast (Fig. 1.2). In mammals, two sequence elements flank the cleavage site and direct poly(A) addition. The hexanucleotide signal sequence AAUAAA (or sometimes a variant of this sequence) is found 10-30nts upstream of the cleavage/poly(A) site and is highly conserved in animals (Proudfoot and Brownlee 1976). A more variable GU-rich downstream element (DSE) is located 20-40nts below the cleavage site (McLauchlan et al. 1985). The sequence variability and distance between these two

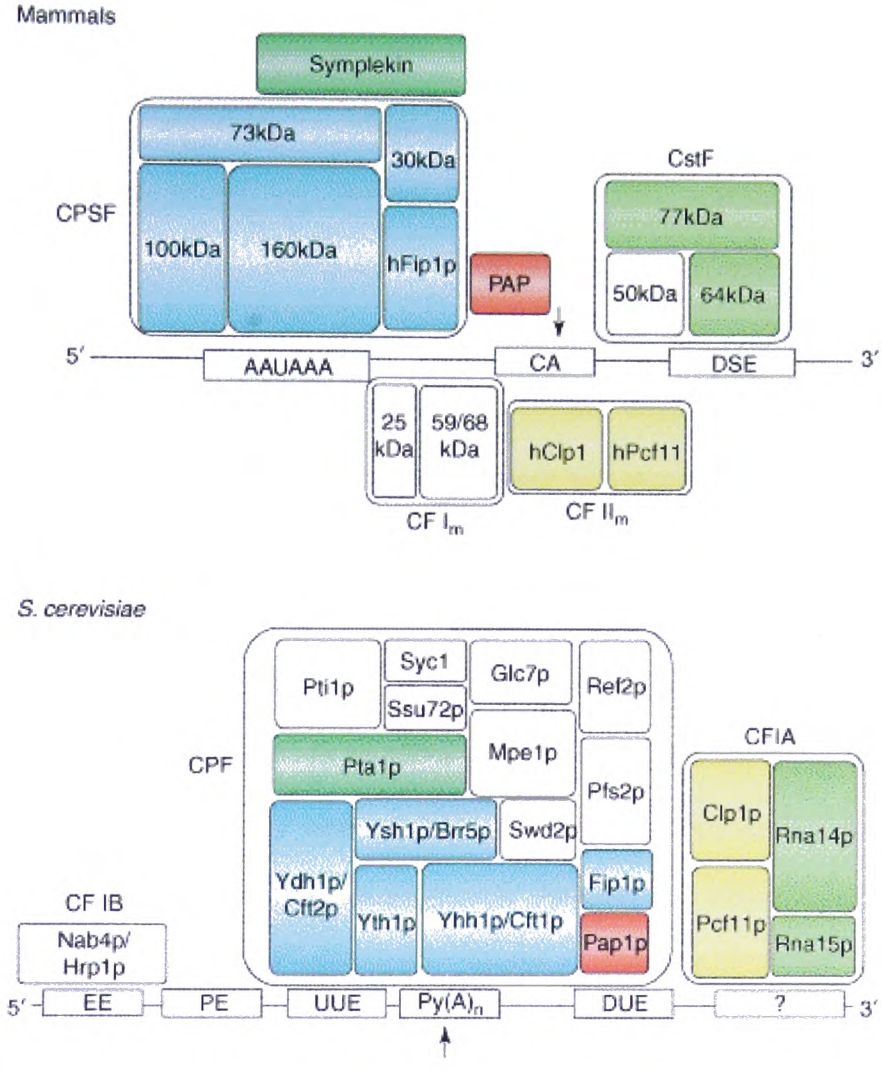


Figure 1.2. Comparison of the mammalian and budding yeast polyadenylation machineries. Subunits homologous between mammals and yeast are colour-coded. Factors that do not have equivalents in both mammals and yeast are shown in white. DSE = Downstream Element, EE = Efficiency Element, PE= Positioning Element, UUE = Upstream U-rich Element, DUE = Downstream U-rich Element, Py(A)n = cleavage/poly(A) site. Figure kindly provided by Nick Proudfoot.

elements determine the strength of the poly(A) signal and specify the site of cleavage, which generally occurs after a CA dinucleotide (Chen et al. 1995). The hexanucleotide sequence is recognised by the 160kDa subunit of CPSF (cleavage and polyadenylation stimulation factor) and the DSE is bound by the 64kDa subunit of CstF (cleavage stimulation factor) (MacDonald et al. 1994; Murthy and Manley 1995). CPSF takes part in both the cleavage and polyadenylation reactions, helping to recruit the poly(A) polymerase, PAP (Murthy and Manley 1995). On the other hand, CstF is required only for the cleavage reaction and together with CPSF, recruits the additional cleavage factors, CFIm and CFIIm (Takagaki et al. 1989). Assembly of the polyadenylation machinery also requires the carboxy-terminal domain of RNA pol II and the scaffold protein, symplekin (Hirose and Manley 1998; Takagaki and Manley 2000).

By comparison with the situation in mammals, the polyadenylation machinery of *S. cerevisiae* is less well-defined. In budding yeast, two variable upstream elements are identifiable, an AU-rich efficiency element (EE) and an A-rich positioning element (PE). Flanking the poly(A) site are two U-rich sequences, the USE and DSE (Dichtl and Keller 2001). Mammalian and yeast polyadenylation complexes contain functionally orthologous components, but also possess unique factors. Three complexes exist in yeast: CPF, CFIA and CFIB (Proudfoot and O'Sullivan 2002). CPF contains many factors functionally homologous to CPSF and symplekin, but also includes the poly(A) polymerase, Pap1p. The subunit composition of CFIA is similar to that of CstF and CFIIm. The yeast CFIB complex, an RNA-binding protein also called Hrp1p, has no known homologue in mammalian cells (Kessler et al. 1997). The mechanism of polyadenylation in *S. pombe* has not been studied in great detail, but one study suggests that mRNA 3′ end formation in fission yeast is broadly similar to that in budding yeast (Humphrey et al. 1991).

The newly-formed poly(A) tail is immediately bound by poly(A) binding protein (PABP; reviewed in Mangus et al. 2003). Nuclear PABPs regulate the ultimate length of the poly(A) tail and are required for some mRNAs to be exported from the nucleus. In the cytoplasm, PABPs facilitate the formation of the 'closed loop' mRNP structure that is crucial for promoting translation initiation and termination, the recycling of ribosomes and increasing mRNA stability (Wells et al. 1998).

1.4 Cytoplasmic polyadenylation

In addition to the essential 3' end processing reaction that occurs in the nucleus, mRNAs can be polyadenylated in the cytoplasm. The mechanism of cytoplasmic polyadenylation is distinct from that in the nucleus and has only been well characterised in animal oocytes and neuronal cells (reviewed in Mendez and Richter 2001). This process requires some of the factors essential for nuclear polyadenylation, but also specific factors required for cytoplasmic polyadenylation. In general, extension of poly(A) tails in the cytoplasm leads to translational activation, whereas deadenylation causes translational repression (Sonenberg et al. 2000). This mechanism of translational control allows for a rapid up-regulation in gene expression, without the need for new transcription.

1.4.1 Cytoplasmic polyadenylation in oocytes and early embryogenesis

Gene expression in vertebrate oocytes is controlled principally at the level of translation, where maternal mRNAs are kept dormant until later in development, when they are translationally activated by cytoplasmic polyadenylation. This process has been well characterised in *X. laevis* oocyte maturation. Immature oocytes arrest at the first meiotic

division until progesterone triggers germinal vesicle breakdown (GVBD) and re-entry into meiosis, forming a mature oocyte. After fertilisation, the oocyte completes the final meiotic division and initiates embryonic cycles of mitosis. During maturation, transcription ceases and the short poly(A) tails (20-40nts) of the maternal mRNAs required for this process are rapidly extended (to over 150nts), allowing translational activation.

The trans-acting factors and cis-acting sequences required for cytoplasmic polyadenylation have been elucidated. Two sequences in the 3′ UTR are necessary, the canonical polyadenylation element, AAUAAA and the more specific CPE (cytoplasmic polyadenylation element), with the consensus sequence UUUUAU (Fox et al. 1989). Three core factors are essential for cytoplasmic polyadenylation: CPSF, CPEB (CPE-binding protein) and a poly(A) polymerase. With similarity to nuclear polyadenylation, AAUAAA is bound by a cytoplasmic form of CPSF (Dickson et al. 1999). The CPE is bound by CPEB, an RNA binding protein containing a zinc finger and two RNA recognition motifs (RRMs) (Hake and Richter 1994). In metazoans, multiple isoforms of CPEB exist, which are likely to have distinct roles in early development. To activate oocyte maturation, progesterone binds to a cell-surface receptor, triggering a signal cascade that activates Eg1, an aurora kinase. Eg1 phosphorylates CPEB at serine 174, which increases its affinity for CPSF, allowing subsequent recruitment of a cytoplasmic poly(A) polymerase (Mendez et al. 2000a; Mendez et al. 2000b) .

The CPE is also required for the active translational repression of maternal mRNAs. Prior to oocyte maturation, the CPE is still bound by CPEB, but CPEB also interacts with another protein, Maskin, which in turn binds the cap binding protein, eIF4E. In doing so, Maskin occludes the binding site for eIF4G, preventing the assembly of a translation initiation complex on CPE-containing mRNAs. Upon oocyte maturation, Maskin

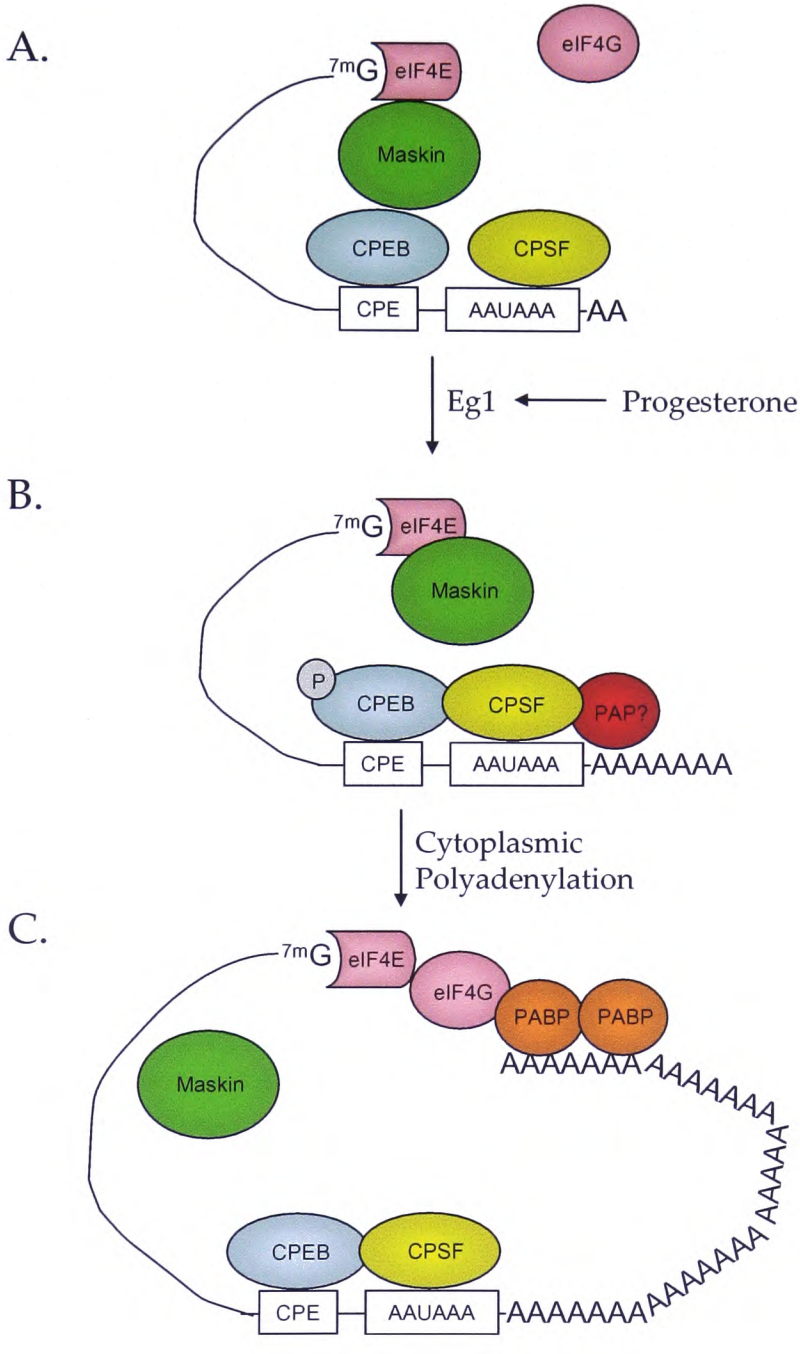


Figure 1.3. The mechanisms of translational repression and activation in the cytoplasm of *X. laevis* oocytes. (A). Maskin binds to CPEB and eIF4E, preventing assembly of the translation initiation complex. (B). Phosphorylation of CPEB increases its affinity for CPSF, which enables it to recruit PAP. (C). Cytoplasmic polyadenylation is activated, PABP binds to the poly(A) tail and binds eIF4G, allowing it to displace maskin. Adapted from Richter, 1999.

is displaced, allowing the binding of eIF4G (Stebbins-Boaz et al. 1999). The mechanism that triggers this exchange is not yet clear; the PABP-eIF4G interaction that occurs as a result of cytoplasmic polyadenylation may help displace Maskin, and recently the phosphorylation of Maskin by MPF has also been implicated in triggering its dissociation from eIF4E (Wakiyama et al. 2000; Barnard et al. 2005). The proposed model for cytoplasmic translational repression and activation in *X. laevis* oocytes is illustrated in Fig. 1.3.

Targets for cytoplasmic polyadenylation during early development include *mos*, *cyclin B1* and *wee1* mRNAs, which are activated in a strict temporal pattern. The number of CPEs, their precise sequence and their proximity to other sequence elements can all affect the timing of an mRNA's translational activation. *mos* is one of the earliest mRNAs to be targeted; it encodes a serine/threonine kinase that triggers a mitogen - activated protein kinase (MAPK) signalling cascade that triggers many of the processes required for maturation, including GVBD and chromosome condensation (Sagata et al. 1989). Mos is also implicated in the adenylation of later-acting mRNAs, including that encoding *cyclin B1*, which contains a Mos response element (MRE) in addition to a CPE (de Moor and Richter 1997). Cyclin B1 dimerises with CDK1 to form maturation promoting factor (MPF), which drives the progression of meiosis (Barkoff et al. 2000). Wee1 must be activated after oocyte maturation, since it is inhibitory to GVBD. Cytoplasmic polyadenylation of *wee1* mRNA after GVBD is required for the extended duration of the first mitotic cycle after fertilisation, which may be required for gamete fusion (Charlesworth et al. 2000).

Post-fertilisation, 90% of CPEB is destroyed; this is triggered by its phosphorylation by MPF (Mendez et al. 2002). However, the remainder is required for CPEB-mediated polyadenylation in early embryogenesis, which ensures the correct temporal and spatial translation of important maternal mRNAs. In *X. laevis*, the remaining

CPEB localises with Maskin and *cyclin B1* mRNA to the spindles and centrosomes of the early embryo; this ensures *cyclin B1* is activated at the correct time and place for mitosis (Groisman et al. 2000). In *D. melanogaster*, the CPEB homologue Orb directs the localisation and translation of *oskar* and *gurken* mRNAs, which set up the body plan in the early fly embryo (Christerson and McKearin 1994).

1.4.2 Cytoplasmic polyadenylation in somatic cells

Cytoplasmic polyadenylation was initially thought to regulate gene expression only during early development; however, there is increasing evidence that it also has a role to play in adult cells. In the mouse, CPEB is mainly found in the testes and ovaries, but some is also localised to the hippocampus and visual cortex of the brain (Wu et al. 1998). This led to the discovery that CPEB-mediated polyadenylation is required for the activation of dendritic mRNAs in neurons, in response to synaptic stimulation.

Glutamate stimulation of N-methyl-D-aspartate (NMDA) receptors in the brain triggers a signalling pathway that activates Aurora kinase, which phosphorylates CPEB and allows cytoplasmic polyadenylation (Wells et al. 2001; Huang et al. 2002). However, few mRNA targets of CPEB-dependent polyadenylation in the brain have been identified. One dendritic mRNA activated in this way encodes a subunit of calcium/calmodulin-dependent protein kinase II (α-CaMKII), essential for many neuronal functions. *α-CaMKII* mRNA contains CPE sequences and is up-regulated in response to light-induced synaptic stimulation in a CPE-dependent manner (Wu et al. 1998). Upon synaptic stimulation, CPEB not only activates the translation of neuronal mRNAs, but also controls their dendritic localisation in rat neurons (Huang et al. 2003). In this study, CPEB co-localised with dynein, kinesin and maskin to transport a CPE-containing GFP reporter mRNA along microtubules.

1.4.3 The cytoplasmic poly(A) polymerase

The poly(A) polymerase responsible for cytoplasmic polyadenylation in animal oocytes was not identified until recently. In humans, at least four isoforms of the canonical poly(A) polymerase exist: PAPI, PAPII, neo-PAP and TPAP. PAPI and PAPII (also called PAP α and β) are two isoforms of the canonical *PAP* gene, created by alternative splicing. Further isoforms have been reported, but are unlikely to encode functional enzymes (Zhao and Manley 1996). The longest isoform, PAPII, is thought to be the major form of poly(A) polymerase enzyme in human tissues. The *neo-PAP* gene (also called PAPγ) encodes a nuclear poly(A) polymerase that is 60% identical to PAPII and is over-expressed in some human tumours (Kyriakopoulou et al. 2001; Topalian et al. 2001). The *TPAP* gene encodes a testes-specific PAP, found in the cytoplasm of spermatocytes (Kashiwabara et al. 2000; Lee et al. 2000). However, it is unlikely that PAPI/II or neoPAP are responsible for cytoplasmic polyadenylation, since they all possess a nuclear localisation sequence (NLS) and undergo inhibitory phosphorylation by CDKs at mitosis, the very time when cytoplasmic polyadenylation is required during oocyte maturation (Colgan et al. 1996). Although TPAP is required for the cytoplasmic polyadenylation of some mRNAs during spermatogenesis, it is thought to act by a CPEB-independent mechanism (Kashiwabara et al. 2002).

Together, these studies indicate that an enzyme other than a canonical PAP is responsible for CPEB-dependent polyadenylation in higher eukaryotes. Recently, evidence has emerged to suggest that a Cid1-like protein is the cytoplasmic poly(A) polymerase activated by CPEB (discussed below). However, since CPEB and maskin are not conserved in fission yeast, the mechanism of Cid1-mediated cytoplasmic polyadenylation in *S. pombe* is likely to differ from that described in early animal development.

1.5 Cid1 is a member of a conserved family of poly(A) polymerases

Apart from Cid1, five other Cid1-like proteins were discovered in *S. pombe*, revealing the existence of a multi-protein Cid1 family. Further sequence analysis revealed that Cid1 families are conserved throughout eukaryotes (Wang et al. 2000a; Read and Norbury 2002). All members of the Cid1 family are related to the canonical PAP, containing similar catalytic NTP transferase and PAP-associated domains. However, apart from these regions, Cid1-like proteins are highly diverged from the classical PAP enzyme, revealing a novel class of non-canonical poly(A) polymerases (Read et al. 2002) (Fig. 1.4). An inundation of recent publications suggests that all members of the Cid1 family are likely to be poly(A) polymerases, however, not all are cytoplasmic. These studies reveal that Cid1-like poly(A) polymerases are involved in a diverse range of biological processes.

1.5.1 S. cerevisiae Trf4/5

Only two Cid1-related proteins exist in budding yeast, Trf4 and Trf5. In contrast to Cid1, both proteins are exclusively nuclear (Walowsky et al. 1999). The lack of a cytoplasmic Cid1-like protein and the reduced number of paralogues suggest that budding yeast lack some of the cellular processes shared by fission yeast and higher eukaryotes. *TRF4* was originally identified as a gene that, when mutated, showed synthetic lethality with mutations in DNA topoisomerase I (*TOP1*), an enzyme that relaxes the supercoiling of the DNA duplex, allowing replication to proceed (Sadoff et al. 1995). Further genetic studies identified a *TRF4* paralogue, *TRF5*; although neither gene is essential on its own, a *trf4Δtrf5Δ* double mutant is inviable, implying that Trf4 and Trf5 have overlapping, essential functions (Castano et al. 1996b). Further analyses indicated a mitotic role for Trf4; Trf4 interacts with

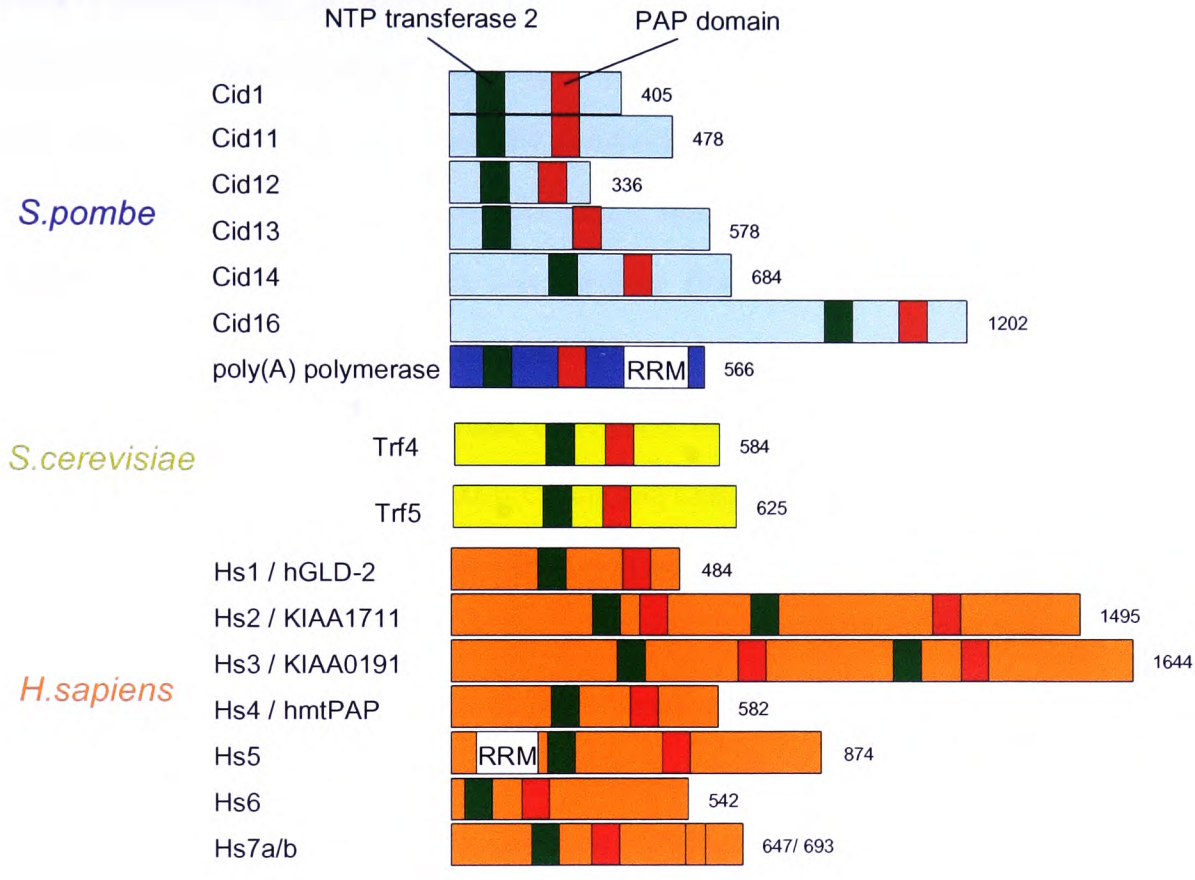


Figure 1.4. Cid1-like proteins are conserved across eukaryotes. The Cid1 family are members of the polymerase β superfamily, which includes poly(A) polymerase (dark blue). Cid1-like proteins all share the catalytic NTP transferase (green) and PAP-associated domains (red) but otherwise are highly diverged from the classical poly(A) polymerase.

a subunit of cohesin, Smc1, and a *trf4-ts top1Δ* mutant is defective in nuclear segregation at the restrictive temperature (Castano et al. 1996a). However, evidence also emerged for an essential role for Trf4 in DNA replication; Trf4 purified from bacteria was reported to have DNA polymerase activity *in vitro*, *trf4-ts trf5Δ* cannot complete S-phase at the restrictive temperature and Trf4 can interact with and stimulate DNA pol ε (Wang et al. 2000b; Edwards et al. 2003). Together, these studies led to the notion that Trf4/5 is the fourth essential DNA polymerase in budding yeast, DNA pol σ, required for the establishment of sister chromatid cohesion (Wang et al. 2000b; Carson and Christman 2001).

Recently, the role of Trf4/5 as a DNA polymerase has come into question, with the publication of numerous independent reports of Trf4 possessing poly(A) polymerase activity (Lacava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005). These studies also elucidated a biological role for Trf4-mediated polyadenylation in the activation of the nuclear exosome. The nuclear exosome is a complex of multiple 3′-5′ exonucleases, required for the degradation and/or processing of numerous RNAs (Mitchell et al. 1997). However, the purified exosome has weak degradation activity *in vitro*, suggesting that other co-factors are necessary for efficient RNA degradation *in vivo*. Trf4 was found to be a member of the TRAMP complex, which can stimulate the activity of the exosome *in vitro* and is required for the processing of numerous exosome substrates *in vivo* (Lacava et al. 2005; Vanacova et al. 2005). Trf4 interacts with an RNA helicase (Mtr4) and one of two functionally redundant putative RNA-binding proteins (Air1 or Air2) to form the TRAMP complex (for Trf4/Air1/Mtr4 polyadenylation complex). In this new model, Air1/2 recruits the TRAMP complex to its target RNAs, which are polyadenylated by Trf4. Poly(A) addition allows recruitment and activation of the exosome, which degrades the RNA in a 3′-5′ direction, facilitated by the helicase activity of Mtr4. It is likely that multiple rounds of TRAMP

polyadenylation and exosome degradation are required for complete destruction of the RNA.

The original report of Trf4 as an activator of exosome-mediated degradation described an incorrectly-modified initiator tRNA ($tRNA^{Met}$) as the substrate for Trf4 polyadenylation (Kadaba et al. 2004). Since then, Trf4 has been shown to target various RNAs for degradation and/or processing in the nucleus, including pre-rRNAs, pre-snoRNAs and intergenic cryptic transcripts (Lacava et al. 2005; Wyers et al. 2005). However, these studies failed to show Trf5 poly(A) polymerase activity *in vitro*. Since then, several groups have been successful in purifying active Trf5 and have shown that it also contributes to rRNA processing, like Trf4 (Haracska et al. 2005; Houseley and Tollervey 2005; Egecioglu et al. 2006). Together, these results suggest that Trf4/5 and the TRAMP complex are part of a nuclear surveillance mechanism in eukaryotes, preventing the export and subsequent expression of aberrant RNAs.

The conflicting reports describing Trf4 enzymatic activity are difficult to reconcile, since it is unlikely that Trf4 possesses both DNA polymerase and poly(A) polymerase activities. Given the solid evidence presented in the recent papers describing the TRAMP complex and the high sequence homology of Trf4/5 with the rest of the Cid1 family, it is more likely that Trf4/5 are poly(A) polymerases. However, the genetic data linking Trf4 to replication and chromosome cohesion cannot be disregarded, yet it is difficult to explain how a nuclear surveillance complex could affect such mechanisms. Perhaps the Trf4/Trf5 poly(A) polymerases have other cellular roles, in addition to that of the TRAMP complex. Alternatively, the TRAMP complex may be required for the correct processing of RNAs whose protein products are essential for replication and cohesion.

1.5.2 The *S. pombe* Cid1 family

The five other members of the Cid1 family in *S. pombe* were named Cid11, Cid12, Cid13, Cid14 and Cid16. Recent publications reveal that members of this family of non-canonical PAPs are located in both the nucleus and cytoplasm in fission yeast. Two independent studies identified Cid13 as another cytoplasmic PAP of *S. pombe* (Read et al. 2002; Saitoh et al. 2002). Cid13 purified from yeast has PAP activity *in vitro,* but Cid13 was originally identified as a suppressor of the HU-sensitivity of *rad3-ts* and *rad26-ΔN73* mutants. Unlike Cid1, Cid13 is likely to function in a checkpoint-independent manner to allow cells to survive replication stress; a *cid13Δ* strain is hypersensitive to HU treatment as a result of reduced cellular levels of dNTPs and over-expression of *cid13*$^{+}$ cannot suppress the S-M checkpoint defect of *rad3Δ.* Therefore, Cid13 was proposed to act by restoring dNTP levels, but the mechanism of how Cid13 achieves this by cytoplasmic polyadenylation remains controversial. One study reported that Cid13 targets the cell-cycle regulated isoform of *suc22* mRNA, which encodes the small subunit of RNR (Saitoh et al. 2002). In this model, Cid13-mediated cytoplasmic polyadenylation of *suc22* increases its stability and translatability, which enhances dNTP synthesis. In support of this, a subtle decrease in the abundance, stability and polyadenylation of *suc22* mRNA was detected in *cid13Δ* mutants. However, another group found no reduction in Suc22 protein levels in *cid13Δ* cells, even in the presence of HU (Read et al. 2002). Therefore, it is likely that Cid13 targets other RNAs in addition to *suc22* to enhance dNTP levels in response to HU treatment.

A recent publication presents strong evidence that Cid14 is the *S. pombe* homologue of Trf4/5 (Win et al., in press). Cid14 is a nuclear enzyme, enriched in the nucleolus. Unlike Trf4/5, Cid14 is non-essential, but a *cid14Δ* strain has a slow growth

phenotype, aberrant nucleolar structure and suffers from chromosome segregation defects. The expression of *cid14*$^{+}$ can complement the temperature sensitivity of a *trf4-ts top1Δ* mutant, whereas other *S. pombe* Cid proteins cannot. In addition, *cid14Δ* has defects in pre-rRNA processing, like *trf4Δ*, and polyadenylated rRNA accumulates in an exosome mutant in a Cid14-dependent manner. Although Cid14 PAP activity has not yet been confirmed *in vitro*, these results indicate that Cid14 is also likely to polyadenylate pre-rRNAs to target them for processing by the exosome, like Trf4/5. This suggests that the mechanism of exosome activation by polyadenylation described in *S. cerevisiae* is conserved in other eukaryotes.

An unexpected role for a Cid1-like protein in heterochromatin formation was discovered when Cid12 was identified as part of the RNA-directed RNA polymerase complex (RDRC), which also includes the RNA-directed RNA polymerase, Rdp1, and the RNA helicase, Hrr1 (Motamedi et al. 2004). RNAi-dependent heterochromatin formation in *S. pombe* (reviewed in Martienssen et al. 2005) requires the RDRC and the RITS (RNA-induced transcriptional silencing) complex. In brief, short interfering RNAs (siRNAs) produced by Dcr1 from centromeric transcripts are loaded into the RITS complex (containing Ago1, Tas3 and Chp1). Together, RDRC and RITS direct centromeric histone methylation by Clr4, leading to Swi6 and cohesin binding and the formation of transcriptionally-silent heterochromatin. *cid12Δ* is defective in centromeric silencing and has severe chromosome segregation defects, providing further evidence for a role in heterochromatin formation (Motamedi et al. 2004; Win and Wang, submitted). The polyadenylation activity of Cid12 is likely to be important for this function, since mutation of residues required for poly(A) polymerase activity also results in loss of centromeric silencing (Win and Wang, submitted). However, the role of the Cid12-mediated poly(A) tail

and its RNA targets are yet to be characterised. In keeping with its proposed role in RNAi, Cid12 was localised mainly to the nucleus by immunofluorescence, but some cytoplasmic localisation was also observed (Motamedi et al. 2004). Cid12 may have other roles, in addition to and independent from its RNAi function. Cid12 interacts with numerous spliceosome proteins, as well as the RDRC complex (Motamedi et al. 2004) and genetic analysis of *cid12Δ* revealed a meiotic defect and a disrupted S-M checkpoint phenotype with *cdc27-P11*, like Cid1 (Win and Wang, submitted).

Although *S. pombe* Cid11 and Cid16 are yet to be characterised, neither is required for cellular viability (S.W. Wang, unpublished results). Their strong sequence similarity to the confirmed PAPs, Cid1 and Cid13, suggest that they are also likely to possess a polyadenylation activity. In light of recent data, it is unlikely that Cid1-like proteins perform redundant functions in *S. pombe*. Therefore, it is likely that each Cid1-like protein targets a different set of RNAs to affect different cellular processes. Encoding such a repertoire of regulatory poly(A) polymerases provides the cell with increased flexibility for the control of gene expression, which is likely to be of great importance in higher eukaryotes.

1.5.3 The Cid1 family in metazoans

The first member of the Cid1 family to be characterised in higher eukaryotes was GLD-2, a cytoplasmic poly(A) polymerase of *C. elegans* (Wang et al. 2002a). *gld-2* was identified as a gene involved in control of germline development and promotes the transition from mitosis to meiosis (Kadyk and Kimble 1998). GLD-2 had weak PAP activity *in vitro* and was localised to the cytoplasm in germline cells and early embryos (Wang et al. 2002a). This

study also identified a GLD-2 interacting partner, GLD-3, a Bicaudal C-like KH domain RNA-binding protein also required for germline development (Eckmann et al. 2002). GLD-3 can stimulate the PAP activity of GLD-2 *in vitro*. Therefore, it was proposed that GLD-2 and GLD-3 form a heterodimeric PAP *in vivo*; GLD-3 may direct GLD-2 to meiotic-specific mRNAs to allow their cytoplasmic polyadenylation, stabilisation and subsequent translation (Wang et al. 2002a). The RNA targets of GLD-2 are yet to be identified, but one possibility is *gld-1*, which encodes a STAR protein that represses the translation of mitotic-specific genes and works in parallel with GLD-2 to promote meiosis (Eckmann et al. 2004). In addition to its role in the germline, GLD-2 is likely to function in somatic cells, since an alternatively-spliced *gld-2* isoform is expressed in adult tissue, but this is yet to be characterised (Wang et al. 2004). Like fission yeast, *C. elegans* has numerous other Cid1-like proteins. A potential Cid12 homologue, RDE-3, is essential for RNAi and fertility, supporting a central role for Cid1-like proteins in gene silencing pathways (Chen et al. 2005).

Five Cid1-like proteins were identified in humans and were named Hs1 - Hs5 (Kwak et al. 2004); however, closer inspection of the annotated human genome reveals the existence of at least seven human Cid proteins (C.J. Norbury, personal communication). Kwak et al. performed an analysis of the PAP activities of Hs1-Hs5 and their mouse homologues using a reporter assay in *X. laevis* oocytes. Since the activity of *C. elegans* GLD-2 was stimulated by an RNA-binding partner, this study used MS2 coat protein to tether the potential PAPs to a luciferase reporter mRNA. This method was successful in showing PAP activity for *C. elegans* GLD-2 and the previously uncharacterised human Cid1-like protein Hs1 and its mouse homologue, Mm1. As a result of this study and their high sequence similarity to *C. elegans* GLD-2, Hs1 and Mm1 were re-named human GLD-2 (hGLD-2) and

mouse GLD-2 (mGLD-2). Recombinant mouse and human GLD-2 were also shown to be active PAPs *in vitro* (Nakanishi et al. 2005; Rouhana et al. 2005).

In an attempt to identify novel factors responsible for cytoplasmic polyadenylation in *X. laevis* oocytes, Richter and colleagues discovered a GLD-2 homologue (xGLD-2) through its co-purification with symplekin (Barnard et al. 2004). In this elegant study, Barnard et al. presented strong evidence that xGLD-2 is the long-sought enzyme responsible for cytoplasmic polyadenylation in metazoan oocytes. Immuno-depletion of oocyte extracts with anti-symplekin antibodies abolished cytoplasmic polyadenylation of a reporter RNA. Adding back recombinant symplekin, CPSF and CPEB did not restore polyadenylation; an additional factor was required, which turned out to be xGLD-2. As well as promoting polyadenylation, recombinant xGLD-2 interacted with known components of the cytoplasmic polyadenylation machinery *in vitro*, including CPEB and CPSF-160. Immuno-staining of a *X. laevis* cell line using xGLD-2 antibodies showed GLD-2 to be localised to the cytoplasm.

In support of these data, an independent study showed that recombinant xGLD-2 had PAP activity *in vitro* and that endogenous xGLD-2 binds to CPEB, CPSF-100 and CPSF-73, but not Maskin *in vivo* (Rouhana et al. 2005). In addition, Rouhana et al. used RNA immunoprecipitation to show that xGLD-2 specifically associates with *cyclin B1* and *c-mos* mRNAs, but not with those encoding actin or ribosomal proteins. However, this study showed xGLD-2 to be localised to both the nucleus and cytoplasm in immature *X. laevis* oocytes.

Together, these studies suggest that xGLD-2 is targeted to its mRNA substrates in the cytoplasm by CPSF and CPEB, with symplekin acting as a scaffold protein to promote these interactions, forming the "G-complex" (for GLD-2). Barnard et al. showed that this

complex is present even before oocyte maturation is stimulated by progesterone. Upon maturation, xGLD-2 is activated by an unknown mechanism and the target mRNA is polyadenylated. Rouhana et al. suggest that translationally-repressed target mRNAs can also exist in an "MP complex" with the repressor proteins Maskin and Pumilio instead of xGLD-2, but how and when mRNAs switch from MP to G complexes is unclear.

xGLD-2 shares 62% amino acid identity with human and mouse GLD-2. In all three species, long and short mRNA isoforms are expressed, which differ in the length of their 3′ UTRs (Rouhana et al. 2005). Northern blot analyses showed that both isoforms of mGLD-2 are expressed in a wide variety of tissues (Nakanishi et al. 2005; Rouhana et al. 2005). In somatic, testicular and cultured cells, mGLD-2 was localised to both the nucleus and cytoplasm by immuno-blot analysis (Nakanishi et al. 2005). In addition, this study identified an NLS in mGLD-2 that was capable of translocating an EGFP fusion protein from the cytoplasm to the nucleus. However, HA-tagged mGLD-2 in oocytes was enriched in the nucleus prior to germinal vesicle breakdown (GVBD), after which it became dispersed in the cytoplasm. Nakanishi et al. also showed that the short isoform of *mgld-2* mRNA is itself subject to cytoplasmic polyadenylation in oocytes.

Although the function of nuclear mGLD-2 is unclear, these studies show that mGLD2 is also likely to have a role in cytoplasmic polyadenylation. mGLD-2 was required for murine oocyte maturation and affected the polyadenylation of *cyclin B1* and *c-mos* mRNAs, like xGLD-2 (Nakanishi et al. 2005). There is also indirect evidence that mouse and human GLD-2 homologues are responsible for localised cytoplasmic polyadenylation of neuronal mRNAs at synapses; mouse and human *gld-2* mRNA is abundant in the brain and co-localises with *CPEB1* and *pumilio1* mRNAs in the hippocampus (Rouhana et al. 2005).

Besides Hs1/hGLD-2, only one other Cid1-like protein has been characterised in human cells. Hs4 is a PAP localised to the mitochondria, also known as hmtPAP (Tomecki et al. 2004; Nagaike et al. 2005; Min, S., unpublished results). In animals, mitochondrial-encoded mRNAs (mtRNAs) are polyadenylated; this process is required to generate complete stop codons for some mtRNAs (Montoya et al. 1981). The enzyme responsible was not thought to be the canonical PAP, which lacks a mitochondrial target sequence. Recently, Hs4 was identified as the mitochondrial poly(A) polymerase required for mtRNA polyadenylation; mtRNA targets of Hs4 include those encoding the oxidative chain enzymes, COXI-III, ATP6/8 and ND3 (Tomecki et al. 2004; Nagaike et al. 2005). Recombinant Hs4 has PAP activity *in vitro* and RNAi knock-down of endogenous Hs4 decreased the poly(A) tail length of mtRNAs, but not those of nuclear-encoded mRNAs. Also, cells with reduced Hs4 have dysfunctional mitochondria with reduced oxygen consumption (Nagaike et al. 2005). Generally, polyadenylation of mRNAs causes increased RNA stability in eukaryotes, but triggers RNA decay in bacteria and chloroplasts (Dreyfus and Regnier 2002). The role of polyadenylation in mitochondria is unclear, since there are conflicting reports of the effect of poly(A) removal on mtRNA stability. Tomecki et al. reported an increase in the stability of *ND3* mtRNA upon Hs4 knock-down, whereas similar experiments by Nagaike et al. showed a decrease in *COXI-III* and *ATP6* mtRNA stability. Therefore, Hs4-mediated polyadenylation may not be a critical regulator of all mtRNAs.

As yet, a Trf4/Cid14 homologue in higher eukaryotes has not been described. However, it is likely that polyadenylation by a nuclear Cid1-like protein is also involved in exosome activation in humans, as a report of adenylated exosome substrates in HeLa cells has been published recently (West et al. 2006).

1.5.4 Summary of the Cid1 family

The Cid1 family describes an entirely novel group of PAPs, with its members being evolutionarily distinct from the canonical PAP, but having a similar catalytic activity. Cid1-like proteins are regulatory PAPs, targeting specific RNAs to control the expression and/or processing of transcripts involved in diverse cellular processes, such as cell cycle regulation and RNAi-dependent gene silencing. Unlike the canonical PAP, the majority of Cid1-like proteins lack an RRM; therefore, it is likely that additional RNA-binding protein partners are required to target their substrates for polyadenylation, such as GLD-3, CPSF or CPEB. As well as bringing the PAP to the 3′ end of a target RNA, such RNA-binding partners could provide sequence specificity. In this way, Cid1-like proteins may interact with a number of binding partners, allowing them to target different substrates in different situations.

As a general consensus, polyadenylation by cytoplasmic members of the Cid1 family seems to be involved in the stabilisation and increased translatability of their target RNAs, whereas polyadenylation of RNAs by nuclear Cid1-like proteins leads to their degradation by the nuclear exosome. In this case, nuclear polyadenylation by Cid1-like proteins is reminiscent of the situation in bacteria, where polyadenylation also targets RNAs for degradation (Dreyfus and Regnier 2002). Perhaps nuclear Cid1-like enzymes retained this bacterial-like ability to target RNAs for degradation, whereas PAP-dependent polyadenylation evolved to allow mRNAs to escape being targeted by the nuclear exosome, perhaps by being bound by PABP.

1.6 Aims of this study

This thesis is concerned with the functional characterisation of Cid1, a novel cytoplasmic poly(A) polymerase of *S. pombe*. Although Cid1 has been characterised biochemically as a poly(A) polymerase and has genetic interactions with checkpoint pathways, the mechanism by which Cid1 affects the replication checkpoint by cytoplasmic polyadenylation is unknown. To determine the biological function of Cid1, two main areas of research were undertaken:

i) Identification of Cid1 RNA targets (Chapter 2)

ii) Identification of Cid1-interacting proteins (Chapters 3 and 4)

1.6.1 Identifying Cid1 Targets

By analogy with other members of the Cid1 family, Cid1 is likely to target specific mRNAs for polyadenylation in the cytoplasm, leading to their increased stability and translatability. In support of this hypothesis, recombinant Cid1 targets a subset of RNAs *in vitro*, whereas canonical PAP polyadenylates almost all mRNAs (Read et al. 2002). Microarray hybridisation and proteomics approaches were used to identify potential target RNAs that are polyadenylated by Cid1 *in vivo*. Confirmation of these RNAs as genuine Cid1 targets was achieved by northern analysis.

1.6.2 Identifying Cid1-interacting Proteins

Proteins rarely act alone when performing their functions *in vivo* and since Cid1 lacks a canonical RRM, it is unlikely to bind to its target RNAs by itself. Therefore, it was hypothesised that Cid1 acts as part of a complex to carry out its role as a cytoplasmic

poly(A) polymerase. Two complementary methods were used to identify members of the Cid1 complex: yeast two-hybrid screening and affinity purification. Mapping the Cid1 interaction network also gave further clues into the biological function for Cid1, since proteins often co-purify with members of the same biological pathway.

Chapter Two

Identification of Cid1 RNA Substrates

2.1 Introduction

As discussed, Cid1 is likely to target specific RNAs for polyadenylation in the cytoplasm. To identify these RNAs, strains with and without Cid1 were compared at the levels of total RNA and total protein, using microarray hybridisation and two-dimensional liquid chromatography (2D-LC), as described below.

2.2 Microarray studies

If Cid1 polyadenylation of an RNA target causes extension of its poly(A) tail, allowing PABP binding and RNA stabilisation, that RNA should become more abundant. Cid1 targets might therefore be identified by comparing differences at the level of total RNA between strains with and without Cid1. Differences in RNA transcripts were detected using *S. pombe* whole-genome microarrays, in collaboration with Jürg Bähler at the Sanger Institute. In brief, total RNA was purified from the two strains to be compared and converted to cDNA, labelled with different fluorescent dyes (Cy3 and Cy5). The pools of cDNA were mixed equally and hybridised to chips spotted with DNA probes unique to every gene in the *S. pombe* genome, in addition to well-known non-genic transcripts, such as rDNA repeats, telomeric and centromeric sequences. After overnight hybridisation and

washing, the microarray chips were scanned for spots with a Cy3:Cy5 signal ratio less or greater than 1.0, indicating a difference at the RNA level for that transcript.

In the first microarray experiment, total RNA from a wildtype strain was compared with a *cid1Δ* strain, both grown under "normal" conditions. Two independent hybridisations were carried out, with a dye-swap to correct for any dye bias. However, no significant differences for any transcript were found, except for *cid1* (data not shown). This was unexpected, as it is rare to detect no differences at the total RNA level when comparing wildtype and mutant strains (J. Bähler, personal communication), especially since the mutant in question lacks an RNA processing factor. This result suggested two possibilities; either Cid1 is inactive and does not affect RNAs during "normal", unperturbed growth, or Cid1 polyadenylation alters a target RNA's translatability without affecting its absolute level. Previous microarray experiments (R. Read, unpublished results), comparing polysome-associated RNA purified from wildtype and *cid1Δ* detected only subtle decreases in the polysome-association (and therefore translatability) of a few transcripts in the absence of Cid1, including those encoding histones, Spd1 and several ribosomal proteins. However, differential polyadenylation of these RNAs in the absence of Cid1 could not be confirmed. Therefore, further microarray experiments were carried out comparing strains with and without Cid1, grown under certain types of replication stress, where Cid1 function is known to be important for checkpoint function (Wang et al. 2000a).

The second microarray experiment compared total RNA from the DNA pol δ mutant, *cdc27-P11* and *cdc27-P11 cid1Δ*. The defective elongation DNA polymerase in *cdc27-P11* causes replication fork stalling and activation of a Chk1-dependent checkpoint (Bermudez et al. 2002; Mochida et al. 2004). *cdc27-P11 cid1Δ* loses viability faster than *cdc27-P11*. A list of transcripts whose expression was altered significantly by the absence of Cid1

Table 2.1 (A). Genes up-regulated significantly in *cdc27-P11 cid1Δ* compared to *cdc27-P11*

Gene ID	Common name	Fold change		Mean change	Comments
		Expt. 1	*Expt. 2*		
SPCC330.05c	*ura4*	6.55	6.73	6.64	
SPBC3E7.02c	*hsp16*	4.94	3.32	4.13	heat shock protein
SPAC186.05c	unknown function	3.87	1.84	2.85	telomere-proximal
SPAPJ760.03c	*adg1*	1.94	2.32	2.13	cell-cycle regulated
SPBC16D10.08c		1.76	2.42	2.09	heat shock protein
SPAC1834.03c	*histone h4.1*	2.11	2.05	2.08	cell-cycle regulated
SPBC1105.11c	*histone h3.3*	2.18	1.94	2.06	cell-cycle regulated
SPAC29B12.03	*spd1*	2.45	1.65	2.05	cell-cycle regulated
SPAC19G12.06c	*histone hta2 (H2A)*	2.06	1.98	2.02	cell-cycle regulated
SPBC8D2.03c	*histone h4.2*	2.04	1.94	1.99	cell-cycle regulated
SPAC750.07c		1.92	1.98	1.95	telomere-proximal
SPBC26H8.11c		2.23	1.60	1.92	
SPAC19G12.16c	*adg2*	1.51	2.20	1.86	cell-cycle regulated
SPCC338.12		1.62	1.88	1.75	
SPAC8C9.03	*cgs1*	1.98	1.50	1.74	
SPAC1834.04	*histone h3.1*	1.92	1.53	1.72	cell-cycle regulated
SPBC36.03c		1.77	1.64	1.71	
SPBC1105.12	*histone h4.3*	1.84	1.58	1.71	cell-cycle regulated
SPCC622.09	*histone htb1 (H2B)*	1.83	1.55	1.69	cell-cycle regulated
SPAC212.08c		1.61	1.77	1.69	telomere-proximal
SPAPYUG7.03c	*mid2*	1.51	1.78	1.64	cell-cycle regulated
SPAC30C2.02	*mmd1*	1.50	1.67	1.58	
SPCC4F11.04c		1.57	1.55	1.56	
SPCC4F11.03c		1.58	1.51	1.55	

Table 2.1 (B). Genes down-regulated significantly in *cdc27-p11 cid1Δ* compared to *cdc27-p11*

Gene ID	Common name	Fold change		Mean change	Comments
		Expt. 1	*Expt. 2*		
SPBPB2B2.01		0.12	0.18	0.15	telomere-proximal
SPAC19D5.03	*cid1*	0.229	0.185	0.20	
SPAC1F8.03c	*str3*	0.16	0.46	0.31	iron transporter
SPAC1039.02		0.23	0.44	0.34	telomere-proximal
SPBC947.04		0.34	0.36	0.35	glycoprotein
SPBPB2B2.06c		0.30	0.41	0.36	telomere-proximal
SPBC29B5.02c	*isp4*	0.11	0.62	0.37	peptide transporter
SPBP8B7.05c		0.53	0.29	0.41	
SPBPB2B2.05		0.32	0.54	0.43	telomere-proximal
SPBC1271.07c		0.42	0.46	0.44	
SPBC359.01		0.34	0.56	0.45	amino acid permease
SPBC359.03c		0.44	0.47	0.46	amino acid permease
SPAC8E11.10	*sou1*	0.50	0.43	0.46	dehydrogenase
SPCPB1C11.03		0.36	0.55	0.46	membrane transporter
SPBC359.04c		0.52	0.40	0.46	
SPAC1F8.06	*fta5/sma5*	0.48	0.43	0.46	kinetochore protein
SPBC359.05		0.57	0.39	0.48	ABC transporter
SPBPB10D8.02c		0.49	0.46	0.48	
SPBC3E7.06c		0.43	0.55	0.49	membrane transporter
SPAC23D3.12		0.58	0.43	0.50	

Table 2.1 Genes affected significantly by the absence of Cid1 in the DNA pol δ mutant, *cdc27-P11*. RNA was extracted from *cdc27-P11* and *cdc27-P11, cid1Δ* after 4 hrs' growth at the restrictive temperature; differences at the total RNA level were detected by whole-genome microarray hybridisation. (A). Genes up-regulated more than 1.5 fold in the absence of Cid1. (B). Genes down-regulated more than 0.5 fold in the absence of Cid1. For each experiment, two hybridisations with independent RNA samples were carried out, with a dye swap.

Table 2.2 (A). Genes up-regulated significantly in HU-treated *cds1Δcid1Δ* compared to HU-treated *cds1Δ*

Gene ID	Common name	Fold change		Mean change	Comments
		Expt. 1	*Expt. 2*		
SPBPB2B2.18		1.91	2.21	2.06	telomere-proximal
SPBPB21E7.04c		2.10	1.98	2.04	
SPBP4G3.03		1.76	1.71	1.73	telomere-proximal
SPAC869.02c		1.81	1.64	1.72	telomere-proximal
SPAC977.02		1.52	1.79	1.65	telomere-proximal
SPBC1348.03		1.50	1.61	1.56	telomere-proximal
SPAC750.04c		1.55	1.54	1.55	telomere-proximal
SPAC15A10.10	*mde6*	1.57	1.54	1.55	Mei4-regulated

Table 2.2 (B). Genes down-regulated significantly in HU-treated *cds1Δcid1Δ* compared to HU-treated *cds1Δ*

Gene ID	Common name	Fold change		Mean change	Comments
		Expt. 1	*Expt. 2*		
SPAC19D5.03	*cid1*	0.32	0.32	0.32	
SPAC212.11	*rqh2*	0.56	0.50	0.53	telomere-proximal

Table 2.2. Genes affected significantly by the absence of Cid1 in HU-treated *cds1Δ*. RNA was extracted from *cds1Δ* and *cds1Δcid1Δ* after 4 hrs' growth in 10mM hydroxyurea; differences at the total RNA level were detected by whole-genome microarray hybridisation. (A). Genes up-regulated more than 1.5 fold in the absence of Cid1. (B). Genes down-regulated more than 0.5 fold in the absence of Cid1. For each experiment, two hybridisations with independent RNA samples were carried out, with a dye swap.

in a *cdc27-P11* mutant is shown in Table 2.1. Compared to the previous microarrays, there were many genes that were both significantly up- and down-regulated by the absence of Cid1 in the DNA pol δ mutant. Interestingly, many cell cycle-regulated genes were significantly up-regulated, including histones, *spd1* and Ace2-dependent transcripts (*adg1, adg2* and *mid2*). Also, many telomere-proximal genes and transcripts predicted to encode secreted membrane proteins were down-regulated in the absence of Cid1.

The third microarray experiment compared total RNA from the replication checkpoint mutant *cds1Δ* and *cds1Δcid1Δ*, both of which had been treated with HU. *cds1Δ* cells undergo Chk1-dependent checkpoint arrest upon HU treatment, whereas *cds1Δcid1Δ* enter mitosis inappropriately, similar to *cds1Δchk1Δ* cells (Wang et al. 2000a). A list of transcripts whose expression was altered significantly by the absence of Cid1 in a HU-treated *cds1Δ* mutant is shown in Table 2.2. Only one transcript apart from Cid1 was down-regulated, *SPAC212.11*, which encodes an uncharacterised RecQ helicase, that we have named Rqh2. Several transcripts were also up-regulated, many of which are encoded by telomere-proximal genes.

Since Cid1 is involved in the checkpoint response to replication problems, the microarray profiles of experiments two and three were compared to identify important genes that were altered during both types of replication stress. A list of genes that were up-regulated or down-regulated in both experiments is shown in Table 2.3. Strikingly, many telomere-proximal genes were affected (both up- and down-regulated) by the absence of Cid1 during replication stress. Other interesting genes down-regulated in both experiments included those encoding a calcineurin-like phosphatase, *cdc18*, a GNAT acetyl transferase and *aes1*.

Table 2.3 (A). Genes up-regulated during replication stress in the absence of Cid1.

Gene ID	Common name	Fold change		Comments
		cdc27-P11	*HU cds1Δ*	
SPBPB2B2.18		1.78	2.06	telomere-proximal
SPAC750.07c		1.96	1.44	telomere-proximal
SPAC212.08c		1.69	1.52	telomere-proximal
SPBC1198.14c	*fbp1*	1.53	1.31	fructose bis-phosphatase
SPAC18B11.04	*ncs1*	1.49	1.41	neuronal calcium sensor-like protein
SPAC212.02		1.48	1.59	telomere-proximal
SPAC750.04c		1.42	1.55	telomere-proximal

Table 2.3 (B). Genes down-regulated during replication stress in the absence of Cid1.

Gene ID	Common name	Fold change		Comments
		cdc27-P11	*HU cds1Δ*	
SPBPB2B2.01		0.15	0.79	telomere-proximal
SPAC1F8.03c	*str3*	0.31	0.80	siderophore-iron transporter
SPAC1039.02		0.34	0.79	calcineurin-like phosphatase (telomeric)
SPAC1B3.16c	*vht1*	0.43	0.71	vitamin H transporter
SPBPB2B2.05		0.44	0.80	telomere-proximal
SPBC359.04c		0.47	0.79	glycoprotein
SPBC8E4.10c		0.47	0.80	
SPAPB21E7.07	*aes1*	0.54	0.77	enhancer of RNAi
SPAC1271.07c		0.43	0.83	GNAT acetyl transferase
cdc18	*cdc18*	0.69	0.86	MCM loader (cell-cycle regulated)

Table 2.3. Genes affected by the absence of Cid1 during both types of replication stress. Genes were said to be affected by the absence of Cid1 in both types of replication stress if they were present in the top 50 most up-regulated (A) or down-regulated (B) genes in both the *cdc27-P11* and *cds1Δ* experiments.

The Sanger Institute holds a database of many *S. pombe* microarray experiments. To gain further insight into Cid1 function, the database was searched using GeneSpring™ software for microarrays with statistically-similar profiles to the *cid1Δ* replication stress profile. A list of these microarrays is shown in Table 2.4. Apart from the amino acid module, the *cid1Δ* profile under replication stress was very similar to that of a *sep15-ts* mutant, with similar genes both up- and down-regulated ($p = 3.57 \times 10^{-8}$ and $p = 3.34 \times 10^{-6}$, respectively). Also, *clr4Δ* had a similar cluster of genes up-regulated ($p = 3.57 \times 10^{-8}$). Sep15 is an essential component of the RNA pol II Mediator complex; a *sep15-ts* mutant is also defective in cell separation (Zilahi et al. 2000). Clr4 is a histone methyl transferase, essential for heterochromatin formation (Ivanova et al. 1998).

2.3 Confirmation of microarray data by northern blotting

Although microarray hybridisation had identified changes in many interesting transcripts during replication stress in the absence of Cid1, these differences at the RNA level had to be confirmed by another method. Northern blotting was used to confirm that the transcripts identified were up- or down-regulated in the absence of Cid1. This method also allowed any differences in transcript size to be detected.

Fig. 2.1 confirms that *adg1*, *histone h4.2* and *spd1* transcripts are up-regulated in *cdc27-P11 cid1Δ* compared to *cdc27-P11*. These transcripts accumulate in the absence of Cid1 during replication stress. Interestingly, for *adg1* and *histone h4.2* transcripts (but not *spd1*), a smaller transcript accumulates in *cdc27-P11 cid1Δ*. Could this be indicative of a smaller RNA species lacking a Cid1-dependent poly(A) tail? This is especially apparent for *adg1*, where the larger species is almost completely absent in *cdc27-P11 cid1Δ*. Fig. 2.2 confirms

Microarray Dataset	Description	P-value (similar genes UP)	P-value (similar genes DOWN)	Ref.
AA module	Gene set affected > 2 fold by changes in amino acid metabolism	-	4.97E-10	unpublished
sep15-ts	Gene set affected > 2 fold between wildtype and *sep15* ts mutant	3.57E-8	3.34E-6	(Lee et al. 2005)
clr4Δ	Gene set affected > 2 fold between wildtype and *clr4Δ*	3.57E-8	-	(Hansen et al. 2005)
sla1-ts	Gene set affected between wildtype and *sla1-ts* mutant (La protein - RNA binding protein involved in tRNA maturation)	-	9.49E-10	unpublished
rpb4-ts	Gene set affected between wildtype and *rpb4-ts* mutant (*rpb4* - RNA pol. II subunit)	-	1.73E-8	unpublished
Ampl2x (elu)	"High amplitude" cell-cycle regulated genes that showed >2 fold difference between peak and trough expression in elutriation experiments	-	5.10E-7	(Rustici et al. 2004)

Table 2.4. Microarray profiles that have significant overlap with the *cid1Δ* microarray profile under replication stress. The *S.pombe* microarray database at the Wellcome Trust Sanger Institute was searched for profiles that showed significant overlap with *cid1Δ* profiles under replication stress using GeneSpring™ software. (-) = no significant overlap

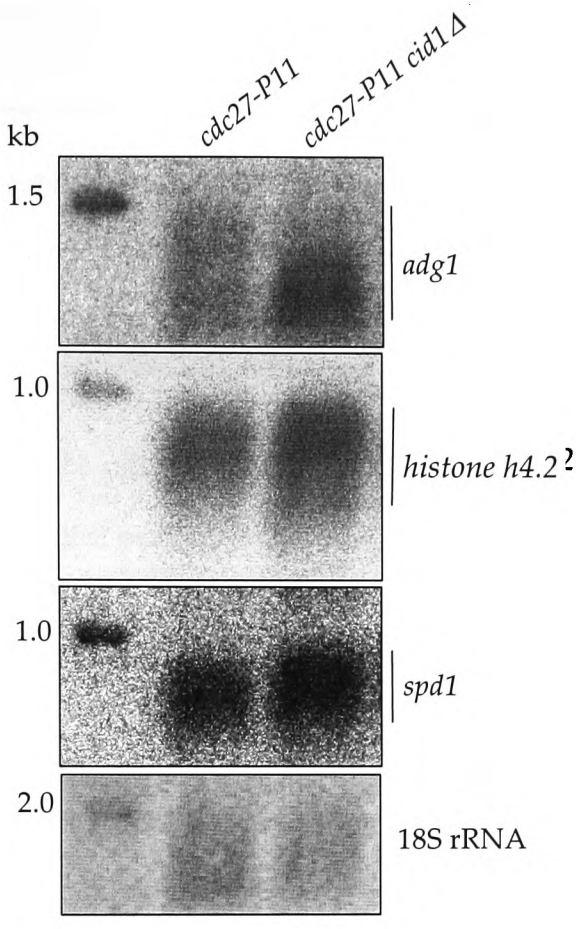


Figure 2.1. Confirmation of gene up-regulation in *cdc27-P11 cid1Δ* by northern blotting. 5μg total RNA from strains grown at 36°C for 4 hrs were separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane, prior to probing with radio-labelled probes specific for *adg1*, histone *h4.2* and *spd1*. Total RNA levels were assessed by methylene blue staining of rRNA.

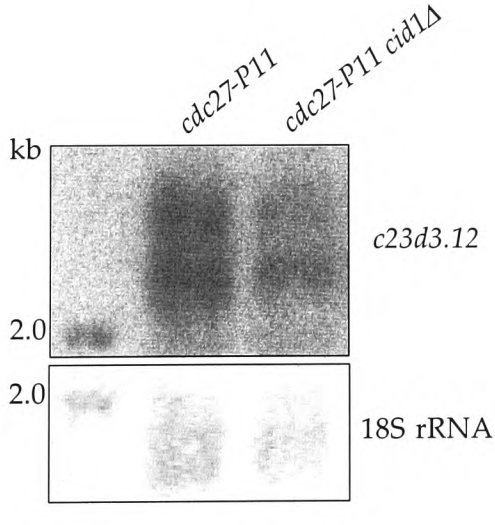


Figure 2.2. Confirmation of gene down-regulation in *cdc27-P11 cid1Δ* by northern blotting. 5μg total RNA from strains grown at 36°C for 4 hrs were separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane, prior to probing with a radio-labelled probe specific for *c23d3.12*, an inorganic phosphate transporter. Total RNA levels were assessed by methylene blue staining of rRNA.

that transcripts are down-regulated in the absence of Cid1 in a DNA pol δ mutant, including *c23d3.12*, encoding an inorganic phosphate transporter. However, only a change in transcript abundance was observed, with no change in transcript size.

Northern blotting revealed that the expression of the novel RecQ helicase gene *rqh2* is highly induced in *cds1Δ* cells (Fig. 2.3), appearing as a major 6kb transcript accompanied by a smear of smaller, perhaps degraded or non-specific RNA. Expression of the *rqh2* transcript was entirely dependent on Cid1, as the signal was completely absent in *cds1Δcid1Δ* cells. The presence of RNA in both lanes was verified by reprobing the membrane for actin expression and staining for total rRNA with methylene blue.

Genes identified as having altered expression in both *cdc27-P11* and HU-treated *cds1Δ* in the absence of Cid1 were also examined by northern blotting (Fig. 2.4). These transcripts were studied to identify any changes in expression between wildtype and *cid1Δ; cdc27-P11* and *cdc27-P11 cid1Δ* at the permissive (25°C) and restrictive (36°C) temperatures; and *cds1Δ* and *cds1Δcid1Δ* with and without HU treatment. Neither the GNAT acetyl transferase, *cdc18* or *aes1* transcript levels altered between wildtype and *cid1Δ*. The GNAT acetyl transferase was barely expressed in wildtype cells, but two major transcripts of 0.8 and 1.2kb were induced in *cdc27-P11* cells at 25°C and 36°C, which was absent in *cdc27-P11 cid1Δ*. A slightly smaller second transcript (1.1kb) was induced upon HU treatment, but this was present in both *cds1Δ* and *cds1Δcid1Δ* cells. The levels of the MCM replication machinery loader, *cdc18*, were not affected greatly by replication stress, but expression of *aes1*, a gene identified as an enhancer of antisense RNA-mediated gene inhibition (Raponi and Arndt 2002), was increased in *cdc27-P11*, but not in *cdc27-P11 cid1Δ*.

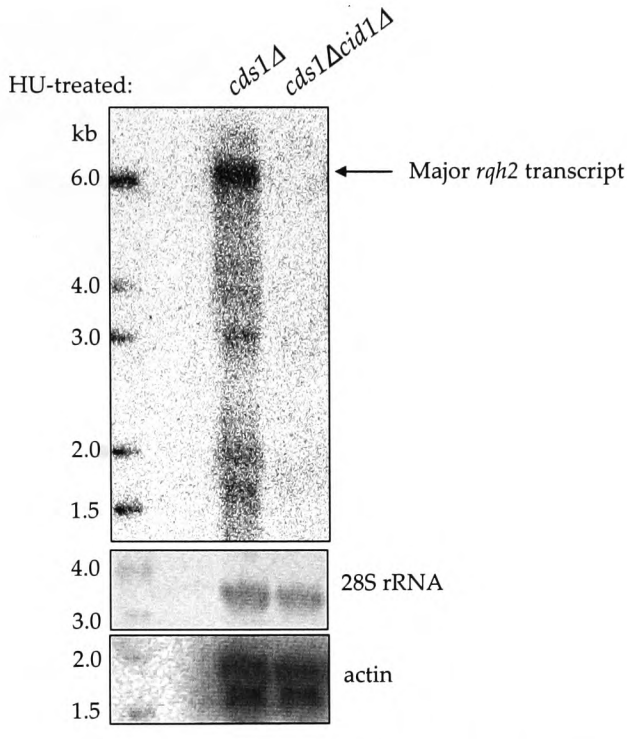


Figure 2.3. Confirmation of gene down-regulation in HU-treated *cds1Δ cid1Δ* by northern blotting. 5μg total RNA from strains grown in 10mM HU for 4 hrs were separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane, prior to probing with radio-labelled probes specific for *rqh2* and *act1*. Total RNA levels were assessed by methylene blue staining of rRNA.

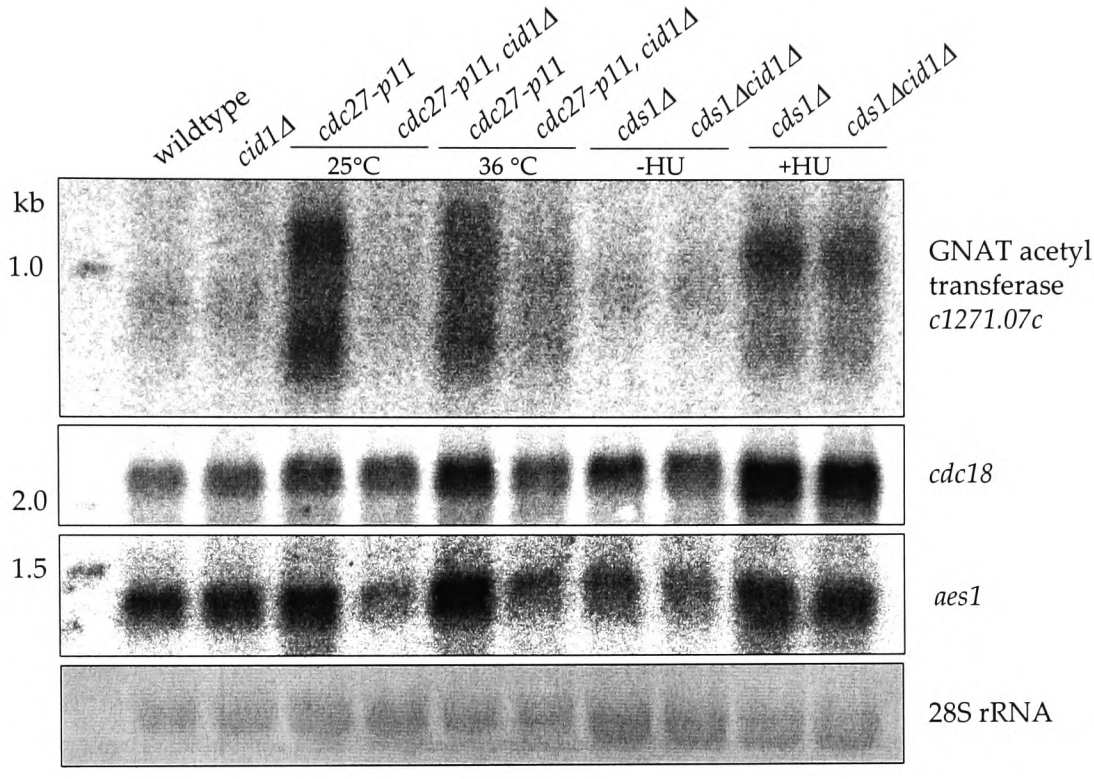


Figure 2.4. Confirmation of gene down-regulation in the absence of Cid1 under both types of replication stress. 5μg total RNA from the above strains were separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane, prior to probing with radio-labelled probes specific for *c1271.07c (GNAT acetyltransferase), aes1* and *cdc18.* Total RNA levels were assessed by methylene blue staining of 28S rRNA.

2.4 *adg1* is polyadenylated in a Cid1-dependent manner in *cdc27-P11* cells

From the microarray and northern analysis, *adg1* seemed a good candidate Cid1 target, since both its transcript level and size were altered in the absence of Cid1. To further investigate the effect of Cid1 on *adg1* expression, northern blotting was carried out using RNA extracted from wildtype, *cid1Δ*, *cdc27-P11* and *cdc27-P11 cid1Δ*, at both the permissive and restrictive temperatures (Fig. 2.5A). An *adg1* transcript of approx. 1.2kb is expressed in wildtype, *cid1Δ*, and *cdc27-P11* and *cdc27-P11 cid1Δ* at 25°C. However, a larger, 1.4kb transcript appears when *cdc27-P11* are shifted to 36°C, which is absent in *cdc27-P11 cid1Δ*. This suggests that the larger *adg1* transcript is only expressed when there are problems with replication, such as in a DNA pol δ mutant, and that its expression is dependent on Cid1. To verify that growth at 36°C did not induce expression of the larger transcript, RNA was extracted from wildtype and *cid1Δ* cells that had been grown at 30°C or shifted to 36°C, and blotted for *adg1* expression (Fig. 2.5B). Only the regular 1.2kb transcript was expressed, indicating that growth at the restrictive temperature does not induce expression of the larger transcript.

To determine whether the difference between the large 1.4kb and the smaller 1.2kb transcript was due to Cid1-mediated polyadenylation, RNA from *cdc27-P11* and *cdc27-P11 cid1Δ* cells was treated with oligo d(T) and RNaseH prior to northern blotting (Fig. 2.5C). An oligo d(T) probe binds to poly(A) tracts, to create DNA-RNA hybrids that are cleaved upon RNaseH treatment so poly(A) tails are completely removed by this procedure. The large 1.4kb *adg1* transcript in *cdc27-P11* cells was reduced to approximately 1.2kb by oligo d(T)/RNaseH treatment, confirming the presence of poly(A). Since the larger

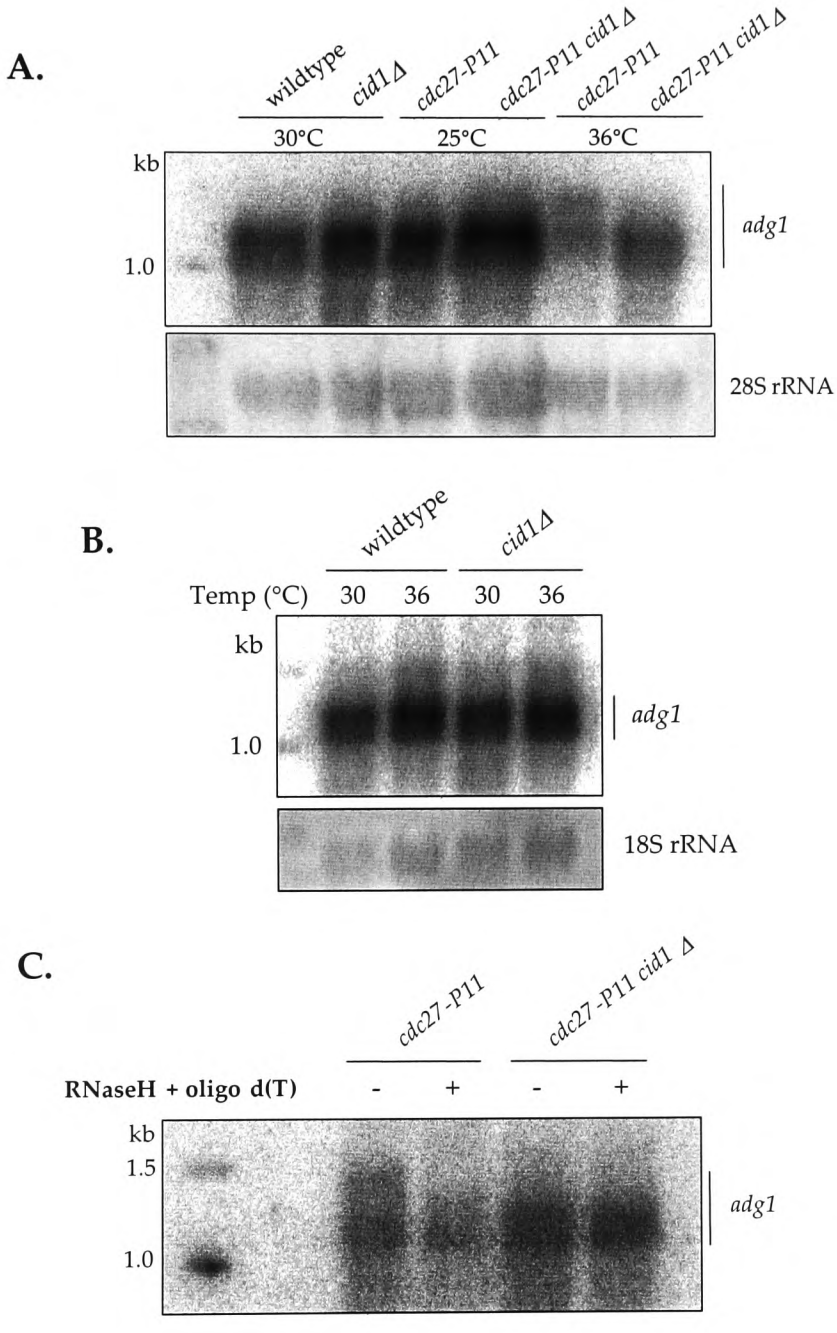


Figure 2.5. *adg1* is polyadenylated in a Cid1-dependent manner in *cdc27-P11* cells. (A). A larger *adg1* transcript appears in *cdc27-P11* cells, which is dependent on Cid1. 5μg total RNA from the above strains, grown at the temperatures indicated, were subjected to *adg1* northern blotting, as before. (B). The size difference of *adg1* is not due to growth at the restrictive temperature. 5μg total RNA from wildtype and *cid1Δ* cells grown at 30°C or 36 °C for 4 hrs were subjected to *adg1* northern blotting, as previously. (C). The size difference with and without Cid1 is due to poly(A). 5μg total RNA from the above strains was treated with RNaseH, with and without oligo d(T), and subjected to *adg1* northern blotting.

transcript was absent in *cdc27-P11 cid1Δ* cells, this suggests *adg1* is polyadenylated by Cid1 in the DNA pol δ mutant.

Since *adg1* is polyadenylated in *cdc27-P11 cells*, it was important to determine whether it was also polyadenylated upon other types of replication stress, such as HU treatment. Northern blotting was carried out with RNA from wildtype, *cid1Δ*, *cds1Δ* and *cds1Δ cid1Δ* cells treated with and without HU (Fig. 2.6). The regular 1.2kb transcript was present in all cells in the absence of HU. However, on HU treatment, the 1.2kb transcript disappeared, replaced with a weakly-expressed larger transcript. In HU-treated *cds1Δ* cells, the larger 1.4kb transcript was stabilised, with a stronger band at 1.4kb than in the other HU-treated samples, including *cds1Δcid1Δ*. This suggests the stabilised larger transcript in *cds1Δ* may be partially dependent on Cid1. *cid1Δ* cells are not especially sensitive to HU, but another deletion mutant of the Cid family, *cid13Δ*, is HU sensitive and has reduced dNTP pools (Read et al. 2002; Saitoh et al. 2002). Perhaps Cid1 and Cid13 have some overlapping functions in the response to HU treatment.

Transcripts of *adg1* are cell cycle-dependent and their expression at the end of mitosis is dependent on the transcription factor, Ace2 (Alonso-Nunez et al. 2005). During exponential growth, the majority of wildtype *S. pombe* are in G2 phase. Upon correct activation of the replication checkpoint, cells arrest in S phase, which may account for the differing levels of *adg1* between cells treated with and without HU. However, the cell cycle-regulation of *adg1* cannot explain the differing transcripts seen in *cdc27-P11* and *cdc27-P11 cid1Δ* in this study. Although *cdc27-P11 cid1Δ* have a reported checkpoint defect, less than 10% of cells leak through to mitosis after 4 hrs' growth at the restrictive temperature (Wang et al. 2000a); therefore the cell cycle distributions of *cdc27-P11* and *cdc27-P11 cid1Δ* should be approximately the same in these experiments.

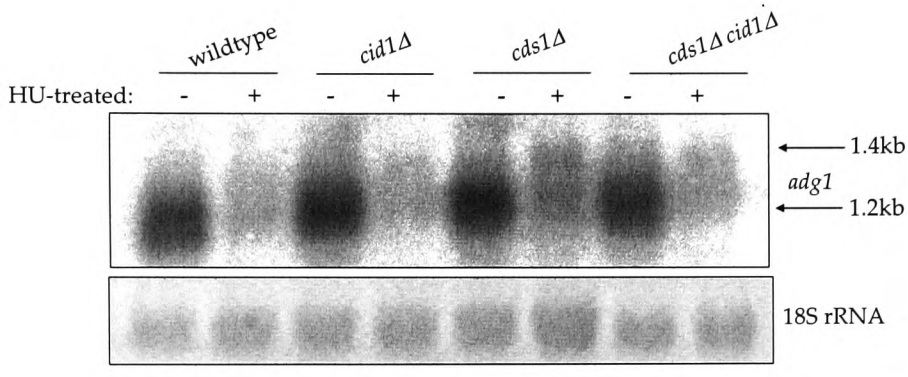


Figure 2.6. *adg1* transcripts are cell-cycle regulated and are down-regulated upon HU treatment. The indicated strains were grown with or without 10mM HU for 4 hrs, prior to RNA extraction and *adg1* northern blotting, as previously. The two sizes of *adg1* transcript are marked with an arrow.

2.5 *rqh2* expression is induced by hydroxyurea and is Cid1-dependent

rqh2 was another good candidate Cid1 target, due to the dramatic induction of *rqh2* expression upon HU treatment in *cds1Δ*, which was completely absent in *cds1Δcid1Δ*. Expression of *rqh2* was studied by northern blotting, using RNA from wildtype, *cid1Δ* and *cds1Δ* cells, with and without HU treatment (Fig. 2.7A). As described previously, *rqh2* was not expressed in wildtype cells under normal conditions (Mandell et al. 2005a); this was also the case in *cid1Δ* cells. However, upon HU treatment, there was weak induction of the 6kb transcript in wildtype, which did not occur in *cid1Δ*. Weak induction of the major *rqh2* transcript also occurred in untreated *cds1Δ* cells, but the strong induction only occurred in HU-treated *cds1Δ* cells and this induction was Cid1-dependent. *rqh2* was first identified as a transcript up-regulated upon telomere crisis in *trt-* cells (Mandell et al. 2005b); work described in this chapter shows *rqh2* is also induced upon HU treatment and that this induction is dependent on the Cid1 poly(A) polymerase.

Since *rqh2* is a telomere-proximal gene, it was possible that mechanisms such as telomere erosion may have lead to loss of the *rqh2* ORF in *cid1Δ* cells, which would explain the lack of *rqh2* expression in *cid1Δ* strains. To verify that the *rqh2* gene was present in the *cid1Δ* genome, PCR using *rqh2*-specific primers was carried out using genomic DNA extracted from wildtype and *cid1Δ* cells (Fig. 2.7B). A band of the expected size was amplified in both samples, indicating that the *rqh2* ORF was present in both strains.

Since *rqh2* is induced upon HU treatment, is it also expressed in a DNA pol. δ mutant? To answer this question, *rqh2* northern blotting was carried out using RNA extracted from wildtype, *cid1Δ*, *cdc27-P11* and *cdc27-P11 cid1Δ* at the permissive and restrictive temperatures (Fig 2.8). As before, there was no expression of *rqh2* in wildtype or

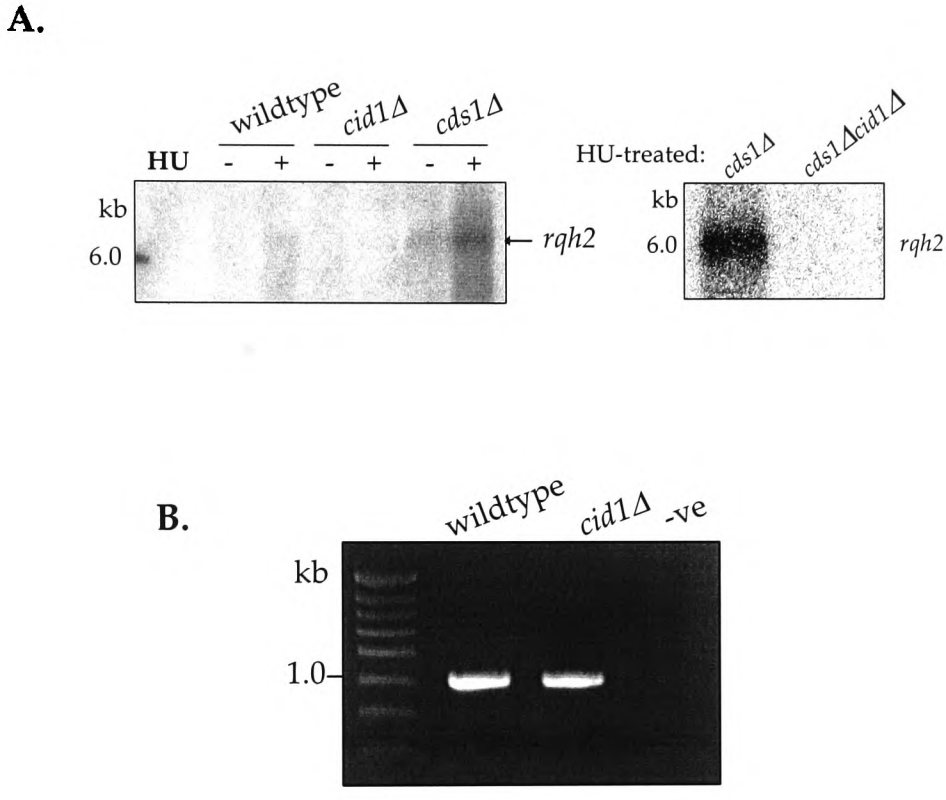


Figure 2.7. *rqh2* expression is induced by hydroxyurea and is Cid1-dependent. (A). The full-length *rqh2* transcript is induced by HU and requires Cid1. Strains were grown with or without 10mM HU for 4hrs, prior to RNA extraction and *rqh2* northern blotting. (B). The *rqh2* gene is present in the *cid1Δ* genome. Genomic DNA was extracted from wildtype and *cid1Δ* strains and subjected to PCR with *rqh2*-specific primers, alongside a no DNA negative control (-ve).

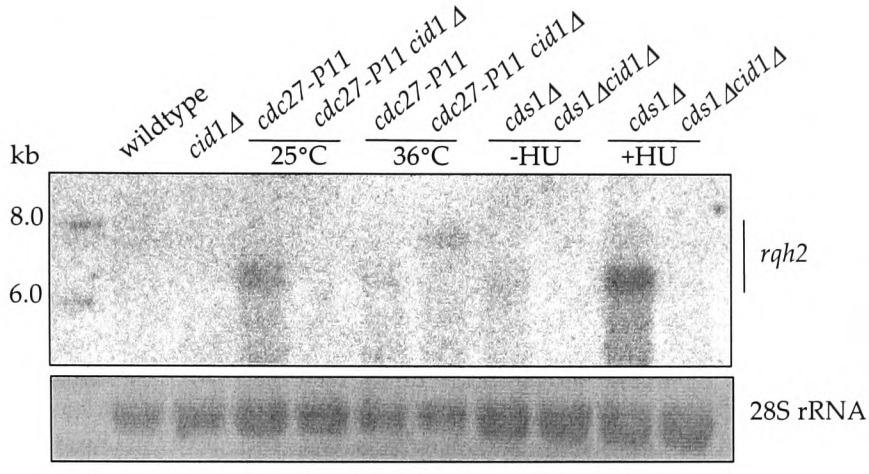


Figure 2.8. *rqh2* expression is induced in *cdc27-P11* cells. The indicated strains were grown at the above temperatures, or with and without 10mM HU for 4 hrs at 30°C, prior to RNA extraction and *rqh2* northern blotting, as before. Total RNA levels were assessed by methylene blue staining of rRNA.

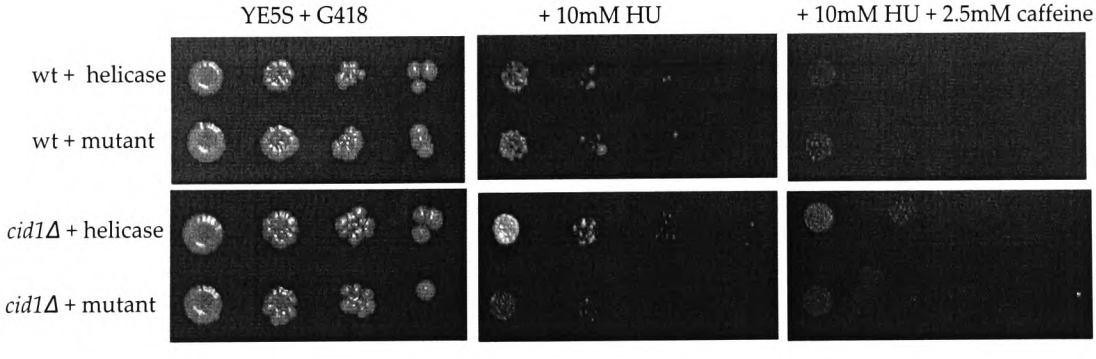


Figure 2.9. Expression of the Rqh2 helicase domain in a *cid1Δ* strain causes hydroxyurea resistance. *S.pombe h-leu1-32* (wt) and *cid1Δ* strains were transformed with pART1 encoding a wildtype or mutated version of the Rqh2 helicase domain and transformants selected with G418. Transformants were grown in liquid culture to mid-exponential phase, 10-fold serial dilutions made and spotted onto agar with or without drug. Plates were photographed after 5 days' growth at 30°C.

cid1Δ cells. The major 6kb *rqh2* transcript was expressed in *cdc27-P11* at 25°C and this induction was dependent on Cid1, as the transcript was absent in *cdc27-P11 cid1Δ*. Unexpectedly, there was weaker induction of *rqh2* in *cdc27-P11* at 36°C than at 25°C; the 6kb transcript was absent in *cdc27-P11 cid1Δ* at 36°C, but there was expression of an unusual, larger 8kb *rqh2* transcript. Perhaps this larger transcript is expression from an alternative promoter or poly(A) site in the absence of Cid1 at high temperatures (O. Rissland, personal communication). Temperature-sensitive DNA pol mutants are likely to suffer from different replication problems at the permissive and the restrictive temperatures, activating the replication checkpoint in different ways, which might explain the difference in *rqh2* expression at 25 and 36°C. However, this work shows that *rqh2* expression is induced by replication stresses that result from both HU treatment and a defective DNA polymerase.

Is the control of *rqh2* expression by Cid1 important for its role in the replication checkpoint? A *cid1Δ* mutant is sensitive to a combination of HU and caffeine, since it is defective in some aspects of the replication checkpoint (Wang et al. 2000a). If Rqh2 is important for the replication checkpoint, Rqh2 over-expression should by-pass the need for Cid1 and prevent the sensitivity of *cid1Δ* to HU and caffeine. To test this hypothesis, plasmids that expressed a native or mutated version of the Rqh2 helicase domain from an *adh1* promoter were obtained (a kind gift of T. Cech). The plasmids were transformed into wildtype and *cid1Δ* strains and transformants tested in a drug resistance assay (Fig 2.9). Unlike the over-expression of Cid1, expressing the native Rqh2 helicase domain in wildtype cells did not cause resistance to HU and caffeine. This suggests that the induction of *rqh2* does not explain the Cid1 over-expression phenotype; additional targets must be important. However, over-expressing the Rqh2 helicase domain, but not the mutant version, caused a *cid1Δ* mutant to have improved growth with HU alone and gave

slight resistance to HU and caffeine. This genetic interaction implicates *rqh2* as an important Cid1 target.

2.6 Proteomic comparison of wildtype and *cid1Δ*

Large-scale transcript profiling and proteomic studies have shown a lack of correlation between mRNA and protein levels in numerous instances (Gygi et al. 1999). So, in parallel with the microarray studies, attempts were made to identify Cid1 targets by comparing the proteomes of wildtype and *cid1Δ* strains. If Cid1 polyadenylation of an RNA target increases its translatability, there should be a subsequent increase in the corresponding protein level. Therefore, Cid1 targets might be identified by comparing differences at the level of total protein between strains with and without Cid1. Differences in the levels of individual proteins were detected using 2D-LC, in collaboration with Alexandre Akoulitchev at the Dunn School. 2D-LC (described in Fig. 2.10) separates a protein lysate in two dimensions into 890 fractions; firstly by ionic charge, secondly by hydrophobicity. Proteins contained in fractions that vary between strains are identified by mass spectrometry.

2D-LC is a novel technique, which had not been used previously to study gene expression in *S. pombe*. After optimisation of the lysate extraction procedure using a non-ionic lysis buffer, a 2D-LC experiment was performed, comparing the total proteomes of wildtype and *cid1Δ* strains, grown under identical "normal" conditions. The merged proteome profiles of the two strains are shown in Fig. 2.11. Two fractions showed major differences at the total protein level, with a greater amount of protein in wildtype than *cid1Δ* (Fig. 2.12A). These fractions were subjected to mass spectrometry to identify their protein

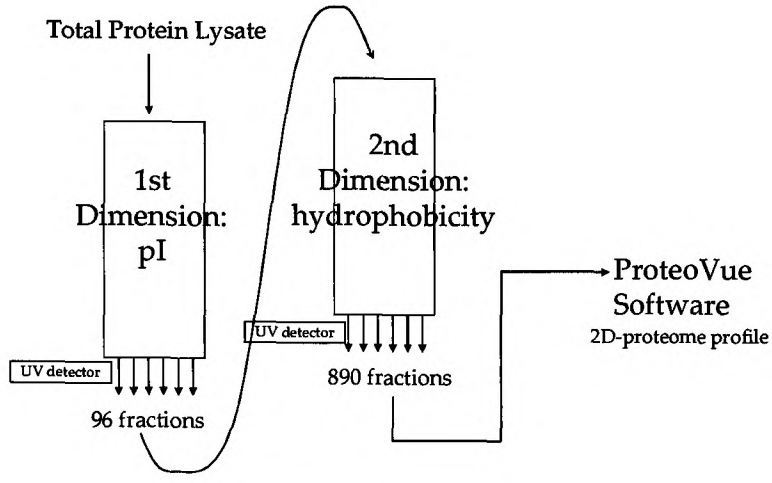


Figure 2.10. Schematic of two-dimensional liquid chromatography. A total protein lysate is separated into 890 fractions in two dimensions, using a fully automated liquid chromatography system, the Proteome Lab™. For each strain, the fractions' UV absorbance data are converted to a 2-D proteome profile, which can then be merged with profiles of other strains to identify differences.

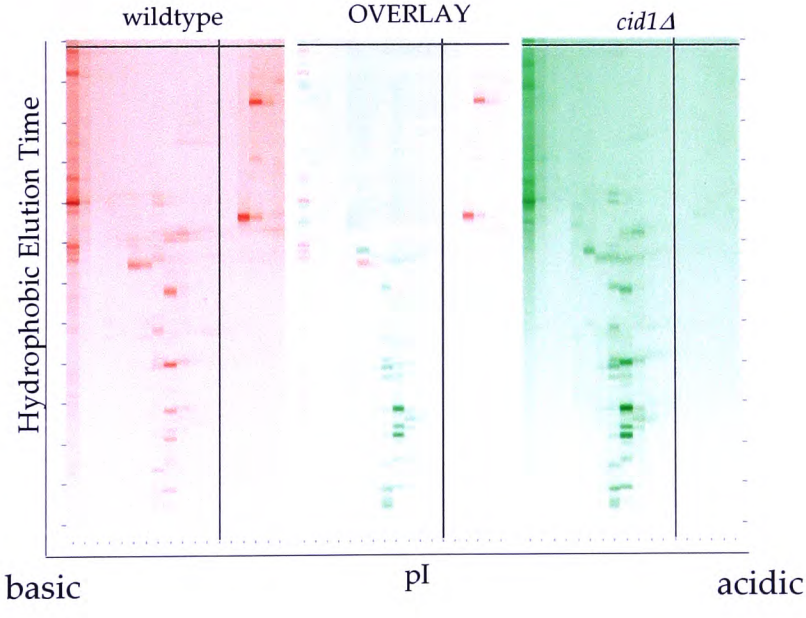


Figure 2.11. Proteome profiles of wildtype and *cid1Δ* strains. The proteome profile of the wildtype strain (red) was merged with the *cid1Δ* profile (green) to generate a merged profile (overlay), which allows differences to be identified.

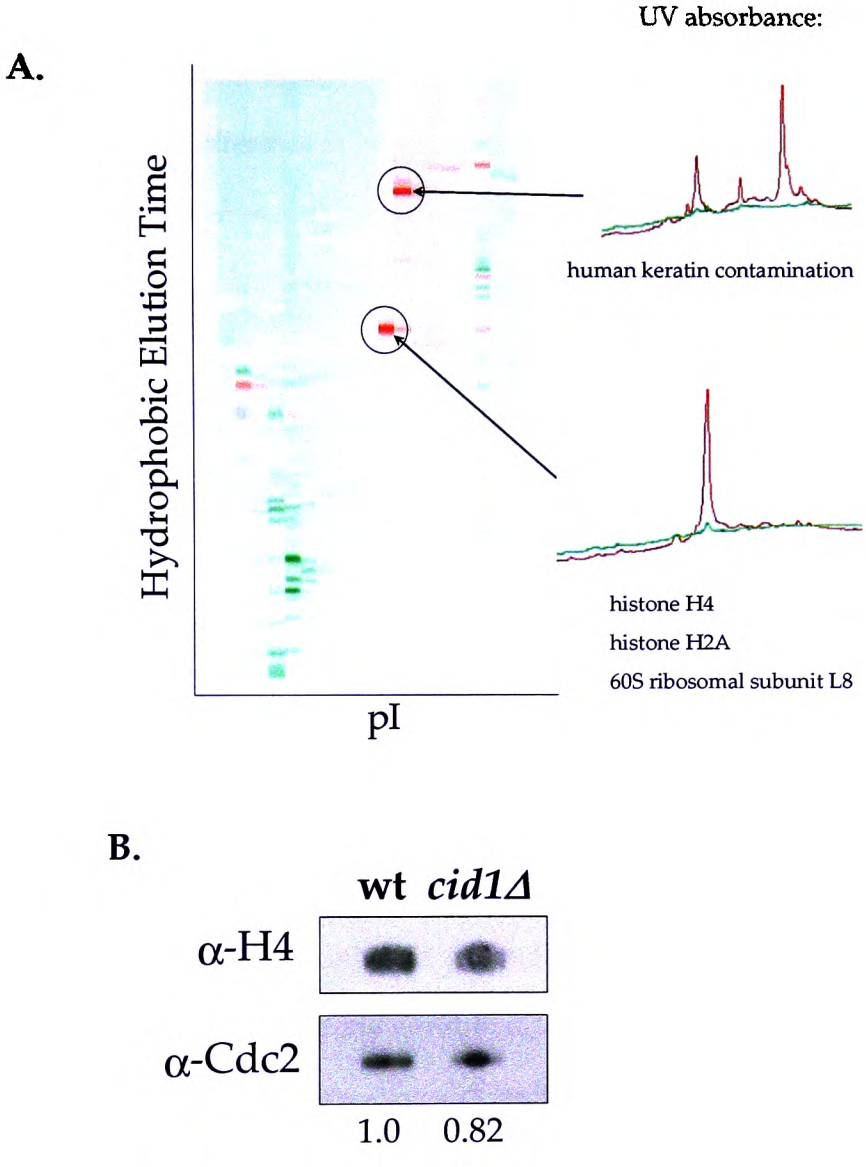


Figure 2.12. 2D-LC identified differences in levels of histone and ribosomal proteins between wildtype and *cid1Δ* lysates. (A). Overlaid wildtype and *cid1Δ* proteome profiles identified two fractions (ringed) with major differences at the protein level, according to UV spectrometry measurements. Proteins residing in those fractions were identified by mass spectrometry. (B). Total histone h4.2 protein levels are unaffected in a *cid1Δ* strain. Total histone h4.2 levels were measured by anti-h4.2 western blotting and quantified by comparison to Cdc2 protein levels, detected using anti-Cdc2 antibodies.

components; one fraction contained histones H4.2, H2A and the 60S ribosomal protein subunit L8, the second fraction contained human keratin, a contaminant.

To confirm a difference in histone proteins between wildtype and *cid1Δ* strains, western blotting was carried out, using anti-histone H4 antibodies (Fig. 2.12B). There was no significant difference in total histone H4 between wildtype and *cid1Δ*. Unfortunately, no antibody was available to detect the *S. pombe* ribosomal protein L8, so any difference between wildtype and *cid1Δ* could not be confirmed. Further work is needed to verify whether the expression of histones or ribosomal proteins is affected by Cid1.

2.7 Discussion

Microarray analysis identified a panel of genes that were affected by the absence of Cid1 during two types of replication stress; replication fork stalling was induced either by HU treatment or by a temperature-sensitive DNA pol δ mutant, *cdc27-P11*. Genes affected fell into two main classes; cell cycle-regulated genes (Ace2-dependent genes, histones) and telomere-proximal genes (including the novel RecQ helicase, *rqh2*). Microarray data also suggested that the Cid1 poly(A) polymerase was inactive in normal, unperturbed cells, since no differences at the total RNA level were detected between wildtype and *cid1Δ* cells grown under normal conditions.

Many cell cycle-regulated genes were up-regulated in the absence of Cid1 in the DNA pol δ mutant *cdc27-P11*, including the Ace2-dependent transcript, *adg1*. This up-regulation was confirmed by northern blotting, which also determined that *adg1* was polyadenylated in a Cid1-dependent manner during replication stress. Ace2 is a transcription factor that controls the expression of a number of genes required for septation

and ensures their cell-cycle dependent expression at the M/G1 transition; Ace2 itself is kept under cell-cycle control by the forkhead transcription factor, Sep1 (Rustici et al. 2004). Adg1 (Ace2-dependent gene-1) is a serine/threonine rich protein of unknown function, but *adg1Δ* cells have a mild septation defect (Alonso-Nunez et al. 2005). Although the transcription of *adg1* is dependent on Ace2, additional post-transcriptional controls may affect its expression, such as Cid1-dependent polyadenylation. The cluster of genes affected by the absence of Cid1 during replication stress had a strong overlap with genes affected by a *sep15-ts* mutant. Sep15 is an essential subunit of the RNA pol II mediator complex, but mutants have defects in cell separation, thought to be due to defects in cell cycle-dependent gene expression (Lee et al. 2005). Why would Cid1 polyadenylate *adg1* during replication stress? *cdc27-P11 cid1Δ* cells exhibit the "cut" phenotype; that is, they septate without completing replication (Wang et al. 2000a). Perhaps Cid1's control of Ace2-dependent transcripts provides the brake between S-phase and septation during replication stress, preventing cell separation before the completion of replication.

Strikingly, all four core histone transcripts were also up-regulated in *cdc27-P11* in the absence of Cid1. Like *S. cerevisiae,* histone mRNAs in *S. pombe* are likely to be polyadenylated, as they are retained on oligo d(T) cellulose (Fahrner et al. 1980 and data not shown). Northern analysis revealed that smaller histone transcripts accumulate in *cdc27-P11 cid1Δ* cells, but it could not be proven that this was due to lack of Cid1-mediated polyadenylation. Histone mRNAs are degraded after the completion of S phase or upon inhibition of replication (Sittman et al. 1983), so perhaps there is a problem with histone mRNA degradation in *cdc27-P11, cid1Δ.* However, western blotting with anti-H4.2 antibodies revealed no difference in total histone H4 protein levels between *cdc27-P11* and *cdc27-P11 cid1Δ* (data not shown). Interestingly, attempts to identify Cid1 targets by 2D-LC

also identified histones H4 and H2A (and ribosomal proteins), but again, changes in histone protein levels between wildtype and *cid1Δ* could not be confirmed by western blotting. Although the level of total histone H4 did not change between wildtype and *cid1Δ*, post-translational modifications of histones was not examined in these strains. The N-terminal tail of histones can be modified in an ever-increasing number of ways, including phosphorylation, acetylation, methylation and ubiquitination (for review, see Peterson and Laniel 2004). Perhaps the difference in H2A and H4 protein identified by 2D-LC was a difference in a modified form of H2A or H4. This hypothesis now seems particularly favourable, since differential expression of a histone acetyltransferase has since been detected by microarray analysis. Further work is needed to verify whether the expression and/or modification of histones are affected by Cid1. 2D-LC is a novel technique and assumes that Cid1 targets protein-encoding mRNAs. At the time of this experiment, it was not known that Cid1 is most likely inactive during normal growth, so was unlikely to reveal major differences at the protein level. It would be useful to repeat the 2D-LC experiment using strains grown under conditions of replication stress. However, the fact that histones are altered at the level of RNA and protein in the absence of Cid1, as identified by two independent methods, should not be disregarded.

Why were so many cell cycle-regulated transcripts affected in *cdc27-P11* in the absence of Cid1? This study showed that a*dg1* is polyadenylated in a Cid1-dependent manner, but further experiments are needed to confirm differential polyadenylation of histone and *spd1* transcripts in the absence of Cid1. Is Cid1 polyadenylation leading to a subsequent increase in translatability of these transcripts, as well as the increased stability shown by northern analysis? Perhaps the role of Cid1 is only to maintain a cytoplasmic pool of cell cycle-regulated transcripts during replication arrest, which otherwise would be

de-adenylated and degraded. Upon recovery from arrest, these transcripts are then available for new rounds of translation.

The Cid1-dependence of *rqh2* expression and the *in vivo* genetic interaction of the Rqh2 helicase domain with *cid1Δ* strongly implicate *rqh2* as a Cid1 target. However, it is not known whether Cid1 directly affects *rqh2* expression by polyadenylating the transcript, or indirectly, by polyadenylating an upstream effector of *rqh2* expression. *rqh2* is a large, unusual gene, with four copies in the *S. pombe* genome, residing at the sub-telomeres of chromosomes I and II (Fig. 2.13). Rqh2 contains a RecQ helicase domain, with similarity to the mammalian BLM and WRN helicases. RecQ helicases have many roles in the maintenance of genomic stability (reviewed in Hickson 2003). Interestingly, the *rqh2* ORF contains regions of homology to centromeric *dh* repeats, which recruit the RNAi machinery to maintain silencing of the *rqh2* ORF itself and the surrounding sub-telomeric region (Kanoh et al. 2005). Therefore, an increase in *rqh2* expression would require that its RNAi silencing pathway be turned off or by-passed in some way. Since Cid1 is required for *rqh2* expression, perhaps the role of Cid1 is to suppress the RNAi pathway, specifically at the telomeres during replication stress? If so, this would explain why the expression of so many telomere-proximal genes was affected by the absence of Cid1, according to the microarray data. The high correlation of genes up-regulated in the *cid1Δ* replication stress and *clr4Δ* microarrays also suggests that Cid1 affects heterochromatin, since Clr4 is the *S. pombe* histone methyltransferase required for establishment of heterochromatin (Ivanova et al. 1998). Interestingly, one of the genes down-regulated by the absence of Cid1 in both types of replication stress encodes a GNAT histone acetyltransferase. Recently, an *S. pombe* histone acetyltransferase has been shown to negatively regulate telomere silencing (Gomez et al. 2005). Cid1 may affect the expression of a variety of genes, such as histone

acetyltransferases, which ultimately lead to a temporary relief in telomeric silencing during replication stress. This "opening up" of telomeric chromatin would allow access for repair proteins and expression of genes that are normally kept silenced (*rqh2*). Rqh2 itself may play a role in the repair of stalled replication forks at the telomere, since other RecQ helicases are known to play a role in the telomere maintenance (Crabbe et al. 2004). An *rqh2* homologue in *Neurospora crassa*, QDE-3, also encoded in the sub-telomere, is required for gene silencing (Cogoni and Macino 1999). As described earlier, Cid12 is also involved in the RNAi pathway and is required for heterochromatin formation (Motamedi et al. 2004). Perhaps Cid1 is in competition with Cid12 activity to promote RNAi at telomeres.

The repair of stalled replication forks at telomeres is a unique problem; unlike the rest of the genome, a fork stalled at the very end of a chromosome cannot be resolved by an oncoming fork travelling in the opposite direction. Also, telomeres have evolved mechanisms to prevent the activation of checkpoint signals in normal conditions, otherwise the DNA ends would be recognised as double-strand breaks. Therefore, it is likely that alternative checkpoint mechanisms are activated in the event of DNA damage or stalled replication forks at telomeres. Although telomeres do not normally trigger checkpoint activation, it is well-known that checkpoint proteins are required for the maintenance of telomere integrity (for review, see d'Adda di Fagagna et al. 2004); *rad3Δ* mutants have shortened telomeres (Matsuura et al. 1999) and many checkpoint proteins bind to telomeres (Nakamura et al. 2002). However, the link between checkpoint proteins and telomeric integrity is as yet, unknown. Perhaps Cid1 is part of this proposed "telomere replication checkpoint"; Cid1 may be required either to activate this special branch of the replication checkpoint, or to activate the repair. From the results of the microarray, a role for Cid1 in the maintenance of telomeric heterochromatin is proposed (Fig. 2.14). Under

normal conditions, Cid1 is inactive and telomeric integrity is maintained; however, upon replication stress, Cid1 is activated and up-regulates a panel of genes that leads to disruption of telomeric silencing, allowing *rqh2* expression and access for repair proteins. Transcription of *rqh2* is required for the establishment of telomeric heterochromatin, but when RNAi is disrupted by Cid1 activation, the expression of *rqh2* is up-regulated; this may acts as a negative feedback mechanism to reconstitute silencing after replication stress. There are reports of stress-activated ATF/CREB transcription factors affecting heterochromatin formation at the mating type locus in *S. pombe* (Jia et al. 2004), and the disruption of heterochromatin by heat-shock (Allshire et al. 1995), so the idea of a checkpoint pathway affecting telomeric heterochromatin is tenable.

However, many questions remain unanswered. Is the *rqh2* transcript a mature, translatable mRNA, or is it simply a by-product of lack of RNAi silencing? Rqh2 northern blotting of polysome-purified RNA samples in *trt-* cells identified *rqh2* transcripts associated with polysomes, suggesting it is translated (Mandell et al. 2005b). If Cid1 does play a role in telomeric silencing, how is telomeric heterochromatin altered in a *cid1Δ* mutant? Does Cid1 affect histone acetylation, methylation and Swi6 binding during replication stress? Further investigation is needed to decipher the exact role of this cytoplasmic poly(A) polymerase in the replication checkpoint, but this study implicates an effect on cell-cycle dependent genes and the maintenance of telomeric silencing.

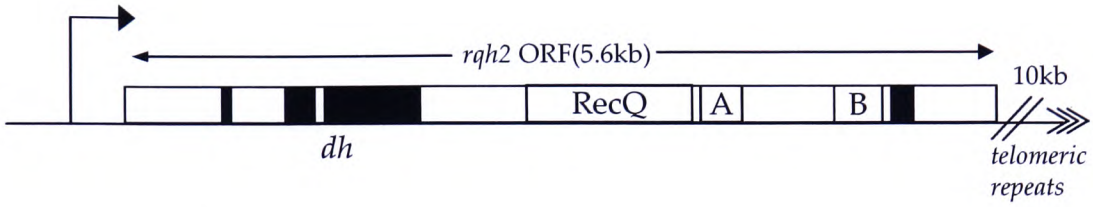


Figure 2.13. Schematic of the *rqh2* ORF. Boxes A and B are the conserved RQC and HDRC domains. Solid black regions are regions of homology with *dh* repeats (adapted from Mandell et al., 2004).

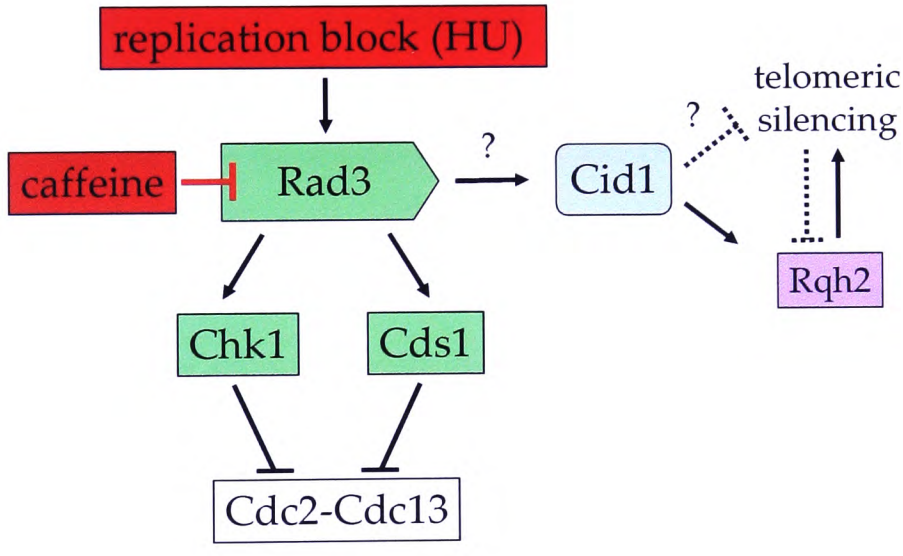


Figure 2.14. Proposed model for the role of Cid1 in telomeric heterochromatin maintenance. Cid1 could be the missing link between the DNA integrity checkpoint and the maintenance of telomeric heterochromatin in *S. pombe*. Transcription of *rqh2* is required to maintain silencing at sub-telomeres, which is altered upon replication stress, in a Cid1-dependent manner.

Chapter Three

Cid1 interacts with Hyd1, a hydrolase with a metallo-β-lactamase motif

3.1 Introduction

The yeast two-hybrid (Y2H) assay is a powerful technique for identifying protein-protein interactions (Fields and Song 1989). This method was used successfully to identify GLD-3, the RNA-binding partner of GLD-2, a Cid1-like protein in *C. elegans* (Wang et al. 2002a). Y2H screening makes use of the fact that many eukaryotic transcription factors are made up of physically-separable subunits, a transcriptional activation domain and a DNA-binding domain. To test an interaction between two proteins, one is fused to the binding domain (the "bait"), the other to the activation domain (the "prey"); then, bait and prey expression plasmids are co-transformed into *S. cerevisiae.* Only when there is a physical interaction between the two proteins can the two domains come together to activate transcription from a number of reporter genes, encoded in the yeast genome. In addition to testing interactions between two individual proteins, this method can also be used to screen cDNA expression libraries for unknown partners for a bait protein. In this way, Y2H screening was used to identify a previously unknown Cid1-interacting protein, as described below.

3.2 Cid1 interacts with Hyd1 *in vivo*

Y2H screening was carried out using full-length Cid1 protein as bait and an *S. pombe* cDNA library as prey (kindly provided by T. Toda). More than 1 x 10^6 transformants (containing bait and prey) were screened for positive interactions by replica-plating onto medium lacking histidine; colony survival indicated activation of the first reporter gene, *HIS3*. Subsequently, surviving colonies were tested for expression of the second reporter gene, *lacZ*, using the β-gal assay; colonies that turned blue were scored as positive. Only two clones survived both rounds of selection; when sequenced, both contained the full-length ORF of the same gene, *SPAC824.07*. According to the *S. pombe* genome database (www.genedb.org/pombe), *SPAC824.07* encodes an uncharacterised protein predicted to have hydrolase activity, so was renamed *hyd1*. The interaction of Hyd1 with Cid1 in the Y2H assay is shown in Fig. 3.1. As discussed in more detail later, Hyd1 is a member of the metallo-β-lactamase family, which includes some important nuclear polyadenylation factors. It was considered possible that the metallo-β-lactamase domain of Hyd1 was the reason for its interaction with Cid1 and that other members of this family would interact with Cid1 also. However, other RNA processing enzymes containing this domain, such as Ysh1 (*S. pombe* CPSF-73) and Cft2 (*S. pombe* CPSF-100) did not interact with Cid1 in the Y2H assay (Fig. 3.1B), suggesting a specific interaction between Hyd1 and Cid1.

As Y2H screening can yield false positives, the Hyd1-Cid1 interaction was further investigated by co-immunoprecipitation. A *hyd1-HA* strain was created, which had the endogenous *hyd1* gene replaced with a HA-tagged version. This was mated with *cid1-GFP*, to create a strain containing tagged versions of both proteins, each expressed from their own promoter, to allow *in vivo* co-immunoprecipitation experiments to be carried out. Cid1-GFP was co-immunoprecipitated using anti-HA antibodies, only in the presence of

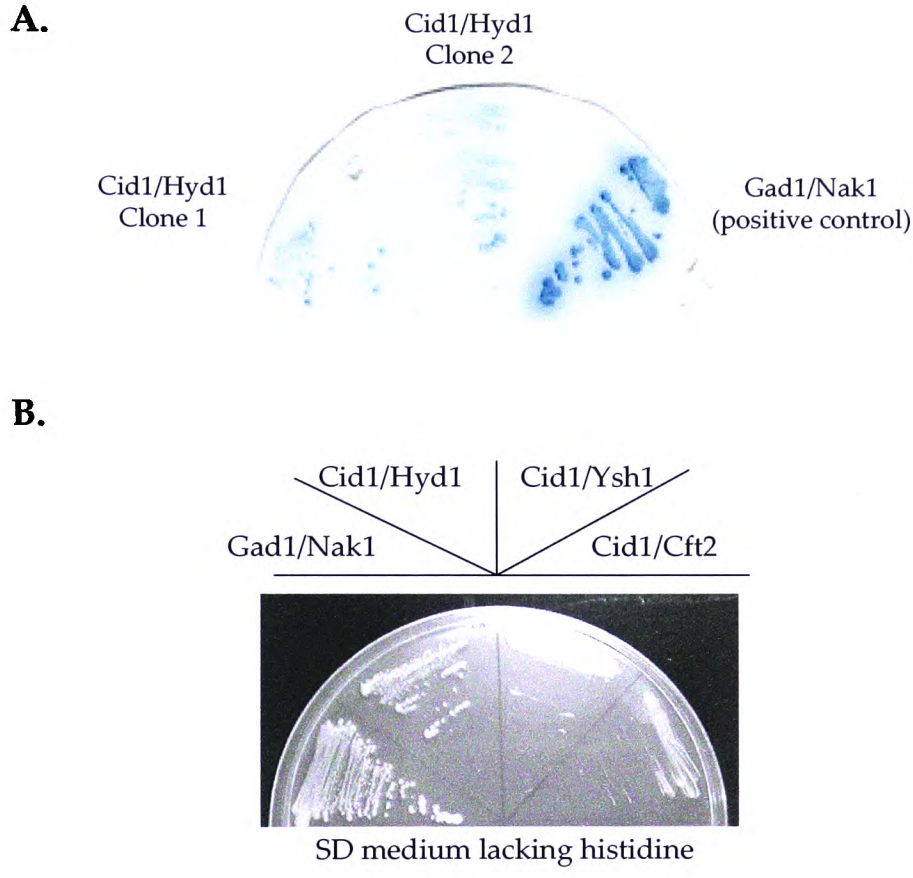


Figure 3.1. Cid1 interacts with Hyd1 in the yeast two-hybrid assay. (A). Cid1/Hyd1 transformants and the positive control, Gad1/Nak1 turned blue in the β-gal assay, indicating activation of the *lacZ*$^+$ reporter gene. (B). The RNA processing metallo-β-lactamase enzymes, Cft2 and Ysh1, do not interact with Cid1 in the yeast two-hybrid assay. Ysh1 and Cft2 were cloned into the prey plasmid and transformed into yeast containing the Cid1 bait plasmid. Ysh1/Cid1, Cft2/Cid1, Hyd1/Cid1 and Gad1/Nak1 (positive control) transformants were streaked onto SD medium lacking histidine; colony formation indicated activation of the *HIS3* reporter gene.

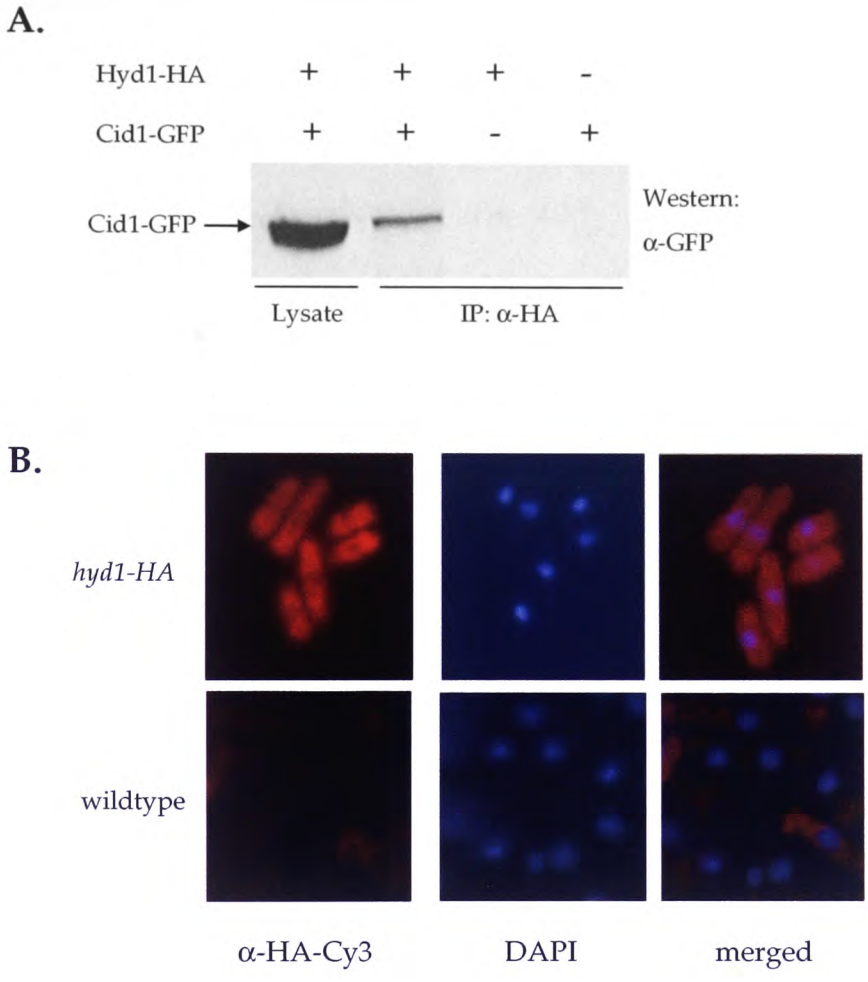


Figure 3.2. Cid1 interacts with Hyd1 *in vivo*. (A). Cid1-GFP co-immunoprecipitates with Hyd1-HA. Protein lysates were prepared from strains containing endogenously-tagged Hyd1 and Cid1. HA-tagged Hyd1 was precipitated with anti-HA antibody and Cid1-GFP was detected with anti-GFP antibody. (B). Hyd1-HA is a cytoplasmic protein, like Cid1. *hyd1-HA* and untagged wildtype cells were subjected to immunofluorescence using anti-HA primary antibody and a Cy3-conjugated secondary antibody. Cells were fixed in methanol and stained with DAPI to visualise nuclei, prior to examination by fluorescence microscopy.

Hyd1-HA (Fig. 3.2A). However, Hyd1-HA could not be co-immunoprecipitated with Cid1-GFP (data not shown). Also, these experiments did not distinguish between an indirect or direct Hyd1-Cid1 interaction.

To determine the location of Hyd1 in the cell, *hyd1-HA* was examined by indirect immunofluorescence, using anti-HA antibodies (Fig. 3.2B). Hyd1 immunostaining was exclusively cytoplasmic and excluded from the DAPI-stained nucleus. Therefore, during normal, mid-exponential growth, Hyd1 is a cytoplasmic protein, like Cid1 (Read et al. 2002).

3.3 Deletion of Hyd1 does not affect the Cid1 phenotype

To determine whether the Hyd1-Cid1 interaction was important for Cid1 function, a *hyd1Δ* deletion mutant was created by replacing the entire *hyd1* ORF with *ura4* by homologous recombination. Hyd1 was not essential for viability, as *hyd1Δ* cells grew indistinguishably from wildtype (data not shown).

The role of Cid1 in the replication checkpoint was initially identified by its ability to confer resistance to HU and caffeine when over-expressed (Wang et al. 1999). If Hyd1 is essential for this role, over-expression of Cid1 in a *hyd1Δ* strain should abrogate the resistance phenotype. To test this, a vector over-expressing Cid1 from the *nmt1* promoter was transformed into wildtype and *hyd1Δ* strains, together with an empty vector negative control (Fig. 3.3). Unexpectedly, the over-expression phenotype of Cid1 was unaffected in a *hyd1Δ* strain. This implied that Hyd1 is not essential for the role of Cid1 in the replication checkpoint. However, it should be noted that over-expression experiments are not

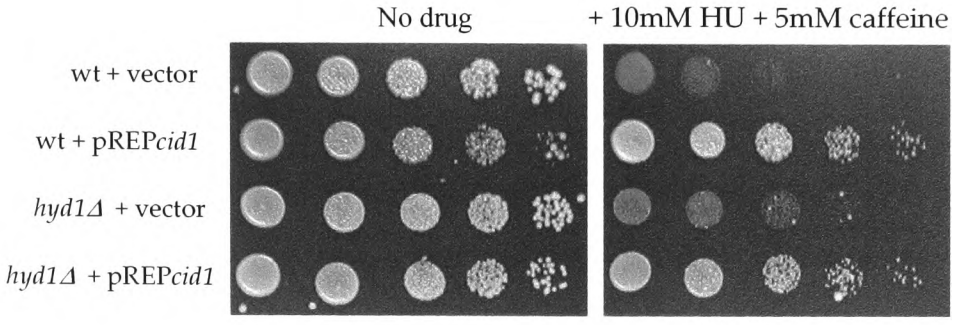


Figure 3.3. Deletion of *hyd1* does not affect the Cid1 phenotype. Wildtype (wt) and *hyd1Δ* strains were transformed with empty pREP3X vector or pREP*cid1*. Overexpression was induced by the removal of thiamine, grown overnight to mid-exponential phase and 10-fold serial dilutions were spotted onto selective medium with or without drug. Plates were photographed after 5 days' growth at 30°C.

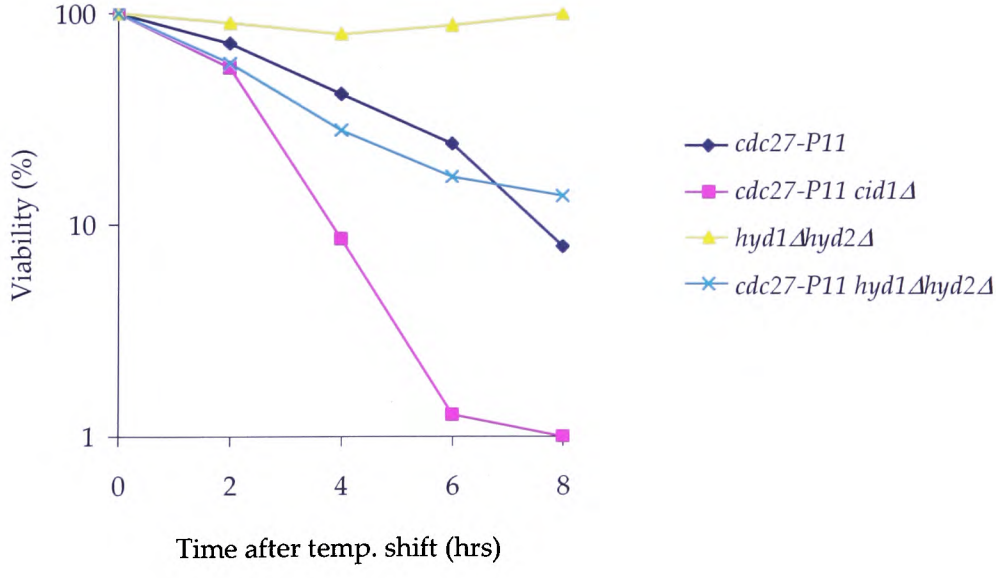


Figure 3.4. *hyd1Δhyd2Δ* does not increase the lethality of *cdc27-P11*, unlike *cid1Δ*. Strains grown to mid-exponential phase were shifted to 36°C; samples of 500 cells were taken at the above time-points, plated onto rich medium and incubated at 25°C for 5 days. Viability was determined as a percentage of cells surviving at time zero.

physiological. Hyd1 may still play a role in Cid1 function, which is dispensable for the over-expression phenotype, but is required for normal Cid1 function *in vivo*.

3.4 A *hyd1Δhyd2Δ* double deletion is sensitive to hydroxyurea

RNA processing enzymes of the metallo-β-lactamase family often act as heterodimers with paralogous proteins to which they have a high degree of sequence similarity (reviewed in Weiner 2005). The *S. pombe* genome was searched for proteins that were potential paralogues of Hyd1, by BLAST analysis. One paralogue was identified, *SPCC13B11.03c*, which encodes an uncharacterised metallo-β-lactamase with 73% similarity (55% identity) to Hyd1 at the amino acid level (data not shown); this was renamed Hyd2. To determine whether deletion of both paralogues would affect the Cid1 phenotype, a double deletion strain was constructed, first by creating *hyd2Δ*, then mating with *hyd1Δ* and selecting for a strain with copies of both *hyd1* and *hyd2* genes replaced with *ura4*$^+$. The *hyd1Δhyd2Δ* strain was viable, but grew weakly on minimal medium (data not shown). This meant that it was difficult to over-express Cid1 using this strain. Therefore, in order to determine the effect of *hyd1Δhyd2Δ* on Cid1 function, an alternative approach was needed.

As described previously, a *cid1Δ* mutant caused enhanced lethality in combination with the DNA pol δ mutant, *cdc27-P11*. If the hydrolase function were important for Cid1 activity, *hyd1Δhyd2Δ* may also have a deleterious effect with *cdc27-P11*. To test this, *hyd1Δhyd2Δ* was mated with *cdc27-P11* to create the strain *cdc27-P11 hyd1Δhyd2Δ*. The viability of *cdc27-P11 hyd1Δhyd2Δ* at the restrictive temperature was determined and compared to that of *cdc27-P11*, *cdc27-P11 cid1Δ* and *hyd1Δhyd2Δ* alone (Fig. 3.4). Unlike *cdc27-P11 cid1Δ*, *cdc27-P11 hyd1Δhyd2Δ* lost viability at the same rate as *cdc27-P11* alone. *hyd1Δhyd2Δ* was not affected by growth at the restrictive temperature. This

suggests that the function of the hydrolases in the replication checkpoint is not as important as Cid1 and that *cdc27-P11* cells can still activate the replication checkpoint in their absence.

Although there was no genetic interaction between *hyd1Δhyd2Δ* and *cdc27-P11*, the resistance phenotype of *hyd1Δhyd2Δ* to various DNA damaging agents was investigated. The role of Cid1 in the replication checkpoint is supported by the fact a *cid1Δ* mutant is more sensitive to treatment with HU and caffeine compared to wildtype (Wang et al. 2000a). It was proposed *hyd1Δhyd2Δ* might have a similar phenotype if it is also involved in the replication checkpoint. Fig. 3.5A reveals that *hyd1Δhyd2Δ* is more sensitive to HU treatment alone than *cid1Δ* or wildtype. It was considered possible that *hyd1Δhyd2Δ* might be inherently sensitive to stress, not just to those agents that activate the replication checkpoint. The DNA damaging agents bleomycin and MMS activate an alternative checkpoint mechanism in which Cid1 is not thought to play a role (Wang et al. 2000a). However, a *hyd1Δhyd2Δ* mutant was as unaffected as wildtype or *cid1Δ* when treated with concentrations of bleomycin and MMS known to kill *rad3Δ* mutants (Wang et al. 2002b)(Fig. 3.5B). This suggests *hyd1Δhyd2Δ* is specifically sensitive to HU. This was confirmed by further experiments, which showed that like *cid1Δ*, *hyd1Δhyd2Δ* was not sensitive to either UV irradiation (Fig. 3.6A) or oxidative stress (Fig. 3.6B).

Although a *hyd1Δ* mutant was not HU sensitive (data not shown), a *hyd1Δhyd2Δ* double mutant was specifically sensitive to HU. This suggests that this mutant is more sensitive to replication inhibition by HU treatment, perhaps because it cannot activate Cid1-dependent replication checkpoint mechanisms. However, it is yet to be determined if *hyd1Δhyd2Δ* is checkpoint-defective after HU treatment.

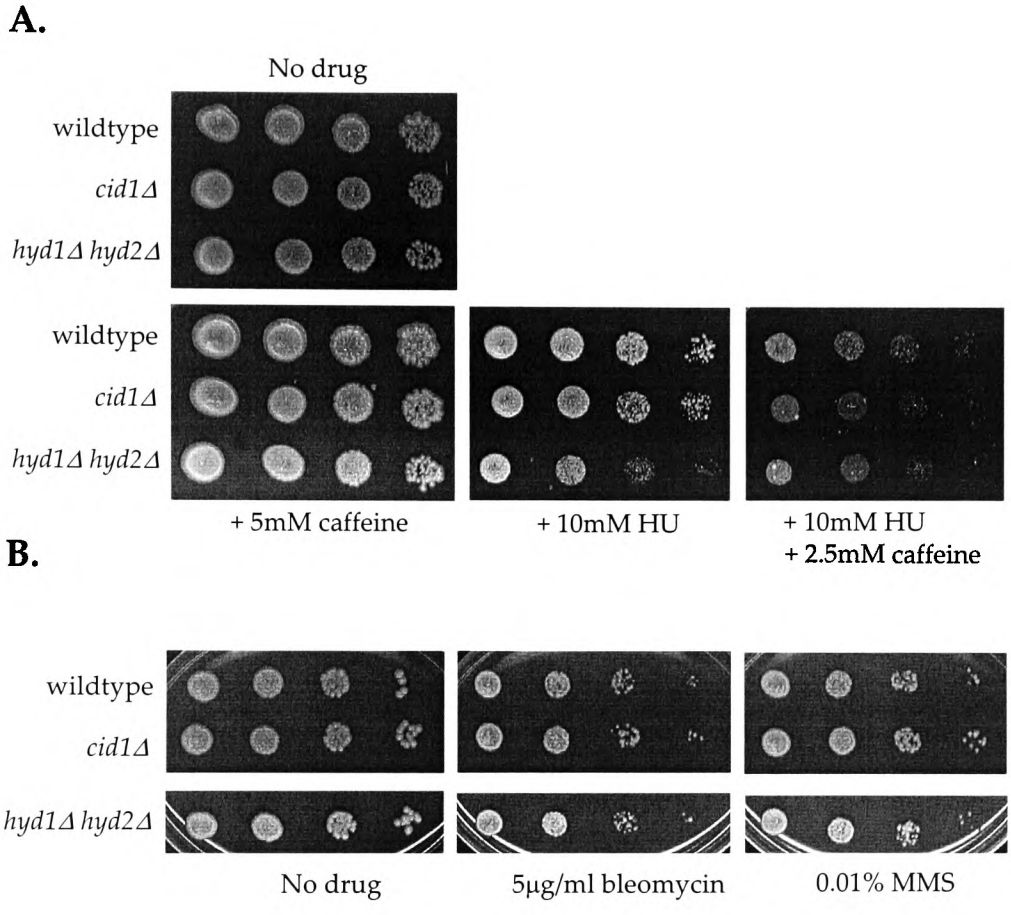
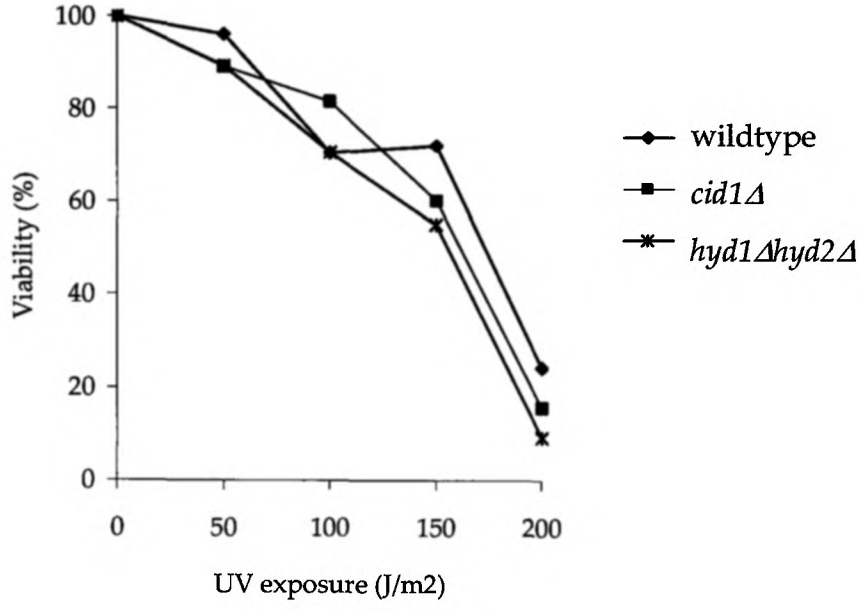


Fig. 3.5. *hyd1Δhyd2Δ* cells are mildly hypersensitive to hydroxyurea. Strains were grown to mid-exponential phase in liquid culture, before 10-fold serial dilutions were made and spotted onto rich agar with or without 10mM HU and/or 2.5mM caffeine (A), with or without DNA damaging drugs (B). Plates were photographed after 5 days' growth.

A.



B.

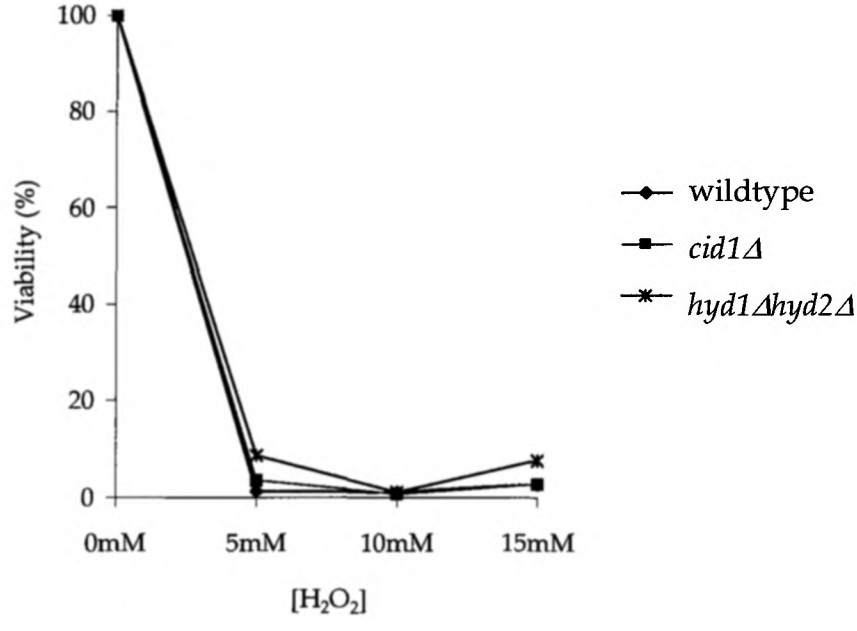


Fig. 3.6. *hyd1Δhyd2Δ* is not sensitive to UV irradiation or oxidative stress. (A). The indicated strains were grown to mid-exponential phase in liquid culture, before samples of 500 cells were plated onto rich medium, irradiated with 0 – 200J/m^2 UV and incubated at 30°C for 5 days. Viability was determined as a percentage of those cells surviving without UV. (B). Mid-exponential phase cultures were treated with 0 – 15mM H_2O_2 for 4 hrs, then 500 cells plated onto rich medium and grown at 30°C for 5 days. Viability was determined as a percentage of those cells surviving without oxidative stress. For both experiments, samples were taken in duplicate.

3.5 Discussion

Y2H screening identified one protein, Hyd1, as a Cid1-interacting protein. There are a number of explanations as to why only a single positive hit was obtained using this method, which has many limitations (Zhu et al. 2003). Cid1 and its partners are likely to be cytoplasmic proteins; as such, they may be difficult to translocate into the nucleus, which is a requirement for transcriptional activation of the reporter genes. Also, Y2H analysis is not always effective in identifying members of RNA processing complexes, since such proteins are likely to interfere with the transcriptional machinery. Although Y2H interactions are detected *in vivo*, this is non-physiological with respect to *S. pombe* proteins, as interactions occur in *S. cerevisiae*. So, the Cid1-Hyd1 interaction was confirmed by an alternative method; Cid1 was successfully co-immunoprecipitated with Hyd1. Deletion of *hyd1* did not affect the Cid1 phenotype, but a double deletion strain of *hyd1* and its paralogue, *hyd2*, was specifically sensitive to the replication inhibitor, HU. Together, these results suggest Hyd1 may play a role in the replication checkpoint function of Cid1; however, further experiments are needed to confirm and clarify such a role.

S. pombe Hyd1 is an uncharacterised protein of 256 aa, belonging to the metallo-β-lactamase family (see Fig. 3.7). This is a large family with members involved in different biological functions, but all are characterised by five conserved sequence motifs, consisting mainly of histidine and aspartate residues (Aravind 1999; Daiyasu et al. 2001). The Hyd1 primary sequence contains all five motifs, including the highly-conserved motif 2 (HxHxDH, x is any residue), important for zinc-binding during catalysis (Fig. 3.7A).

The β-CASP family is a sub-family of metallo-β-lactamases consisting of DNA and RNA processing enzymes; members include CPSF-73 and CPSF-100 (Callebaut et al.

A.

Hyd1

```
1     MSFHITPIWM WKGTGNNYAY LLTCDKTKIT AIVDPAEPES VIPVIKEKTA KKEIDLQYIL
61    TTHHHYDHAG GNEDILSYFP SLKVYGGKNA SGVTYTPKDK EIFKVGEVQV EALHTPCHTQ
121   DSICYYVSSP SKRAVFTGDT LFTSGCGRFF EGDAKQMDYA LNHVLAALPD DTVTYPGHEY
181   TKSNAKFSST IFSTPELTKL VDFCKNHEST TGHFTIGDEK KFNPFMCLGL ESVQKAVGSS
241    DPITVMKKLR DMKNAA
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B.

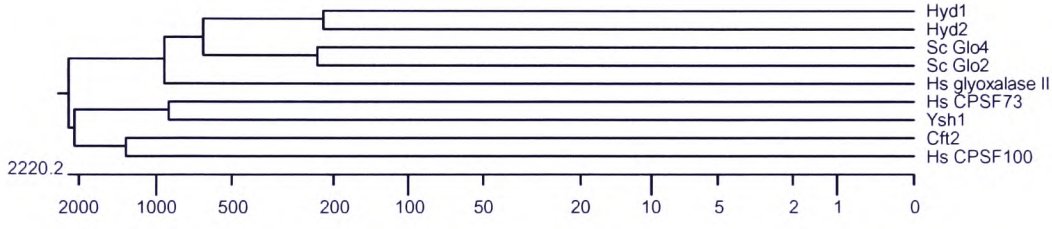


Figure 3.7. Hyd1 is a member of the metallo-β-lactamase superfamily. (A). Amino acid sequence of Hyd1; the five conserved metallo-β-lactamase motifs are shaded, with the important HxHxDH zinc-binding motif shaded in red. (B). Cladogram to show the evolutionary relationships between Hyd1, Hyd2 and other metallo-β-lactamases. Evolutionary distances were calculated by the number of sequence substitutions, indicated by the numbers below. Phylogenetic trees were constructed using ClustalW™ software.

2002). CPSF-73 has been proposed to be the factor responsible for cleavage of mRNAs prior to poly(A) addition (although this is contentious), whereas CPSF-100 lacks a histidine in motif 2, so is predicted to be catalytically dead (Ryan et al. 2004). Many eukaryotic 3' end RNA processing reactions require cleavage of the RNA by a metallo-β-lactamase prior to production of a mature RNA species; for example, tRNAs are cleaved by ELAC prior to the addition of CCA residues and mRNAs are cleaved (probably by CPSF-73) before poly(A) addition. Most of these enzymes act as homo- or hetero-dimers in the cleavage reaction (reviewed in Weiner 2005). Therefore, it is conceivable that the metallo-β-lactamase domain of Hyd1 and/or Hyd2 plays an important role during Cid1-dependent polyadenylation, perhaps in a cleavage reaction, as a dimer with Hyd2.

However, Hyd1 and Hyd2 are smaller proteins than β-CASP family members, and lack the large β-CASP insertion of approximately 200 aa between motifs 4 and 5 (Callebaut et al. 2002). Indeed, Hyd1 and Hyd2 show a greater similarity to the glyoxalase II group of metallo-β-lactamases than to β-CASP members (Fig. 3.7B). According to the *S. pombe* genome database, Hyd1 and Hyd2 are the predicted homologues of *S. cerevisiae* glyoxalase II enzymes, Glo2 and Glo4 (Bito et al. 1997). Glyoxalase II enzymes take part in the reaction to detoxify methylglyoxal, a metabolite synthesised *in vivo* during glycolysis. However, the exact role of the glyoxalase system is unknown, since the effect of methylglyoxal on the cell is unclear (reviewed in Thornalley 1990). Perhaps Hyd1 and Hyd2 are true glyoxalase II enzymes, but have alternative functions in Cid1-mediated polyadenylation? Metabolic enzymes have been reported to play additional roles in the cell, sometimes in controlling gene expression; for example, a large-scale proteomic analysis in *S. cerevisiae* revealed the mitochondrial arginine biosynthetic enzyme Arg5 to possess an unexpected DNA-binding activity (Hall et al. 2004).

Chapter Four

Purification of the Cid1 complex

4.1 Introduction

As discussed, Y2H screening has a limited capability to identify interactions, so Cid1 may have other protein partners, in addition to Hyd1. Also, an RNA-binding protein partner for Cid1 had not yet been found. To identify further Cid1-interacting proteins, a biochemical approach was used; tandem affinity purification of Cid1, followed by mass spectrometry, allowed for the isolation from *S. pombe* cells of an intact, multi-subunit Cid1 complex that had poly(A) polymerase activity.

Affinity purification using epitope-tagged proteins has been successful in various large-scale proteomic interaction experiments (reviewed in Yanagida 2002). However, the presence of non-specific contaminants in purifications is a major problem; a two-step TAP (tandem affinity purification) method developed by Séraphin and co-workers allowed for purification in high yields, but with reduced levels of contamination (Rigaut et al. 1999). TAP purification has also been applied in proteome-wide studies (Gavin et al. 2002) and was the method of choice for Trf4 purification from *S. cerevisiae* (Lacava et al. 2005; Vanacova et al. 2005). The TAP method is described in Fig. 4.1. Briefly, the protein of interest is fused to the TAP tag, consisting of a protein A domain and a calmodulin binding domain (CBD) linked by a peptide encoding a tobacco etch virus protease (TEV) cleavage site. Native protein lysate is prepared from cells expressing the TAP fusion protein, which

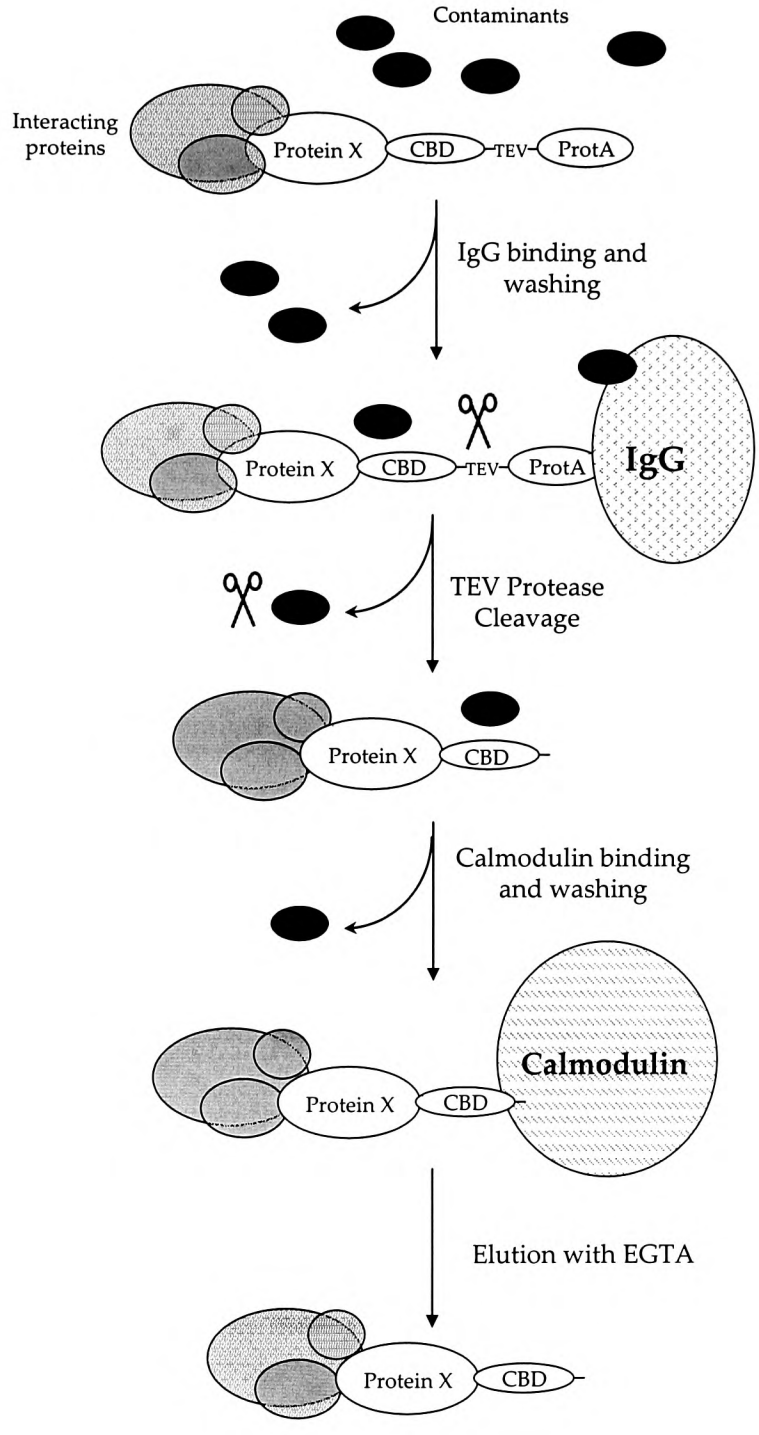


Figure 4.1. Schematic of the TAP method. Adapted from Rigaut et al., 1999.

is bound to IgG sepharose in the first instance. After washing to remove contaminants, the TAP complex is cleaved from the beads using TEV protease. A second round of binding and washing using calmodulin beads removes remaining contaminants. The TAP-purified complex is released from the beads using EGTA, which sequesters away calcium ions to prevent binding.

As the affinity-purified protein is eluted as a complex, purified directly from *S. pombe* cells *ex-vivo*, the TAP complex can be analysed further using biochemical methods. Individual protein components can be identified by SDS-PAGE, followed by tryptic digestion and mass spectrometry of individual protein bands (Steen and Mann 2004), or any nucleic acids specifically associated with the complex can be purified (Verdel and Moazed 2005). TAP-purified complexes can retain enzymatic activity, which can be tested using *in vitro* activity assays (Motamedi et al. 2004). Purification of the Cid1 complex using the TAP method was optimised so that a number of interacting proteins were identified by ESI-qTOF mass spectrometry. The Cid1 complex purified from "normal" *S. pombe* cells (exponentially-growing cells, not exposed to replication stress) was also tested for *in vitro* PAP activity, described below.

4.2 Tandem Affinity Purification of Cid1

Firstly, a *cid1-TAP* strain was obtained that expressed a C-terminal TAP fusion of Cid1 at endogenous levels (a kind gift from S.W. Wang). *cid1-TAP* was not checkpoint defective, indicating that TAP-tagged Cid1 was functional. However, endogenous Cid1-TAP was expressed too weakly to purify sufficient protein for identification by mass spectrometry (data not shown). Therefore, Cid1 was cloned into the *pREPNTAP* plasmid (a gift of K.

Gould), which allowed over-expression of N-terminally TAP-tagged Cid1 from the *nmt1* promoter, upon removal of thiamine. Over-expression of the NTAP-Cid1 protein and NTAP tag alone was verified by western blotting using an anti-Fc antibody, which detected the protein A moiety of the TAP tag (Fig. 4.2A). The resulting NTAP-Cid1 protein was functional, since it caused resistance to HU and caffeine when over-expressed, like untagged Cid1 (Fig. 4.3). TAP purification of over-expressed NTAP-Cid1 only purified non-specific contaminating proteins (data not shown); the over-expression of NTAP-Cid1 by the *nmt* promoter was too strong. Therefore, the expression of NTAP-Cid1 was reduced by adding thiamine to the culture medium, which repressed the *nmt1* promoter, as described (Javerzat et al. 1996) (Fig. 4.2B). Adding 0.1μM thiamine reduced the expression of NTAP-Cid1 significantly and was used in all subsequent NTAP-Cid1 purifications.

Results of the first successful NTAP-Cid1 purification (NTAP-Cid1A) are shown in Fig. 4.4, alongside a negative NTAP only control. Proteins were separated by SDS-PAGE and stained with Sypro Ruby. Many protein bands were present in the Cid1 purification that were absent from the negative control, indicating specific Cid1-interacting proteins; these bands were excised from the gel and identified by mass spectrometry. Not all bands contained sufficient protein for identification, but proteins that were identified are summarised in Table 4.1. Cid1-interacting proteins identified in this experiment included; SPCC550.14 (an uncharacterised RNA-binding protein), Pab1 (Poly(A) binding protein), SPAC23G3.06 (a Nop5-like protein) and Cid1. Contaminating proteins that were also present in the negative control included a translation elongation factor (EF1-α) and residual TEV protease.

SPCC550.14 is an uncharacterised protein, but according to the *S. pombe* genome database (www.genedb.org/pombe), it is a predicted multi-KH domain RNA-binding

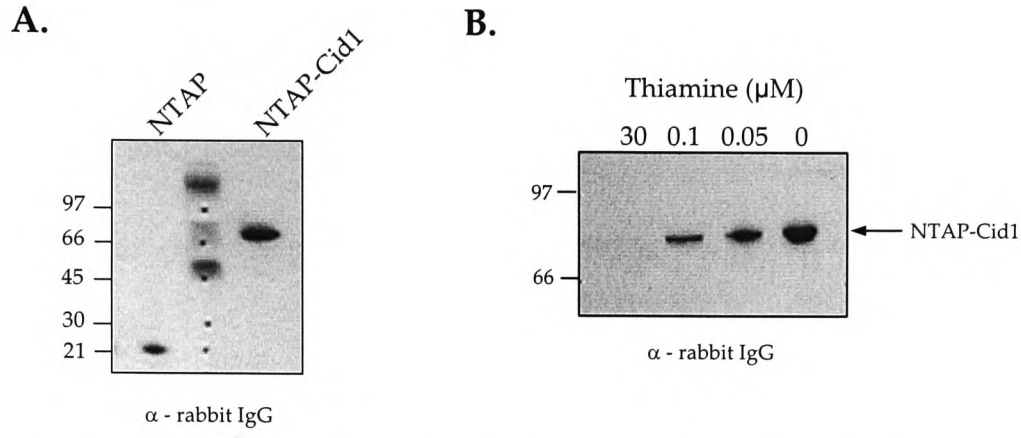


Figure 4.2. Overexpression of N-terminally TAP-tagged Cid1. (A). Cid1 was cloned into the *pREPNTAP* vector and transformed into *S. pombe*. Expression of TAP-tagged Cid1 was induced in liquid culture by the removal of thiamine. (B). The overexpression of NTAP-Cid1 was reduced by the addition of 0 - 30μM thiamine to the final culture. Expression of NTAP tag alone and NTAP-Cid1 was verified by western blotting with anti-Fc antibodies.

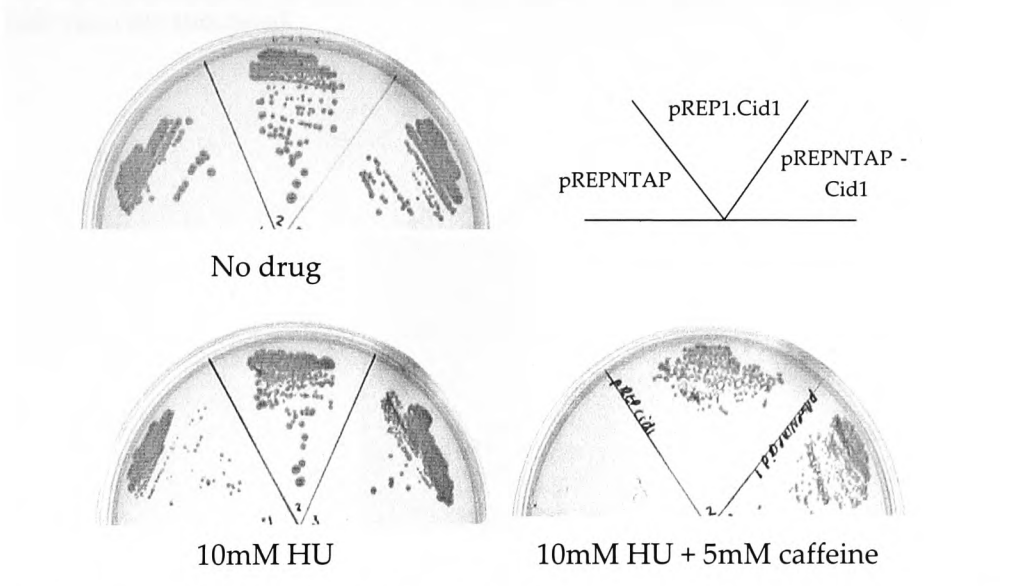


Figure 4.3. An N-terminal TAP-fusion of Cid1 is functional. *S. pombe h⁻ leu1-32* was transformed with plasmid *pREPNTAP* overexpressing TAP tag alone, untagged Cid1 or TAP-tagged Cid1. Transformants were streaked onto minimal medium with or without drug. Plates were photographed after 5 days' growth at 30°C.

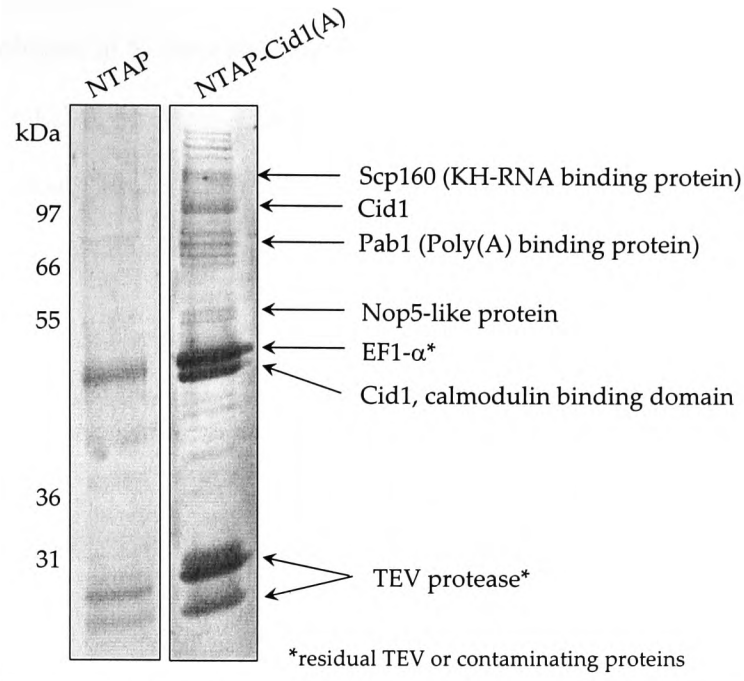


Figure 4.4. TAP purification of NTAP-Cid1. TAP complexes purified from an NTAP-Cid1 lysate and a negative control NTAP lysate expressing TAP tag alone were separated on a 4-15% SDS-PAGE gel and stained with Sypro Ruby. Protein bands were cut out and identified by mass spectrometry. Bands that contained enough protein for identification are annotated.

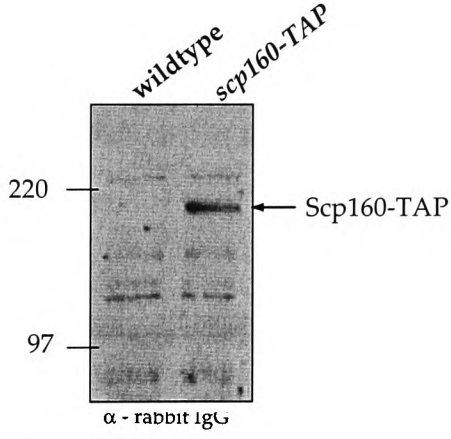


Figure 4.5. A C-terminal TAP fusion of Scp160. *scp160-TAP* was created by fusing the 3'end of *scp160* with the TAP tag by homologous recombination. A protein lysate was prepared from *scp160-TAP* cells and expression of the TAP-tagged protein checked by western blotting using anti-Fc antibodies.

protein and the orthologue of *S. cerevisiae* Scp160, so it was re-named Scp160. A Cid1-like protein in *C. elegans*, GLD-2, binds to a KH-domain protein, GLD-3, to achieve binding to specific RNA targets (Wang et al. 2002a). Therefore, it was proposed that Scp160 may be an RNA-binding protein partner for Cid1. To investigate the Scp160-Cid1 interaction further, a *scp160-TAP* strain, expressing a TAP-tagged version of Scp160, was created by homologous recombination (Fig. 4.5). A second set of TAP purifications was carried out, using strains expressing NTAP-Cid1, Scp160-TAP and a TAP tag alone negative control; results are shown in Fig. 4.6 and summarised in Table 4.1. The single protein present in the negative control (Hsp70) showed that this purification was more stringent than previous TAP experiments. This is likely to be the result of a change in wash buffer composition; imidazole and reducing agents were added to the wash buffers in later experiments. Unfortunately, this meant that the Cid1-Scp160 interaction was lost, but additional Cid1-interacting proteins were identified, including: Tcg1, ribosomal proteins, actin and Mlo3. The *S. pombe* genome database annotates Tcg1 as an ssDNA binding protein with two RRMs, predicted to bind telomeric DNA and Mlo3 as an RNA annealing factor with a single RRM. Interestingly, the Scp160-TAP purification contained a similar set of proteins to this NTAP-Cid1 purification (NTAP-Cid1 B), including Pab1, Tcg1 and ribosomal proteins. This suggests that Cid1 and Scp160 complexes have a significant overlap in their subunit composition.

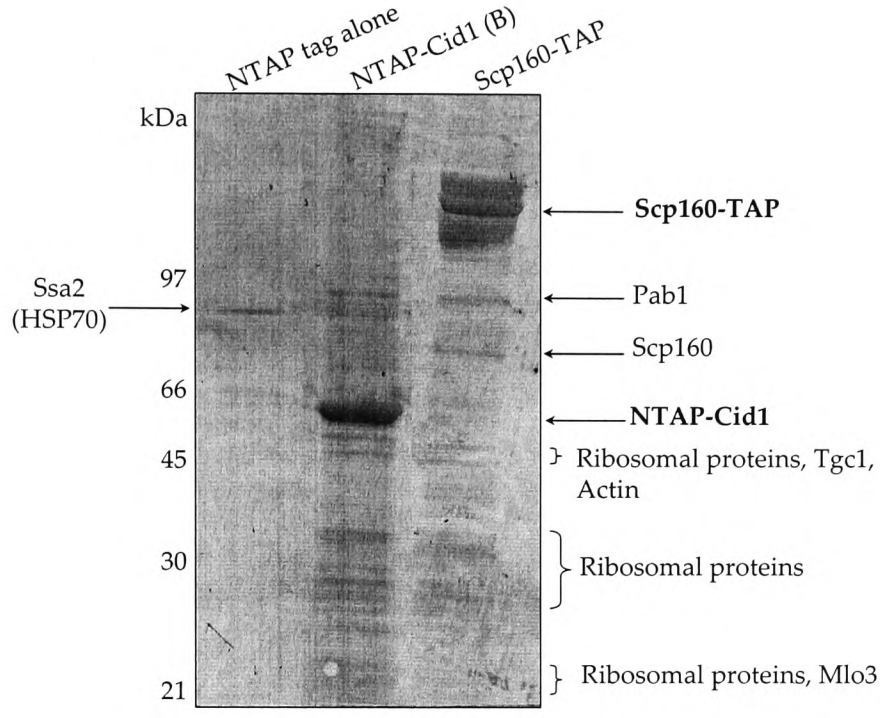


Figure 4.6. Cid1 and Scp160 complexes share components. TAP purifications were carried out using protein lysate extracted from *NTAP*, *NTAP-Cid1* and *scp160-TAP* strains. The resulting TAP complexes were separated on a 4-15% SDS-PAGE gel and stained with Sypro. Protein bands were cut out and identified by mass spectrometry. Bands that contained enough protein for identification are annotated.

Common name	*Gene ID*	*MW (kDa)*	*No. of unique peptides identified*		
			NTAP-Cid1 (A)	**NTAP-Cid1 (B)**	**Scp160-TAP**
Cid1	SPAC19D5.03	46.2	14	18	-
Scp160	SPCC550.14	141.2	5	-	31
Pab1	SPAC57A7.04C	71.5	4	13	9
Nop5	SPAC23G3.06	55.7	3	-	-
Tcg1	SPBC660.11	37.8	-	2	1
Mlo3	SPBC1D7.04	21.7	-	1	-
ribosomal proteins (various)	various	various	-	various	various
Actin	SPBC32H8.12c	41.7	-	3	-

Table 4.1. Summary of TAP results. The table includes proteins identified as significant hits in TAP-tagging experiments, which were absent from the negative TAP tag only purification. (-) = not identified in this experiment.

4.3 Scp160 is an RNA-binding partner of Cid1

As the Cid1-Scp160 interaction appeared to be weak by two-step TAP purification, Scp160 was tagged with HA in order to test the *in vivo* interaction by one-step co-immunoprecipitation with Cid1-GFP. Scp160-HA was indeed co-immunoprecipitated with Cid1-GFP, when both were expressed at endogenous levels (Fig. 4.7). However, Cid1-GFP could not be precipitated with Scp160 (data not shown).

Scp160 was also tagged with GFP by homologous recombination, to determine its cellular localisation. Expression of the Scp160-GFP fusion protein was verified by western blotting using anti-GFP antibodies (Fig. 4.8A). *scp160-GFP* cells were examined by fluorescence microscopy (Fig. 4.8B). Scp160-GFP localised to the cytoplasm, but unlike the diffuse fluorescence observed for Cid1-GFP (Read et al. 2002), Scp160-GFP appeared to be localised in a reticular pattern in the cytoplasm and around the nuclear periphery. This is a similar pattern to that observed for proteins of the endoplasmic reticulum, such as BiP (Pidoux and Armstrong 1992). However, experiments attempting to co-localise Scp160 with ER or Golgi proteins were inconclusive (data not shown).

4.4 An *scp160Δ* mutant phenocopies the over-expression of Cid1

To determine if Scp160 is an RNA-binding protein important for Cid1 function, a *scp160Δ* deletion mutant was created by replacing the entire *scp160* ORF with *ura4*$^+$ by homologous recombination. In budding yeast, *SCP160* is non-essential, but *scp160Δ* cells have reduced viability, abnormal morphology and an increased DNA content, a complex phenotype that is not complemented by a plasmid carrying the wildtype gene (Wintersberger et al. 1995).

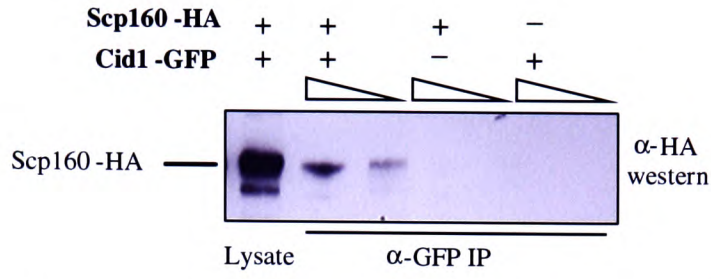


Figure 4.7. Scp160-HA co-immunoprecipitates with Cid1-GFP *in vivo*. Protein lysates were prepared from strains containing endogenously-tagged Scp160-HA and Cid1-GFP. GFP-tagged Cid1 was precipitated with anti-GFP antibody and protein G sepharose beads. After washing four times in 1% NP40 buffer, beads were resuspended in 40µl 2 x SDS sample buffer; between 10-30µl was loaded on a 10% SDS-PAGE gel. Co-immunoprecipitated Scp160-HA was detected with anti-HA antibody.

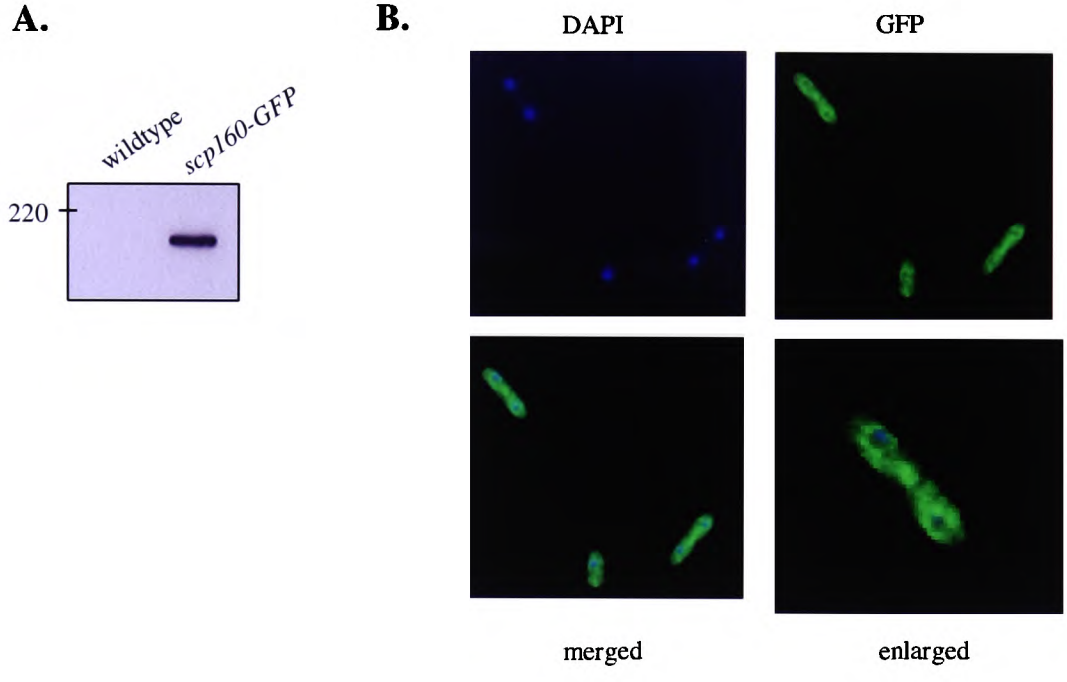


Figure 4.8. Scp160-GFP localises to specialised structures in the cytoplasm. *scp160-GFP* was created by fusing the 3'end of *scp160* with the GFP ORF by homologous recombination. (A). A protein lysate was prepared from *scp160-GFP* cells and expression of the fusion protein verified by western blotting with anti-GFP antibodies. (B). *scp160-GFP* cells from a mid-exponential phase culture were fixed in 100% methanol and visualised by fluorescence microscopy.

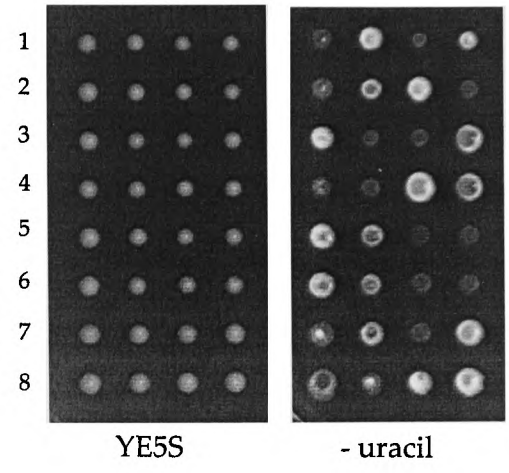


Figure 4.9. Scp160 is non-essential in fission yeast. One copy of the *scp160* gene was replaced with *ura4+* in diploid cells by homologous recombination. *scp160::ura4*$^{+}$ diploids were left to sporulate and the resulting four-spored asci were dissected onto YE5S medium (left). *scp160Δ* spores were identified by replica-plating onto minimal medium lacking uracil (right); the *ura4*$^{+}$ marker segregated 2:2, as expected.

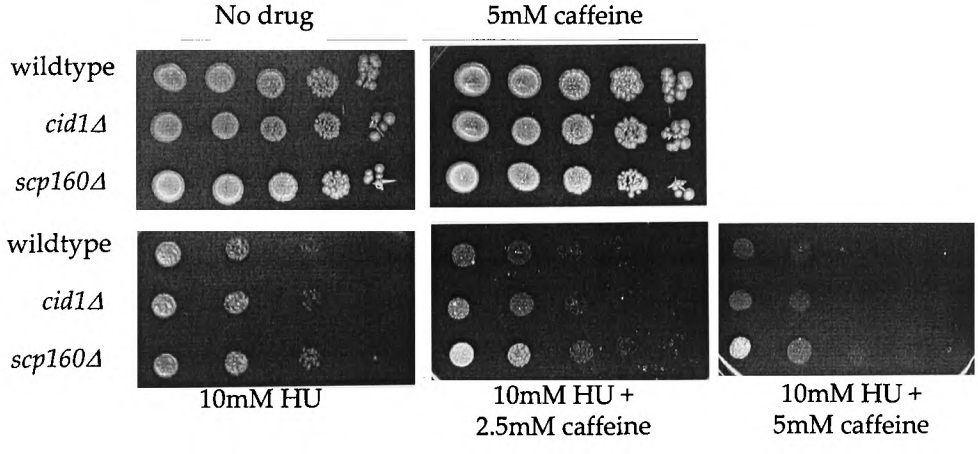


Figure 4.10. *scp160Δ* phenocopies the overexpression of Cid1. Strains were grown to mid-exponential phase in liquid culture and 10-fold serial dilutions were spotted onto rich medium with or without the indicated drugs. Plates were photographed after 5 days' growth at 30°C.

Scp160 is not an essential protein in fission yeast, since an *scp160Δ* strain grew indistinguishably from wildtype (Fig. 4.9).

Since *cid1Δ* mutants show slight hypersensitivity to HU in combination with caffeine (Wang et al. 2000a), the drug sensitivity phenotype of *scp160Δ* was investigated (Fig. 4.10). Unexpectedly, *scp160Δ* cells were more resistant to HU and caffeine than wildtype. Like the *cid1Δ* sensitivity phenotype, *scp160Δ* resistance was specific to a combination of HU and caffeine, and the strain was not resistant to any other DNA damage agents (UV, MMS, bleomycin) or stress (heat-shock, H_2O_2 treatment) (Fig. 4.11). These results implied that deletion of Scp160 has the same effect as over-expressing Cid1, consistent with the possibility that Scp160 is a negative regulator of Cid1. To determine whether Cid1 protein levels were affected in a *scp160Δ* strain, *cid1-HA* was mated with *scp160Δ* and Cid1 protein was quantified by western blotting using anti-HA antibodies (Fig. 4.12). Cid1 levels were unchanged between *cid1-HA* and *cid1-HA scp160Δ* strains, which implies that Scp160 does not regulate Cid1 by affecting its protein levels.

To further investigate the potential negative effect of Scp160 on Cid1 function, *scp160Δ* was combined with the DNA pol δ mutant, *cdc27-P11*, which shows a genetic interaction with *cid1Δ*. The viability of *cdc27-P11 scp160Δ* at the restrictive temperature (36°C) was determined and compared to that of *cdc27-P11*, *cdc27-P11 cid1Δ* and *scp160Δ* alone (Fig. 4.13). As expected, the *cdc27-P11 cid1Δ* mutant lost viability faster than *cdc27-P11* at the restrictive temperature; however, *cdc27-P11 scp160Δ* retained viability slightly better than *cdc27-P11* alone. The *scp160Δ* strain was unaffected by growth at 36°C. This suggests that the absence of Scp160 may help *cdc27-P11* to survive replication stress, perhaps by increasing Cid1 activity. Although the effect of *scp160Δ* is subtle, the drug resistance

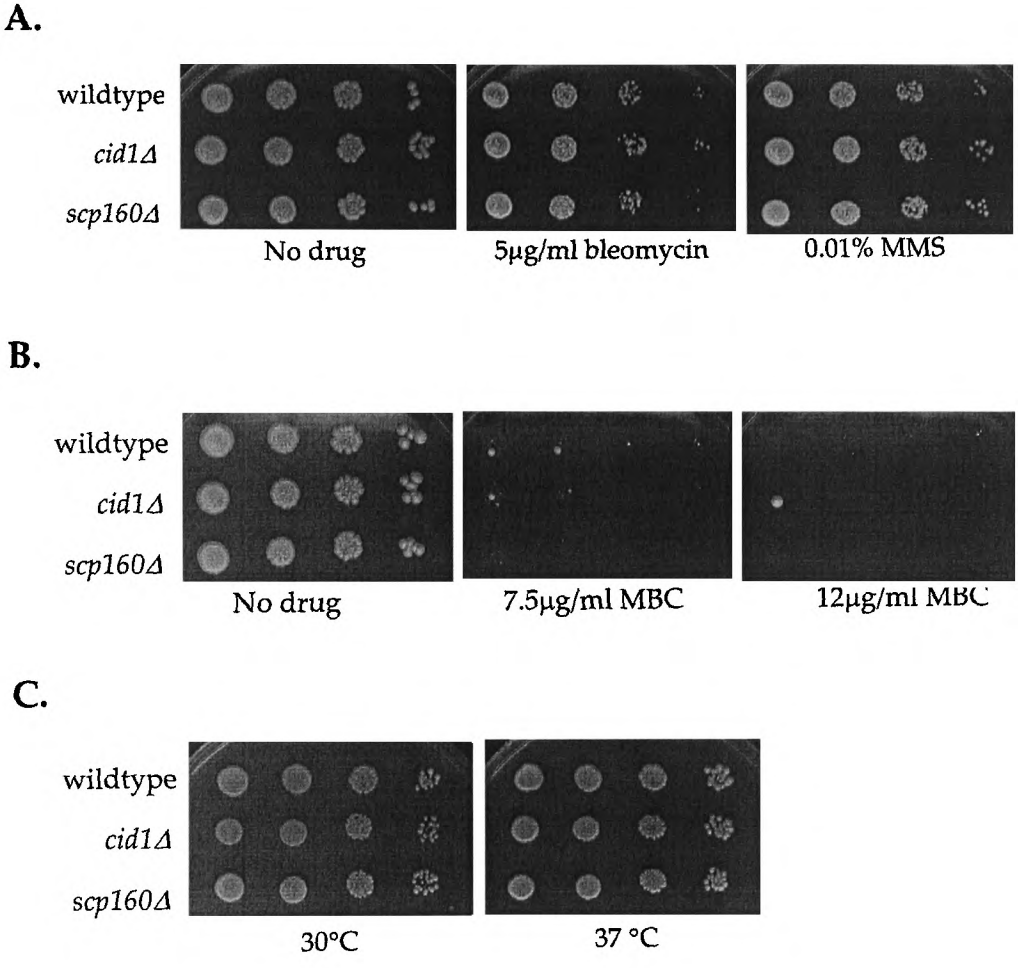
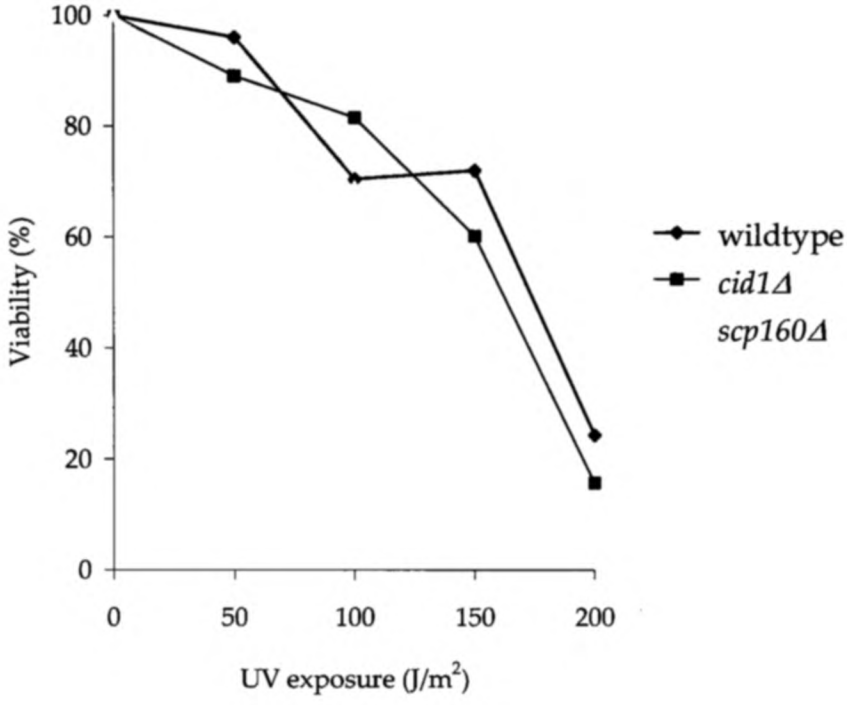
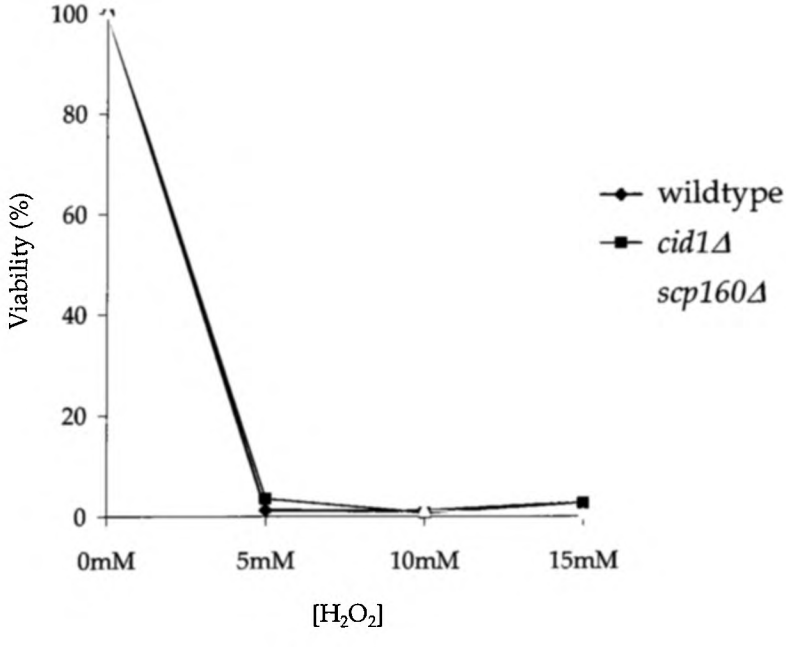


Figure 4.11. *scp160Δ* is specifically resistant to HU and caffeine. *scp160Δ* is not resistant to DNA damaging agents (A), MBC (B), high temperatures (C), UV irradiation (D) or oxidative stress (E). For DNA damage, MBC and temperature assays, strains were grown overnight to mid-exponential phase and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 5 days' at 30°C or 37°C. For UV assays, 500 cells were plated onto rich medium in duplicate and irradiated with 0 – 200J/m^2 UV and incubated at 30°C for 5 days. The viability was determined as a percentage of cells surviving without UV. For oxidative stress assays, cultures were treated with 0 – 15mM H_2O_2 for 4 hrs, then 500 cells plated onto rich medium in duplicate and grown at 30°C for 5 days. The viability was determined as a percentage of cells surviving without oxidative stress.

D.


100
80
60
40
20
0
Viability (%)
0
50
100
150
200
UV exposure (J/m²)
wildtype
cid1Δ
scp160Δ

E.


100
80
60
40
20
0
Viability (%)
0mM
5mM
10mM
15mM
[H₂O₂]
wildtype
cid1Δ
scp160Δ

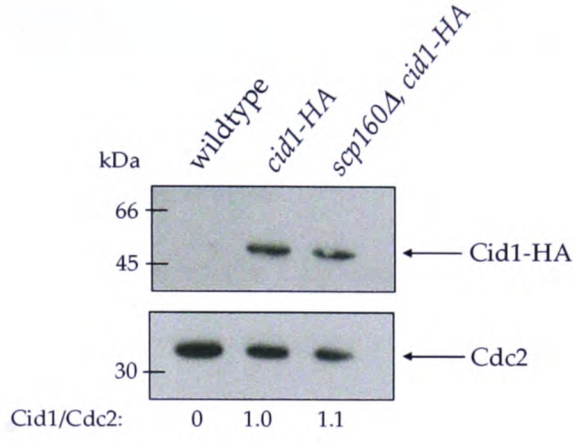


Figure 4.12. Cid1 protein levels are unaffected in *scp160Δ*. Protein lysates prepared from the above strains were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-HA and anti-Cdc2 antibodies. Cid1-HA protein levels were quantified by comparison to Cdc2 levels.

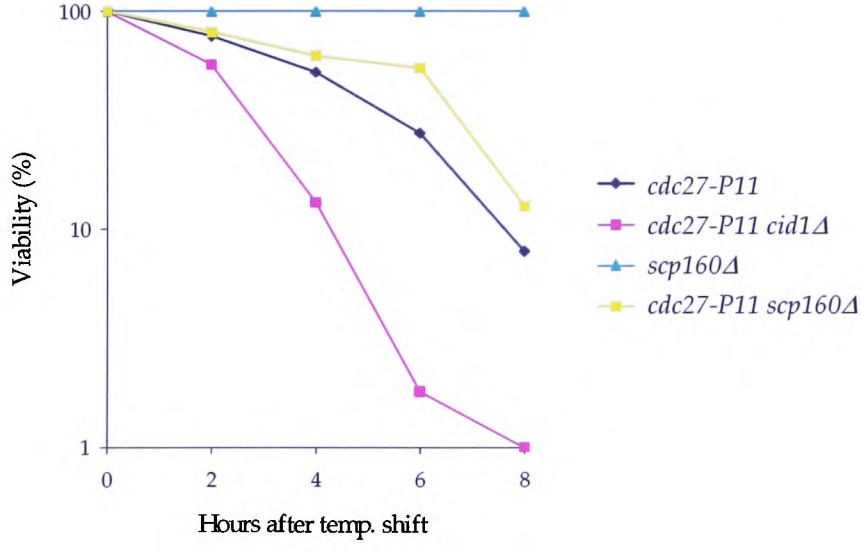


Figure 4.13. *scp160Δ* does not affect the viability of the DNA pol δ mutant, *cdc27-P11*. Strains grown to mid-exponential phase were shifted to 36°C for 8 hrs; samples of 500 cells were taken at the above time-points in duplicate, plated onto YE5S medium and incubated at 25°C for 5 days. Viability was determined as a percentage of those cells surviving at time zero.

phenotype and effect on *cdc27-P11* are both consistent with the hypothesis that Scp160 is a negative regulator of Cid1.

4.5 TAP-purified Cid1 has PAP activity *in vitro*

The mild purification procedures used in the TAP method helps to preserve a protein complex's integrity, so it is possible to retain enzymatic activity. Since previous microarray experiments determined that Cid1 was likely to be inactive in unstressed cells and a potential negative regulator had been identified, it was important to determine whether the Cid1 complex purified from "normal" *S. pombe* cells had any measurable PAP activity *in vitro*. PAP activity can be assayed by measuring the number of adenosine residues added to a radio-labelled $(A)_{15}$ RNA primer, in the presence of ATP and magnesium or manganese ions (Wahle 1991).

The PAP activity of recombinant Cid1 (rCid1) was compared to that of TAP-purified Cid1 complex, TAP-purified Scp160 complex and a negative NTAP only purification by PAP assay, carried out by O. Rissland (Fig. 4.14). As described previously, rCid1 purified from bacteria had robust PAP activity on its own (Lanes 2-4; Read et al. 2002). In comparison, TAP-purified Cid1 had relatively weak, non-processive PAP activity, since it could only add a few residues to the $(A)_{15}$ primer (Lane 9). An equal volume of Scp160-TAP complex or the negative control NTAP complex did not show any poly(A) polymerase activity (Lanes 7 and 11). Unfortunately, this assay was not quantitative, since the protein concentration of the TAP complexes after dialysis into PAP buffer was too low to be measured by Bradford assay. However, this result determined that the Cid1 complex purified from normal *S. pombe* cells has PAP activity *in vitro*, even when present at very low levels.

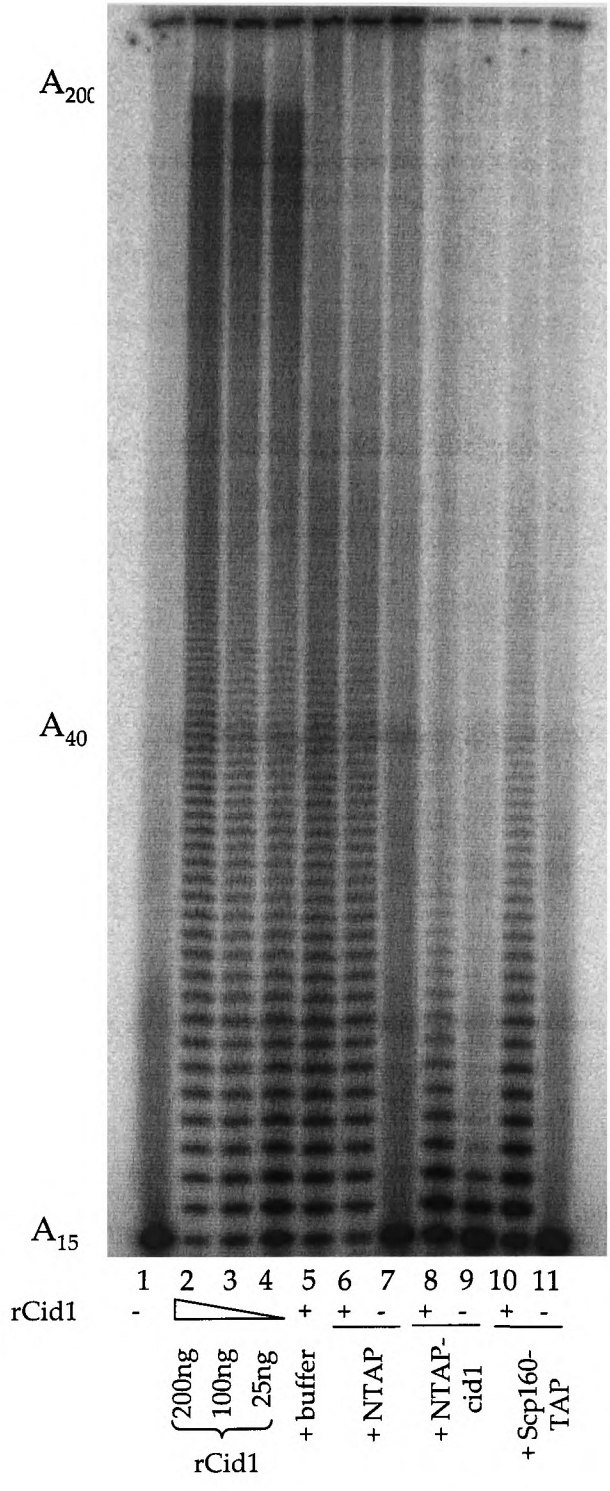


Figure 4.14. Affinity-purified Cid1 has PAP activity *in vitro*. 4µl of TAP elution from NTAP, NTAP-Cid1 and Scp160-TAP experiments (with or without 25ng rCid1) were tested for PAP activity using a radiolabelled RNA primer. Reactions were incubated at 37°C for 45 mins, then products were separated on a 12% polyacrylamide/urea gel and visualised by phosphoimager. PAP assays and sequencing gel carried out by O. Rissland.

Adding important regulatory proteins to a PAP reaction can affect PAP activity. For example, recombinant Trf4 purified from bacteria has no PAP activity *in vitro* unless its regulatory protein partners (Air1 or Air2) are added to the reaction, either as a TAP-purified complex or recombinant protein (Vanacova et al. 2005). The effect of NTAP-Cid1 and Scp160-TAP complexes on the activity of rCid1 was tested by adding each complex to rCid1 in a PAP reaction, performed by O. Rissland. The Cid1 complex reduced the activity of rCid1 substantially (Fig. 4.14, compare Lanes 5 and 8). The Scp160 complex also reduced rCid1 activity, but not as dramatically (Lane 10). Importantly, adding the negative TAP control had no effect (Lane 6). Together, these preliminary experiments suggest that although the Cid1 complex is an active PAP, it may be kept partially inhibited by other members of the complex. Perhaps the inhibitory component of the Cid1 complex is Scp160. However, further experiments are needed to confirm the inhibitory effect of Cid1 and Scp160 complexes on Cid1 activity.

4.6 Discussion

The TAP method was optimised to allow efficient purification of Cid1 and identification of co-purifying protein partners. Various Cid1 TAP purifications identified previously unknown Cid1-interacting proteins, summarised in Table 4.1. Fig. 4.15 is a model of the Cid1 complex, including novel Cid1 interactions discovered in this study. Although experimental variability was evident between TAP purifications, Scp160 was independently confirmed as interacting with Cid1 and Pab1 was immunoprecipitated in both NTAP-Cid1 purifications. Other interesting potential Cid1-interactors include ribosomal proteins and the RNA binding proteins, Tcg1 and Mlo3. The affinity-purified Cid1 complex had weak PAP activity when tested in a biochemical *in vitro* PAP assay. This was the first demonstration that Cid1 complex purified *ex vivo* is an active PAP *in vitro*.

Scp160 is a large putative RNA-binding protein of 140kDa with 11 tandemly-repeated hnRNP K-homology (KH) domains (www.genedb.org/pombe). Taken together, the TAP data and co-immunoprecipitation experiments indicate that Cid1 binds specifically to Scp160 *in vivo*. However, both experiments suggested that the interaction in unstressed *S. pombe* cells is weak and/or transient. It would be interesting to determine whether the interaction is RNA-dependent, or if it is affected in other growth conditions, such as replication stress. Further genetic and biochemical analyses indicated that Scp160 may be a negative regulator of Cid1. In *S. cerevisiae*, Scp160 is an mRNP component associated with specific mRNAs, localised to ER-bound polyribosomes (Li et al. 2003). *S. cerevisiae* Scp160 is an effector of the mating response pathway (Guo et al. 2003), but also interacts with Pab1 and Eap1, an eIF4E-binding protein (Lang and Fridovich-Keil 2000; Mendelsohn et al. 2003); this suggests a role for Scp160 in translational control in response to signalling pathways.

However, the function of Scp160 in *S. pombe* is likely to be distinct from that of *S. cerevisiae*, as there is no suggestion that an *scp160Δ* strain in *S. pombe* has a mating defect. Scp160 shows significant similarity to the vigilin family of RNA-binding proteins in vertebrates (Weber et al. 1997). Interestingly, vigilins have roles in heterochromatin formation; a vigilin of *D. melanogaster*, DDP1, (which can complement the increased DNA content phenotype of *scp160Δ* in budding yeast) binds specifically to inosine-edited RNAs and is required for heterochromatic silencing (Wang et al. 2005). Perhaps Scp160 binds specifically to RNAs polyadenylated by Cid1?

The interaction of Cid1 with Pab1 and ribosomal proteins is consistent with its proposed role in translational control by cytoplasmic polyadenylation. Pab1 was also identified as interacting with *S. pombe* Cid13 (Saitoh et al. 2002). Tcg1 interacted with both Cid1 and Scp160 in TAP experiments. Tcg1 is a small RNA-binding protein that can also bind to ssDNA; it binds single-stranded telomeric sequences *in vitro* and can complement *cdc13-1*, a *ts*-mutant of a telomeric binding protein in *S.cerevisiae* (Pang et al. 2003). Although only one unique Mlo3 peptide was identified by TAP, a role for this putative RNA annealing factor in aiding the Cid1 complex to bind to target RNAs is feasible. Other Cid1-interacting proteins identified by TAP analysis included SPAC23G3.06 and actin. SPAC23G3.06 is an uncharacterised protein, but predicted to be a ribonucleoprotein component with similarity to *S. cerevisiae* Nop5, involved in rRNA processing. *S. cerevisiae* Trf4 also associated with a Nop5-like protein, Nop53, in a large-scale affinity purification (Ho et al. 2002). The functional relevance of the interaction of Cid1 with the cytoskeletal protein actin is unclear. Actin provides a structural framework for cell shape, polarity and septum formation during cytokinesis, but actin filaments are also required for mRNP

localisation in *S. cerevisiae* (reviewed in Chartrand et al. 2001). Therefore, actin could aid Cid1 in the localised translation of specific RNAs.

Although Hyd1 was identified as interacting with Cid1 by yeast two-hybrid, Hyd1 was not pulled down with Cid1 by TAP. Perhaps the Cid1-Hyd1 interaction is too weak to withstand the high stringency TAP method. Such limitations of the TAP method imply that other important Cid1-interacting proteins remain to be identified.

The experiments described in Chapter 2 suggest that Cid1 does not target RNAs during unperturbed growth. If so, Cid1 may associate with different sets of proteins under different conditions; the Cid1 complex purified from unstressed cells in this study is likely to be the inactive Cid1 complex. Further TAP experiments, using lysate purified from cells exposed to replication stress, may isolate the active Cid1 complex. This study used the TAP method to purify a catalytically-active Cid1 complex from yeast cells and to identify the protein components that associated with Cid1. However, TAP could also be used to purify RNAs that are specifically associated with the complex. RNAs purified in this way could then be identified by microarray analysis, by finding RNAs that are enriched in affinity-purified fractions compared to a negative control (Gerber et al. 2004). This "RIP-CHIP" method (RNA immunoprecipitation and microarray chip analysis) is being pursued to identify potential Cid1 RNA targets.

This study shows that Cid1 exists as an active poly(A) polymerase complex *in vivo*, which was successfully purified by the TAP method to identify novel Cid1-interacting proteins. Although the function of each Cid1 partner is unclear, they provide interesting connections with the proposed role of Cid1 in telomeric heterochromatin maintenance. The interaction of Cid1 with the telomere binding protein, Tcg1, supports a role for Cid1 in telomere biology. It is interesting that Tcg1 can bind not only ssDNA, but also RNA.

During unperturbed growth, Tcg1 bound at telomeres may prevent the activation of checkpoint mechanisms, as has been proposed for telomere binding proteins in *S. cerevisiae* (Michelson et al. 2005). Tcg1 could also play a role during replication stress, as a telomeric sensor of stalled replication forks or by targeting RNAs for polyadenylation by Cid1. Also, the Scp160-Cid1 interaction may help the Cid1 complex influence heterochromatic silencing. By analogy with the vigilins, Scp160 may direct heterochromatin formation, which could be influenced by its interaction with Cid1.

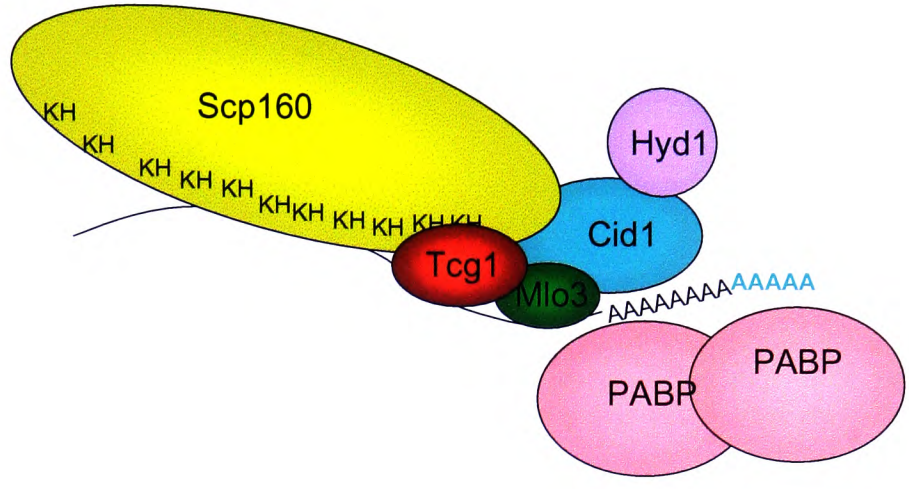


Figure 4.15. Schematic of the Cid1 complex.

Chapter Five

Conclusions

This study strongly supports the hypothesis that Cid1 acts on specific mRNAs in response to replication stress. In addition, potential Cid1 targets and partners were identified, which suggest a role in controlling the expression of telomeric and cell-cycle dependent genes.

5.1 Cid1 is a cytoplasmic poly(A) polymerase activated by replication block

Previous work implicated Cid1 in the replication checkpoint and further biochemical analysis showed that Cid1 possesses poly(A) polymerase activity *in vitro* (Wang et al. 2000a; Read et al. 2002). Microarray and northern analyses carried out in this study indicate that Cid1 does not significantly alter the abundance of RNAs during normal growth, but affects a subset of RNAs during replication stress. This suggests that Cid1 is a poly(A) polymerase *in vivo* that acts during replication stress, supporting a role for Cid1 in the replication checkpoint. Further biochemical experiments demonstrated that Cid1 purified from unstressed, exponentially-growing *S. pombe* cells had significant PAP activity *in vitro.* Therefore, Cid1 may be subject to additional regulation, since it is unlikely to polyadenylate its targets *in vivo* during unperturbed growth.

5.2 Cid1 affects cell cycle-dependent and telomeric transcripts

RNAs affected by Cid1 during replication stress included cell cycle-dependent and sub-telomeric transcripts, as identified by microarray analysis. A number of cell-cycle dependent genes were up-regulated, including those of *adg1* and histone genes, which accumulated as smaller transcripts in the absence of Cid1. Many telomere-proximal genes were either up- or down-regulated by loss of Cid1 function during replication stress; therefore, a role for Cid1 in controlling telomeric gene expression can be proposed. This study also found that expression of a telomeric RecQ helicase, Rqh2, was induced in a Cid1-dependent manner during replication stress. Genetic experiments revealed that the control of Rqh2 expression by Cid1 may contribute to its checkpoint function.

5.3 Identification of novel Cid1-interacting proteins

A comprehensive proteomic study was completed to identify Cid1 interacting partners. Yeast two-hybrid screening identified a previously uncharacterised metallo-β-lactamase, Hyd1, as interacting with Cid1. Tandem affinity purification isolated a multi-subunit Cid1 complex existing in unperturbed *S. pombe* cells, which included: a large multi-KH RNA-binding protein (Scp160), a telomere-binding protein (Tcg1), ribosomal proteins and poly(A) binding protein. These subunits are most likely part of the inactive Cid1 complex, which could prevent Cid1 from polyadenylating its target RNAs during normal growth.

5.4 Future Directions

The role of the Cid1 poly(A) polymerase in response to replication block will be investigated further. The mechanism of how Cid1 is activated in response to replication block is yet to be determined; this could be addressed by searching for evidence of any Cid1 post-

translational modifications or identifying any additional proteins that interact with Cid1 during replication stress.

Now that potential targets have been identified, the exact role of the Cid1 poly(A) tail in translational control can be explored. It is not yet confirmed whether Cid1 polyadenylation causes stabilisation or degradation of its substrate RNA, however the up-regulation of *rqh2* in a Cid1-dependent manner suggests a stabilising effect. Studying the polysome-association of target RNAs under different conditions may help to confirm this.

The significance of the association of Cid1 with Hyd1 will also be explored. Hyd1 may possess a cleavage activity that is associated with Cid1 polyadenylation in the cytoplasm. If so, maybe the cleavage activity (rather than Cid1 itself) is regulated in response to replication block. An example of post-transcriptional mRNA cleavage in response to stress has been reported in mammalian cells recently (Prasanth et al. 2005). The 3′ ends of potential Cid1 target mRNAs will be mapped in order to identify any novel cleavage sites.

The unexpected role for Cid1 in the maintenance of telomeric heterochromatin will be examined. *S. pombe* reporter strains for telomeric silencing exist (Allshire et al. 1995), which could be used to assess the effect of Cid1 and its potential target, Rqh2, on heterochromatin formation. Chromatin immunoprecipitation could also be utilised to determine if Cid1 influences the binding of any telomeric silencing factors, such as Swi6 or Taz1 (Kanoh et al. 2005). If such a role is confirmed, a cytoplasmic poly(A) polymerase that affects heterochromatic regions in response to replication stress would be an important novel regulator of eukaryotic gene expression.

Chapter Six

Materials & Methods

Many of the methods below have been described previously (Maniatis 1982; Ausubel 1999). Chemicals were provided by Sigma, enzymes by Roche and radioisotopes by Amersham, unless stated otherwise. Common buffers and solutions, plasmids and oligonucleotide sequences are described in Appendices A, B and C respectively.

6.1 *S. pombe* Methods

6.1.1. Growth and maintenance of S. pombe

Standard methods for fission yeast growth and genetic manipulations were followed (Moreno et al. 1991, http://www.sanger.ac.uk / PostGenomics / S_pombe / docs / nurse_lab_manual.pdf). *S. pombe* was maintained on YE5S agar or minimal agar with the appropriate supplements; liquid cultures were grown in YE5S medium or supplemented Edinburgh Minimal Medium (EMM2). Strains were stored in YE5S medium with 25% glycerol at -70°C. Strains used in this study are shown in Table 6.1 and media recipes are given in Appendix D.

6.1.2. *Determination of cell concentration*

The concentration of yeast cultures was determined by measuring the absorbance at 600nm (A_{600}) using an Eppendorf Biophotometer. An A_{600} of 1.0 was taken as the equivalent of 2 x 10^7 cells per ml.

Table 6.1. ***S. pombe*** **strains used in this study.**

Strain	*Genotype*	*Source*
wildtype	*h- leu1-32*	Lab stock
CN235	*h- leu1-32, ura4D-18, ade6-M210*	Lab stock
CN236	*h- leu1-32, ura4D-18, ade6-M216*	Lab stock
cdc27-P11	*h- cdc27-p11, leu1-32 ura4-D18*	P. Fantes
cdc27-P11 cid1Δ	*h- cdc27-p11, cid1::ura4+ leu1-32 ura4-D18*	Wang et al. 2000
cdc27-P11 hyd1Δhyd2Δ	*h- cdc27-p11, hyd1::ura4+ hyd2::ura4+ leu1-32 ura4-D18 ade6*	This study
cdc27-P11 scp160Δ	*h- cdc27-p11 scp160::ura4+ leu1-32 ura4-D18 ade6*	This study
cds1Δ	*h- cds1::ura4+ leu1-32 ura4-D18*	A.Carr
cds1Δ cid1Δ	*h- cid1::LEU2 cds1::ura4+ leu1-32 ura4D-18*	Wang et al. 2000
cid1-GFP	*h- cid1-GFP::kanMX6 leu1-32*	Read et al. 2002
cid1-TAP	*h- cid1-TAP::kanMX6 leu1-32*	S.W.Wang
cid1Δ	*h- cid1::ura4+ leu1-32 ura4-D18*	Wang et al. 2000
hyd1-HA	*h+ hyd1-HA::kanMX6 leu1-32 ura4-D18 ade6*	This study
hyd1-HA cid1-GFP	*h+ hyd1-HA::kanMX6 cid1-GFP::kanMX6 leu1-32 ura4-D18 ade6*	This study
hyd1Δ	*h+ hyd1::ura4+ leu1-32 ura4-D18 ade6*	This study
hyd1Δhyd2Δ	*h- hyd1::ura4+ hyd2::ura4+ leu1-32 ura4-D18 ade6*	This study
hyd2Δ	*h- hyd2::ura4+ leu1-32 ura4-D18 ade6*	This study
scp160-GFP	*h+ scp160-GFP::kanMX6 leu1-32 ura4-D18 ade6*	This study
scp160-HA	*h+ scp160-HA::kanMX6 leu1-32 ura4-D18 ade6*	This study
scp160-HA cid1-GFP	*h+ scp160-HA::kanMX6 cid1-GFP::kanMX6 leu1-32 ura4-D18 ade6*	This study
scp160-TAP	*h+ scp160-TAP::kanMX6 leu1-32 ura4-D18 ade6*	This study
scp160Δ	*h- scp160::ura4+ leu1-32 ura4-D18 ade6*	This study
scp160Δ cid1-HA	*h- scp160::ura4+ cid1-HA::kanMX6 leu1-32 ura4-D18 ade6*	This study
ski2Δ	*h- ski2::ura4+ leu1-32 ura4-D18 ade6*	This study

6.1.3 *Transformation by the lithium acetate procedure*

1 x 10^9 cells grown to mid-exponential phase were harvested by centrifugation (4000 x *g*, 3 mins, RT), washed twice with dH_2O, once with 1ml LiAc/TE (100mM LiAc, 100mM TE pH7.5) and resuspended in 500µl LiAc/TE. For each transformation, 100µl cells were mixed

with 2µl herring sperm DNA and 100ng transforming DNA then incubated for 10 min at RT. 260µl PEG/LiAc/TE (50% PEG_{4000}, 100mM LiAc, 100mM TE pH7.5) was added to each reaction, mixed gently and incubated at 30°C, 1 hr, with shaking. 43µl DMSO was added prior to heat-shock (5 mins, 42°C). Cells were harvested by centrifugation (2000 x *g*, 2 mins, RT), washed once with 1ml dH_2O and resuspended in 500µl dH_2O. 50 - 250µl cells were plated onto selective agar and incubated at 25-36°C for 3-5 days.

6.1.4 Genomic DNA extraction

5 x 10^7 cells grown to mid-exponential phase were harvested as above (6.1.3), washed with 500µl dH_2O and resuspended in 200µl DNA extraction buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl pH8.0, 1mM EDTA pH8.0). 200µl phenol: chloroform: isoamyl alcohol (25:24:1) and one pellet volume of acid-washed glass beads were added, vortexed for 2.5 mins and centrifuged (16,000 x *g*, 5 mins, RT). The aqueous layer was removed to a fresh tube, to which 1ml 100% ethanol was added and mixed. Samples were centrifuged as before; the resulting pellet was air-dried, resuspended in 50µl TE and stored at -20°C.

6.1.5 RNA extraction

1 x 10^8 cells grown to early exponential phase were harvested by centrifugation (4000 x *g*, 2 mins, 4°C). Cells were washed in 1ml pre-chilled DEPC water and transferred to 2ml Eppendorf tubes. Pellets were resuspended in 750µl chilled TES (10mM Tris-Cl pH7.5, 10mM EDTA, 0.5% SDS), prior to the immediate addition of 750µl phenol-chloroform (5:1, pH4.7) and vortexing for 5 secs. Samples were incubated at 65°C for 1 hr, vortexing 10 secs every 10 mins. Samples were incubated on ice for 1 min, vortexed for 20 secs and centrifuged (16,000 x *g*, 5 mins, 4°C). 700µl of the aqueous phase was added to 700µl

phenol-chloroform and mixed by inversion (no vortexing). Samples were centrifuged as before and 700µl of the aqueous phase transferred to 700µl chloroform: isoamyl alcohol (25:1) and mixed by inversion. Samples were spun as previously and 500µl of the aqueous phase added to 1.5ml 100% ethanol (-20°C) and 50µl 3M sodium acetate pH5.2. Samples were vortexed for 10 secs, incubated at -70°C for 30 mins and centrifuged (16,000 x *g*, 10 mins, RT). The RNA pellet was washed with 500µl 70% ethanol, air-dried and resuspended in 100µl DEPC water. RNA samples were stored at -70°C.

6.1.6 Native protein extraction

Cells harvested from mid-exponential phase cultures were washed once in dH_2O and resuspended in an equal volume of NP40 buffer. Cells were frozen and ground in liquid nitrogen using a mortar and pestle or a Retsch RM100 motorised grinder, until 75% lysis was achieved, as monitored by light microscopy. The cell lysate was clarified by centrifugation (4000 x *g*, 10 mins, 4°C); the resulting supernatant was removed to a fresh tube and stored at -70°C.

6.1.7 Denatured protein extraction

5 x 10^7 cells grown to mid-exponential phase were harvested as above (6.1.3). Pellets were washed once in 1ml stop buffer (1 x PBS, 50mM NaF, 10mM NaN_3), once in 1ml ice-cold 20% TCA, resuspended in 200µl 20% TCA and transferred to screw-cap Eppendorf tubes. A pellet volume of acid-washed glass beads was added and samples processed three times in a Bioline bead-beater (full speed, 20 secs), incubating for 5 mins on ice between runs. 400µl 5% TCA was added and the supernatant removed to a fresh tube prior to centrifugation (16,000 x *g*, 10 min, 4°C). The supernatant was discarded and the protein pellet resuspended

in 100µl TCA sample buffer (200mM Tris-Cl pH8.0, 1% SDS, 1% β-mercaptoethanol, 5% glycerol, 0.005% bromophenol blue). Lysates were stored at -20°C.

6.1.8 *Over-expression using the nmt1 promoter*

Proteins were over-expressed in *S. pombe leu1-32* strains using variants of the pREP3X vector, which drives expression from the thiamine-repressible *nmt1* promoter (Maundrell 1993). Plasmids were transformed into yeast as described (6.1.3) and selected using the *LEU2* marker by plating onto minimal agar lacking leucine. Expression was repressed by adding 30µM thiamine to the medium. When required, expression was induced by removing thiamine from overnight liquid precultures by washing four times in minimal medium. Full induction was achieved after a further 18 hrs' growth in liquid culture lacking thiamine.

6.1.9 *Gene deletion and tagging by homologous recombination*

Deletion mutants and tagged strains were created using the one-step targeted recombination method, as described (Bähler et al. 1998).

6.1.10 *Crosses and tetrad dissection*

Strains of opposite mating types were crossed by mixing cells on ME4S agar; nutrient starvation induced conjugation after 2 days at 25°C. Diploid cells were treated with 2% glusulase (30°C, 1hr) to dissolve asci, which were dissected using an MSM micromanipulator (Singer Instruments) onto YE5S agar. Spores of the required genotype were identified by replica-plating onto selective agar.

6.1.11 *Immunofluorescence*

5 x 10^8 cells grown to mid-exponential phase were harvested by vacuum filtration onto a GF-C filter (Whatman) and fixed in 100% methanol at -20°C. Cells were transferred to an Eppendorf tube, washed four times with chilled 1x PBS and resuspended in 1ml 1 x PBS containing 1M sorbitol and 0.5mg/ml lyticase, pre-warmed to 37°C. Samples were incubated at 37°C for 10 mins, then washed three times in chilled 1 x PBS. Cells were permeabilised in 1 x PBS containing 1% Triton X-100 for 30 secs, then washed a further three times in 1 x PBS. Cells were resuspended in 1ml PBS/BAL (1 x PBS, 1% BSA, 10% NaN_3, 100mM lysine) and incubated for 30 mins (RT, with rotation), before adding primary antibody and incubating overnight. Cells were washed three times in PBS/BAL prior to adding secondary antibody and incubating in the dark for 2-4 hrs (RT, with rotation). Cells were washed three times with PBS/BAL, once in 1 x PBS and resuspended in 100µl 1 x PBS. A 3µl aliquot was dried onto a polylysine-coated slide (BDH) and 10µl mounting medium with DAPI (Vectashield) added. Slides were examined using a CCD-fluorescence Axioplan-2™ microscope (Zeiss) and Metamorph™ software (Molecular Devices).

6.1.12 *Drug resistance assays*

1 x 10^7 cells grown to mid-exponential phase were harvested as above (6.1.3) and resuspended in 500µl dH_2O. Four serial dilutions (1:10) were made in dH_2O, so that 5µl was the equivalent of 10^5, 10^4, 10^3, 10^2 and 10 cells. 5µl from each strain's dilution series was plated out onto agar with and without drug (for appropriate concentrations, see Appendix D) and left to dry before incubating at 25-36°C for 3-5 days.

6.1.13 *UV resistance assays*

Liquid cultures were grown to mid-exponential phase and diluted serially to obtain 500 cells, which were plated onto YE5S agar and left to dry for 30 mins. Plates were exposed to between 0 - 200 J/m^2 UV (254nm) using a UVC 500 Crosslinker (Hoefer), then incubated at 25-36°C for 3-5 days. Colonies were counted and cell viability was calculated as a percentage of cells surviving without UV treatment.

6.1.14 *Viability assays using temperature-sensitive strains*

Overnight cultures were grown to early exponential phase at the permissive temperature in YE5S medium. Cultures were shifted to the restrictive temperature and samples of 500 cells taken at time points (0 - 8 hrs), in duplicate, and plated onto YE5S agar. After 3 - 5 days' incubation at the permissive temperature, colonies were counted and cell viability calculated as a percentage of colonies formed at time zero.

6.1.15 *Peroxide sensitivity assays*

0 - 15mM H_2O_2 was added to overnight cultures grown to mid-exponential phase and left to grow for a further 3 hrs. Cells were harvested as before (6.1.3) and washed in dH_2O. Serial dilutions were made to obtain 500 cells, which were plated onto appropriate agar and incubated at 25-36°C. After 3-5 days, colonies were counted and cell viability calculated as a percentage of colonies formed in the absence of H_2O_2.

6.2 Bacterial Methods

TOP10F' chemically-competent *E.coli* (F' *mcr*AΔ (*mrr-hd*RMS-*mcr*BC) ϕ80*lacZ* M15 Δ*lac*X74 *rec*A1 *ara*Δ139Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) (Invitrogen) was used to amplify plasmid DNA.

6.2.1 *Transformation of* E.coli

An aliquot of 50μl competent cells was thawed on ice, to which 100ng plasmid DNA or 2-5μl of a ligation reaction added. The reactions were incubated on ice for 30 mins, prior to heat-shock (42°C, 90 secs) and returned to ice for 5 mins. 900μl LB medium was added to each tube and incubated at 37°C for 1 hr with shaking. 50 - 250μl of each transformation was plated onto LB-agar containing selective antibiotic and incubated overnight at 37°C.

6.2.2 *Purification of plasmid DNA*

Large-scale preparations of plasmid DNA were carried out using a Plasmid Maxi kit (Qiagen), according to manufacturer's instructions. Briefly, a bacterial culture was grown overnight to mid-exponential phase in LB medium containing selective antibiotic and harvested by centrifugation (6000 x *g*, 15 mins, 4°C). The cell pellet was resuspended in alkaline lysis buffer P1, neutralised with buffer P2 and adjusted to high-salt with the addition of buffer P3. After incubating on ice for 20 mins, the lysate was centrifuged (20,000 x *g*, 30 mins, 4°C) and the supernatant bound to a pre-equilibrated silica-gel column (Qiagen Tip-500). The column was washed twice with buffer QC (containing 80% ethanol) and purified plasmid eluted with low-salt buffer QF and precipitated with 100% isopropanol. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in dH_2O. DNA stocks were stored at -20°C.

6.3 DNA Methods

6.3.1 *Determination of DNA concentration*

The concentration of DNA preparations was determined by measuring the absorbance at 260nm using an Eppendorf Biophotometer. An A_{260} of 1.0 was taken to be the equivalent of 50µg/ml dsDNA. Pure DNA had an OD_{260}/OD_{280} ratio of 1.8.

6.3.2 *PCR using a DNA template*

PCR reactions comprised: 100ng DNA template, 5µl 2.5mM dNTPs (Roche), 1-2 units Expand™ Taq Polymerase, 5µl 10 x Expand buffer, 0.5µl each 100µM oligonucleotide primers and made up to 50µl with dH_2O. Reactions were thermally cycled in a GeneAmp PCR system 9700 (Perkin-Elmer) as follows: 94°C for 5 mins, followed by 25 cycles of [94°C for 30 secs, annealing temperature for 1 min, 72°C for 1 min per kb] and a final 7 min hold at 72°C, before chilling to 4°C. The annealing temperature (T_m) varied depending on the GC/AT ratio of the primers, calculated using the equation: $T_m = 4(G + C) + 2(A + T)°C$.

6.3.3 *Colony PCR from yeast cells*

A single colony of yeast was resuspended in 60µl dH_2O and boiled at 98°C for 5 mins then cooled on ice. The colony PCR reaction was set up as follows: 20µl boiled cells, 8µl 2.5mM dNTPs (Roche), 1-2 units PicTaq Polymerase (CRUK), 5µl 10 x Expand buffer (Roche), 1µl 25mM $MgCl_2$, 2.5µl each 100mM primers and made up to 50µl with dH_2O. Thermal cycling was carried out as above (6.3.2), except the extension time was increased by 20 secs every cycle and the number of cycles was increased to 30.

6.3.4 *Agarose Gel Electrophoresis*

0.2 volumes of 6 x DNA loading dye were added to samples, prior to loading on 1% agarose gels containing 0.5μg/ml ethidium bromide, prepared with 1 x TBE buffer. Electrophoresis was carried out at 2 - 10V/cm², in 1 x TBE running buffer. DNA was visualised using a UV transilluminator (UVP). Hyperladder I (Bioline) was used as a size marker.

6.3.5 *Restriction Enzyme Digestion*

Digests comprised: 0.2 - 1μg DNA, 2μl 10 x restriction buffer, 5-10 units restriction enzyme(s) and made up to 20μl with dH_2O. Reactions were incubated at the optimum temperature (usually 37°C) for 1-2 hrs and digestion verified by agarose gel electrophoresis.

6.3.6 *Ethanol Precipitation*

To concentrate nucleic acids, 2.5 volumes of 100% ethanol (-20°C) and 0.1 volumes of 3M sodium acetate pH5.2 were added, mixed and incubated at -70°C for 30 mins. Samples were centrifuged (16,000 x *g*, 10 mins, RT), washed carefully in 500μl 70% ethanol (4°C), air-dried and resuspended in dH_2O.

6.3.7 *Phenol Extraction*

To remove protein and lipid contaminants, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to nucleic acid solutions and mixed by inversion. Samples were centrifuged (16,000 x *g*, 5 mins, 4°C) and the aqueous layer removed to a fresh tube.

6.3.8 *DNA Sequencing*

Dideoxy-DNA sequencing was carried out using the BigDye™ Terminator Cycle Sequencing Kit (Applied BioSystems). 3μl BigDye reagent was mixed with 200ng DNA

template, 3.2pmol oligonucleotide primer and made up to 10µl with dH_2O. Reactions were thermally cycled in a GeneAmp PCR system 9700 (Perkin-Elmer) as follows: 96°C for 4 mins, followed by 25 cycles of [96°C for 10 secs, 50°C for 5 secs, 60°C for 4 mins]. Samples were ethanol precipitated and DNA pellets stored at 4°C, prior to analysis using an ABI PRISM 3000 DNA Sequencer, carried out by Julian Robinson at the Dunn School of Pathology. The resulting sequence data was analysed using Sequencher™ software (Gene Codes Corp.).

6.3.9 Gel extraction

DNA was purified from agarose gel slices using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's protocol. Briefly, DNA was bound to a silica-gel column in the presence of high salt, impurities removed with wash buffer (containing 80% ethanol) and the purified DNA eluted with 30µl dH_2O.

6.3.10 Ligation

Digested and purified DNA fragments were ligated together as follows: 25 - 100ng each vector and insert DNA, 10 units T4 DNA ligase, 1µl 10 x ligase buffer and made up to 10µl with dH_2O. Reactions were incubated at 16°C overnight and transformed into bacteria, as described (6.2.1).

6.4 RNA Methods

When working with RNA, gloves and barrier tips were used. "RNA only" solutions were prepared with autoclaved dH_2O, pre-treated with 0.1% DEPC.

6.4.1 Determination of RNA concentration

The concentration of RNA preparations was determined by measuring the absorbance at 260nm using an Eppendorf Biophotometer. An A_{260} of 1.0 was taken to be the equivalent of 40µg/ml RNA. Pure RNA had an OD_{260}/OD_{280} ratio of 2.0.

6.4.2 RT-PCR

1µg of RNA was mixed with 0.5µl 100µM *RT* primer in 5µl DEPC dH_2O, denatured at 65°C for 5 mins, then cooled to 42°C before adding 14µl *RT* mix containing: 4µl 5 x *RT* buffer (Promega), 1µl RNasin (Promega), 2µl 0.1M DTT, 1µl 10mM dNTPs (Roche), 1µl AMV reverse transcriptase (Promega) and 4µl DEPC dH_2O. Reactions were incubated at 42°C for 1 hr, then heat-inactivated (65°C, 15 mins). 1 - 2µl of the resulting cDNA was used in a subsequent PCR reaction (see 6.3.2).

6.4.3 RNA Gel Electrophoresis

5 - 10µg RNA was mixed with 3.6µl formaldehyde, 10µl deionised formamide, 2µl 10 x MOPS and made up to 20µl with DEPC dH_2O. Samples were denatured at 65°C for 10 mins, chilled on ice and 4µl of 6 x RNA loading dye was added, prior to loading on a 1% agarose/6% formaldehyde gel, prepared with 1 x MOPS. Electrophoresis was carried out at 65V for 5 - 7 hrs, using 1 x MOPS as the running buffer. Perfect RNA™ 0.2 - 10kb (Novagen) was used as a size marker.

6.4.4 *Northern Blotting*

RNA gels were soaked in DEPC dH_2O for 20 mins, then placed on a paper wick soaked in 20 x SSC buffer. A Hybond-N^+ nylon membrane (Amersham) was placed in contact with the gel and layers of Whatman 3MM paper, paper towels and a weight were placed on top. The northern blot was left overnight to allow the RNA to transfer to the membrane by capillary action. The RNA was cross-linked to the membrane using a UVC 500 Crosslinker (Stratagene) at full power for 30 secs. To verify successful transfer, membranes were stained with methylene blue (0.03% methylene blue, 0.3M sodium acetate) and destained with dH_2O. Membranes were stored at 4°C.

6.4.5 *Hybridisation of DNA probes to Northern Blots*

DNA probes were amplified from *S. pombe* genomic DNA by PCR (6.3.2). 100ng gel-purified PCR product was labelled with α-^{32}P-dCTP using the Rediprime II™ kit (Amersham). Briefly, DNA was denatured (98°C, 5 mins), chilled on ice and added to a buffered mixture of dATP, dGTP, dTTP, exonuclease-free Klenow enzyme and random primers. 5µl α-^{32}P-dCTP was added, mixed and incubated at 37°C, 10 mins, then quenched by the addition of 5µl 0.2M EDTA pH8.0. Labelled DNA probe was purified using a Quick Mini-Spin column (Roche) by centrifugation (1,500 x *g*, 4 mins, RT). The eluate was collected in a screw-cap Eppendorf tube and stored at -20°C, until ready for hybridisation.

Membranes were placed in a 150ml hybridisation bottle (Amersham) with 10ml ExpressHyb™ solution (Clontech) and pre-hybridised at 60°C for 30 mins in a rotary oven. The DNA probe was denatured and cooled on ice, before adding to the ExpressHyb solution and hybridised at 60°C, overnight. The next day, the hybridisation solution was discarded and the membrane washed twice in low stringency buffer (2 x SSC, 0.05% SDS) at RT, 20

mins. The membrane was washed in high stringency buffer (0.1 x SSC, 0.1% SDS) at 50°C until the activity was less than 50cpm. The membrane was wrapped in plastic film and exposed to a phosphor-screen (Molecular Dynamics) overnight. Screens were scanned using a FLA5000 phosphoimager (Fuji) and AIDA™ analysis software (Raytest GmbH). If required, membranes were stripped with boiling 0.5% SDS solution and re-probed.

6.4.6 5' end-labelling of RNA

RNA was 5' end-labelled with γ-^{32}P-ATP in a T4 polynucleotide kinase (PNK) reaction, which comprised: 200ng RNA, 5-10 units PNK, 5µl γ-^{32}P-ATP (16pmol), 1µl 10 x PNK buffer and made up to 10µl with DEPC dH_2O. Reactions were incubated at RT for 30 mins then 20µl stop buffer added (20mM Tris-Cl pH7.5, 0.1M NaCl, 10mM EDTA). Radio-labelled RNA was purified using a Quick Mini-Spin column (Roche) by centrifugation (1,500 x *g*, 4 mins, RT) and the resulting eluate was ethanol precipitated and resuspended in 20µl DEPC dH_2O.

6.4.7 Poly(A) Polymerase Assay

Purified protein was tested for PAP activity as follows: 4.5µl protein (up to 200ng) was mixed with 2µl 5 x PAP buffer (100mM Tris-HCl pH 7.0, 250mM KCl, 3.5mM $MnCl_2$, 1mM EDTA, 500 µg/ml acetylated BSA, 50% glycerol) 10mM $MgCl_2$, 1µl RNasin (Promega), 10mM ATP and 1µl 5' end-labelled $(A)_{15}$ RNA primer and reactions incubated at 37°C for 20-40 mins. 20µl stop buffer (20mM Tris-Cl pH7.5, 0.1M NaCl, 10mM EDTA) was added, before samples were ethanol precipitated, ready for PAGE analysis (see 6.4.8).

6.4.8 *Sequencing Gel Electrophoresis*

Radio-labelled RNA samples were separated on 12% polyacrylamide gels, made with: 25g urea, 5ml 10 x TBE, 8.75ml dH_2O and 15ml acrylamide: bisacrylamide (19:1, Biorad), polymerised with 200µl 10% APS and 20µl TEMED. The gel was pre-run at 35W for 30 mins, using 1 x TBE as the running buffer. Samples were resuspended in 5µl formamide loading dye (95% deionised formamide, 0.005% BPB, 0.005% XC) and denatured (98°C, 4 mins) prior to loading. 15% gels were run at 35W for 1.5 hrs. Gels were transferred to Whatman 3MM paper, dried and exposed to a phosphor-screen, as above (see 6.4.5).

6.4.9 *Microarray Hybridisation*

Total RNA was extracted from two different *S. pombe* strains as described (6.1.5). Whole-genome microarray analysis was carried out in collaboration with Jürg Bähler at the Wellcome Trust-Sanger Institute, Cambridge.

20µg total RNA in 13.9µl dH_2O from the "reference" and "sample" strains was converted to cDNA, labelled with Cy3 or Cy5, by mixing with 9.6µl Labelling Mix (GibcoBRL) containing: an anchored oligo d(T) primer, DTT, dNTPs, reverse transcriptase and Cy3- or Cy5-dCTP. Reactions were incubated at 42°C for 1.5 hrs in the dark and treated with 0.1M NaOH and 0.1M HCl to remove any remaining RNA. The labelled cDNAs were purified using AutoSeq G-50 columns (Amersham) and pooled. The cDNA mixtures were ethanol precipitated and resuspended in 39µl hybridisation buffer (5 x SSC, 6 x Denhardt's solution, 60mM Tris-Cl pH7.6, 0.12% sarkosyl, 48% formamide) and mixed with 6µg poly(A) DNA. The hybridisation mixtures were denatured (100°C, 5 mins) before pipetting onto coverslips and hybridised to microarrays in 15 x SSC, incubated at 49°C for 16 hrs.

Microarrays were washed in Solution 1 (2 x SSC) for 5 mins at RT, then washed twice in Solution 2 (0.1 x SSC, 0.1% SDS) for 15 mins at RT. After a final wash in Solution 3 (0.1 x SSC) for 5 mins at RT, arrays were dried by centrifugation (1000 x *g*, 1 min, RT) and scanned with a GenePix 4000B laser scanner. Data was analysed using GenePix Pro and GeneSpring software (Silicon Genetics).

6.5 Protein Methods

6.5.1 Determination of Protein Concentration

The concentration of total protein in a sample was determined by Bradford assay. BSA protein standards between 1 - 25μg/ml were used to generate a standard curve. 800μl 20% Bradford solution (Biorad) was mixed with 200μl of a standard (or an appropriately diluted sample). After incubating at RT for 2 mins, the absorbance at 595nm was measured using an Eppendorf Biophotometer. A standard curve was plotted with A_{595} against concentration; a sample's concentration was determined from the standard curve.

6.5.2 Denaturing protein gel electrophoresis (SDS-PAGE)

Protein samples were heated (98°C, 5 mins) prior to loading onto mini SDS-PAGE gels, prepared with: a 6-15% separating gel (375mM Tris-Cl pH8.8, 0.1% SDS, 6-15% acrylamide: bisacrylamide (37.5:1, Biorad)) and a 6% stacking gel (125mM Tris-Cl pH6.8, 0.1% SDS, 6% acrylamide: bisacrylamide (37.5:1, Biorad)), polymerised by adding a final concentration of 0.03% APS and 0.2% TEMED. Gels were run at 50-150V, using 1 x SDS running buffer (25mM Tris, 190mM glycine, 0.1% SDS).

6.5.3 Total Protein Staining

SDS-PAGE gels were stained for total protein with Coomassie stain (0.1% coomassie brilliant blue, 10% acetic acid, 20% methanol), removed by destaining solution (10% acetic acid, 40% methanol). For increased sensitivity, gels were stained with Sypro (Biorad), a fluorescent dye visualised at 532nm using the FLA5000 scanner (Fuji).

6.5.4 *Western Blotting*

SDS-PAGE gels were soaked briefly in transfer buffer (48mM Tris, 39mM glycine, 1.3mM SDS, 10% methanol) before proteins were transferred to Hybond™ nitrocellulose membrane (Amersham) using a semi-dry blotter (Hoefer), at a constant current of 0.02A for 1-2 hrs. Membranes were blocked with 1 x PBS-T containing 10% milk (30 mins, RT, with shaking) before adding primary antibody, diluted in 1 x PBS-T with 3% milk and incubated with the membrane (overnight, 4°C, with shaking). The next day, the membrane was washed three times with 1 x PBS-T (15 mins, RT, with shaking). If required, a secondary HRP-conjugated antibody, diluted in 1 x PBS-T with 3% milk, was added to the membrane (1 hr, RT, with shaking). The membrane was washed three times as previously, before incubating with ECL solution (Amersham) for 1 min to detect bound antibody. ECL contains luminol, which is oxidised by HRP to emit chemiluminescence, detected by exposure to Hyperfilm™ x-ray film (Amersham). Antibodies used in this study are given in Table 6.2.

Name	*Type*	*Use (concentration)*	*Source*
Anti-Cdc2	Mouse monoclonal	WB (1:800)	Y100 (J. Gannon)
Anti-GFP	Mouse monoclonal	WB (1:1000)	CRUK 3E1
Anti-GFP	Rabbit polyclonal	IP (1:125)	Molecular Probes
Anti-HA	Mouse monoclonal	IP (1:200) IF (1:100)	Covance HA-11
Anti-HA-HRP	Mouse monoclonal	WB (1:1000)	Roche
Anti-histone H4	Rabbit polyclonal	WB (1:2500)	Abcam Ab10158
Anti-mouse-Cy3	Goat polyclonal	IF (1:800)	Sigma
Anti-mouse-HRP	Goat polyclonal	WB (1:1000)	Sigma
Anti-rabbit-HRP	Goat polyclonal	WB (1:1000)	Sigma
CN43 (anti-Cid1)	Rabbit polyclonal	WB (1:500)	Lab stock

Table 6.2. Antibodies used in this study.
(WB = western blot, IP = immunoprecipitation, IF = immunofluorescence)

6.5.5 *Protein identification by electro-spray ionisation mass spectrometry (ESI-MS)*

Bands of interest were excised from protein gels with a clean scalpel and diced into 1mm^2 squares (stored at 4°C). The gel squares were washed three times in 100µl fresh ambic solution (50mM ammonium bicarbonate in 50% acetonitrile) for 20 mins, soaked in 100µl 100% acetonitrile for 20 mins and air-dried. Proteins were digested by the addition of 50µl trypsin solution (20ng/µl in ambic) then incubated overnight at 37°C, with shaking. The supernatant was removed to a fresh tube, to which 50µl 0.1% formic acid was added and agitated for 20 mins to extract the digested peptides. Samples were dried to completeness in a Speed-Vac (Thermo Finnigan). Samples were reconstituted in 5µl 0.1% formic acid and injected into an electro-spray Q-ToF (quadrupole time-of-flight) mass spectrometer (MicroMass) by Dr. Benjamin Thomas at the Dunn School of Pathology. Peptide identities were searched using MASCOT (www.matrixscience.com) against the SwissProt database.

6.5.6 *Co-immunoprecipitation of tagged proteins*

Native protein lysate was prepared from 2 x 10^9 cells of a tagged strain, as described (6.1.6). 200µl protein-G sepharose beads were washed three times in 5ml NP40 buffer and resuspended in 200µl NP40 buffer. 1ml lysate was pre-cleared by incubation with 20µl of protein-G sepharose (15 mins, 4°C, with rotation). The beads were removed by centrifugation (16,000 x *g*, 10 secs, 4°C) and the supernatant removed to a fresh tube. The clarified lysate was divided in two and each incubated with immunoprecipitating antibody (1 hr, 4°C, with rotation). 5 - 20µl protein-G sepharose was added and incubated for a further 30 mins, as before. The beads were harvested as before and the supernatant discarded. The beads were washed four times in 1ml NP40 buffer containing 1% IGEPAL, then resuspended in 30µl 2 x SDS sample buffer, ready for SDS-PAGE analysis (6.5.2).

6.5.7 *Tandem Affinity Purification (TAP)*

1×10^{11} cells of TAP-tagged strain, grown to an A_{600} of 1.0, were harvested by centrifugation (4000 x *g*, 5 mins, 4°C). Cell pellets were snap-frozen in liquid nitrogen and stored at -70°C. Native protein lysate was obtained by grinding in NP40 buffer, as described (6.2.6). An additional 2 volumes of NP40 buffer were added to the lysate before centrifugation (16,000 x *g*, 10 min, 4°C). The cell debris was discarded and the supernatant transferred to two 50ml tubes (Falcon), ready for IgG binding.

1ml IgG FastFlow™ Sepharose (Amersham) was washed three times with 10ml NP40 buffer and resuspended in 500µl NP40 buffer. 400µl IgG beads were added to each tube and incubated overnight on a rotating wheel, 4°C. The beads were collected by centrifugation (1000 x *g*, 5 mins, 4°C), transferred to a 10ml Polyprep column (Biorad), washed three times with 10ml NP40 buffer and once with 10ml TEV Cleavage Buffer (TCB: 10mM Tris-Cl pH 8.0, 150mM NaCl, 0.5mM EDTA pH8.0, 0.1% IGEPAL, 1mM DTT).

The column was sealed at the bottom, then 1ml TCB and 30µl TEV protease (Invitrogen) were added. The column was sealed and incubated overnight on a rotating wheel, 4°C. The next day, the TEV eluate was collected into a new sealed column and the beads washed with a further 500µl TCB. An extra 5µl 1M $CaCl_2$ was added to the TEV eluate, before diluting with 3ml Calmodulin Binding Buffer (CBB: 10mM Tris-Cl pH 8.0, 150mM NaCl, 2mM $CaCl_2$, 1mM MgAc,1mM imidazole, 10mM β-mercaptoethanol).

500µl calmodulin beads (Stratagene) were washed three times with 5ml CBB, then resuspended in 250µl CBB. 200µl beads were added to the column containing the TEV eluate. The column was sealed and incubated for 3 hours on a rotating wheel, 4°C. The column was washed three times with 1ml CBB containing 0.1% IGEPAL. TAP complexes

were eluted from the beads using 200µl Calmodulin Elution Buffer (CEB: 10mM Tris-Cl pH8.0, 150mM NaCl, 20mM EGTA, 1mM magnesium acetate, 1mM imidazole, 10mM β-mercaptoethanol, 10% glycerol) into an Eppendorf tube, on ice. This was repeated four times to collect five 200µl fractions, which were stored at -70°C.

For SDS-PAGE analysis (6.5.2), the most concentrated fractions were pooled and adjusted to 25% (v/v) trichloroacetic acid (TCA). The tube was incubated on ice for 30 mins, vortexing periodically, and then centrifuged (16,000 x *g*, 30 mins, 4°C). The supernatant was discarded and the protein pellet washed carefully with ice-cold 100% acetone before centrifuging again (16,000 x *g*, 5 min, 4°C). The pellet was air-dried and resuspended in 15µl 2 x SDS sample buffer, ready for analysis. For PAP activity assays, the most concentrated fractions were dialysed overnight into 1 x HBS (40mM HEPES pH7.0, 200mM NaCl) using a Slide-A-Lyser™ cassette (Pierce) before assaying as described (see 6.4.7).

6.5.8 Two-dimensional Liquid Chromatography (2D-LC)

2D-LC comparing the total proteomes of different *S. pombe* strains was carried out using the Proteome Lab™ System (Beckman-Coulter). For each strain, a 400ml culture grown to mid-exponential phase was harvested by centrifugation (4000 x g, 5 mins, 4°C). Protein lysate was obtained by grinding under liquid nitrogen in a non-ionic PF-2D lysis buffer (250mM Tris pH8.0, 6M urea, 2M thiourea, 10% glycerol, 2% octylglucoside, 5mM TCEP, 1.25mM protease inhibitor cocktail). The lysate was clarified by centrifugation (16,000 x g, 1 hr, RT) and the supernatant removed to a fresh tube. The sample was desalted using a PD-10 column (Amersham), which also allowed buffer exchange into 3.5ml START buffer (Beckman Coulter). The total protein concentration was determined by Bradford assay and the sample diluted to obtain 5mg total protein in 2ml START buffer. The sample was now

ready for 2D-LC analysis and was injected into the Proteome Lab by Dr. Benjamin Thomas at the Dunn School of Pathology. Proteins were resolved into 890 fractions, by isoelectric point in the first dimension and by hydrophobicity in the second dimension; the UV absorbance of each fraction was measured to create a 2-D proteome map for each sample, using ProteoVue™ software (Beckman-Coulter). Maps from two different strains could be compared and any fraction that differed between strains was further analysed by mass spectrometry (6.5.5).

6.6 *S. cerevisiae* Methods

6.6.1 *Yeast two-hybrid screening*

S.cerevisiae strain L40a (*trp1.leu2.his3* (*LYS2::lexA-HIS3*)(*URA3::lexA-lacZ*)) was used for yeast two-hybrid screening, grown on SD agar with the appropriate supplements (Bio101). The yeast two-hybrid system used pBTM116 as the bait plasmid (encoding the *LexA* DNA binding domain) and pGADGH as the prey plasmid (encoding the GAL4 activator domain). Large-scale transformations of L40a with both bait and prey plasmids were carried out by the lithium acetate method (see 6.1.3); co-transformants were selected on SD medium lacking tryptophan and leucine. Clones that activated the first reporter gene, *HIS3*, were detected by replica-plating onto SD lacking tryptophan, leucine and histidine. These clones were tested for the expression of the second reporter gene, *lacZ*, by lifting colonies onto filter paper, freeze-thawing in liquid nitrogen and placing onto filter paper pre-soaked in β-gal assay solution (1.5% X-GAL, 0.2% β-mercaptoethanol in Z buffer). Colonies were incubated at 30°C and positive clones turned blue (indicating *LacZ* expression) within 24 hrs.

6.6.2 *Plasmid extraction from* S.cerevisiae

Plasmid DNA was extracted from positive yeast two-hybrid clones, as follows. 1.5ml culture was grown overnight in selective medium and harvested by centrifugation (16,000 x *g*, 1 min, RT). The cell pellet was resuspended in 200μl DNA extraction buffer (see 6.1.4), to which 200μl phenol: chloroform: isoamyl alcohol (25:24:1) and glass beads were added, vortexed for 2 mins and centrifuged (16,000 x *g*, 5 mins, RT). 1-5μl of the aqueous layer was used to transform *E.coli* (see 6.2.1), or 15μl to transform yeast (see 6.1.3).

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Appendices

APPENDIX A. Common Buffers and Solutions

1 x PBS: 0.14mM NaCl, 3.4μM KCl, 10μM Na_2HPO_4, 1.8μM KH_2PO_4

1 x PBS-T: 1 x PBS, 0.3% Tween-20

1 x TE: 10mM Tris, 1mM EDTA, pH7.5

1 x TBE: 10mM Tris, 10mM H_3BO_3, 2mM EDTA, pH8.3

20 x SSC: 3M NaCl, 0.3M sodium citrate, pH7.0

6 x DNA loading dye: 30% sucrose, 100mM EDTA pH8.0, 0.01% BPB

6 x RNA loading dye: as above, but made with DEPC dH2O and autoclaved.

10 x MOPS: 40% 1M MOPS pH7.0, 3.3% sodium acetate pH5.2, 2% 0.5M EDTA pH8.0

2 x SDS sample buffer: 60mM Tris-Cl pH6.8, 1% SDS, 1% β-ME, 5% glycerol, 0.01% BPB

NP40 buffer: 6mM Na_2HPO_4, 4mM NaH_2PO_4, 150mM NaCl, 0.1% IGEPAL, 2mM EDTA pH8.0, 50mM NaF, 0.1mM Na_3VO_4, 10% glycerol
(before use, add protease inhibitors and reducing agents: 1mM DTT, 1 Complete™ tablet (Roche), 1mM PMSF, 1mM benzamidine)

X-Gal solution : 20mg/ml X-GAL in DMF

Buffer Z: 60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$

APPENDIX B. Plasmids

Plasmids used in this study are described below:

Name	*Source*	*Use*	*Reference*
pREP3X series	S. Forsburg	*S. pombe* overexpression vector using the *nmt1* promoter	Maundrell et al. 1993
pREP3X-*cid1*	SW. Wang	*cid1* cDNA cloned into pREP3X using *NdeI* and *BamHI*	Wang et al. 2000
pREPNTAP	K. Gould	pREP3X vector used for N-terminal TAP tagging	Gould et al. 2004
pREPNTAP-*cid1*	This study	*cid1* cDNA cloned into pREPNTAP using *SalI* and *NotI*	
pART1-kanMX	T. Cech	*S. pombe* expression vector using the *adh1* promoter, with *LEU2* marker replaced with *kanMX*	Mandell et al. 2005
pART1-kanMX-helicase	T. Cech	Rqh2 helicase domain cloned into pART1-kanMX using *BamHI* and *SacI*	Mandell et al. 2005
pART1-kanMX-helicase	T. Cech	Rqh2 helicase domain cloned into pART1-kanMX using *BamHI* and *SacI*	Mandell et al. 2005
pBTM116-*cid1*	SW. Wang	*cid1* cDNA cloned into pBTM116 using *BamHI* and *PstI*	
pGADGH-library	T.Toda	*S. pombe* cDNA library cloned into pGADGH using *EcoRI* and *XhoI* (1.25×10^7 recombinants)	
pGADGH-ysh1	This study	*ysh1* cDNA cloned into pGADGH using *BamHI* and *XhoI*	
pGADGH-cft2	This study	*cft2* cDNA cloned into pGADGH using *BamHI* and *XhoI*	
pFA6a series	J. Bähler	Used for templates for GFP or HA C-terminal tagging by homologous recombination	Bä hler et al. 1998
pREP42	Lab stock	*ura4* cloned into pREP3X; used for template for deletion by homologous recombination	
pPC128	P. Russell	pFA6a-based plasmid used for C-terminal TAP tagging	

APPENDIX C. Oligonucleotides

Oligonucleotide sequences used in this study are described below:

No.	*Name*	*Sequence (5′-3′)*
	5′ hyd1 del	CTAAGATTTTGTTTCTTAGTAGAGTAGGAAGTTTAGTATATTCATTTTTTTTTAA AGTGCATAAGATAATAAGAAACGCGAAATCCCACTGGCTATATGT
	3′ hyd1 del	AATTCAAAGTCGGTCTAAGCAGCGTTTTTCATGTCACGCAATTTCTTCATAACA GTGATTGGATCACTACTGCCCACTGCAATTCTAAATGCCTTCTGAC
	5′ hyd1 tag	TAGAATCGGTACAAAAAGCAGTGGGCAGTAGTGATCCAATCACTGTTATGAA GAAATTGCGTGACATGAAAAACGCTGCTCGGATCCCCGGGTTAATTAA
	3′ hyd1 tag	GCAATTTACTCTGACACCGCTAAACAGCCTTCAATTCGTATATGTGATCCAATT GAAAAATAGATCAACTATATTCTTGCGAATTCGAGCTCGTTTAAAC
	5′ hyd2 del	TTTCAATCTATCCTTGGTTCTTCAACGTGCGTAGTTCATCCATCACCTTGATAGG ATCGTTAAGACCCAGATGCTTTTGCAAATCCCACTGGCTATATGT
	3′ hyd2 del	TAATAACGAATATGTCTTTTCAAATATTACCTATCCCAATGTGGGTAGGAACTC AAGATAATTATGCATATCTTTTGTTGAATTCTAAATGCCTTCTGAC
	5′ scp160 del	ATGGAACATCTCTCTAATCTTGAGCAACCAACAACGATGGACTCATATGATTT TCAGAAACTGACTAACGAAATCCCACTGGCTATATGT
	3′ scp160 del	AATGTAAGTACTAAATAAAGGCTCCAAAATTTTACCTTACTCTTGATTTTGGTT TTCTTGAAGTCTCTCGAATTCTAAATGCCTTCTGAC
	5′ scp160 tag	AAGTCGAGCTGGCGTTGTCAAGGCTAAAGATCTGATTTTCGAGAGACTTCAAG AAAACCAAAATCAAGAGCGGATCCCCGGGTTAATTAA
	3′ scp160 tag	CAATAAGGAAAATGCTCGATTCAAGCAATTTCCGTCTCCTTAAAAAATGACAT GTAGTAATAAAAGAGCGGAATTCGAGCTCGTTTAAAC
	5′ rqh2	TTCAGAAATGCTAAAGTCGC
	3′ rqh2	GTCAGTATAATTTGATGATGG
	5′ adg1	TCTATCTTCCAAACCTTGTGC
	3′ adg1	CTCAGTGTCATAAGAAAGAGG
	5′ adg2	ATCAGCGGGCTATTAATTAGC
	3′ adg2	AACTAGTGAAGGTAGTCATGC
	5′ h4.2	GGATTGGGAAAGGGTGGTGC
	3′ h4.2	AAGGAAGTGACAGTCTTACG
	5′ cdc18	CCGTCCTATAATTCTACTGCC
	3′ cdc18	TGGGTTATATAATTCATTGGC
	5′ aes1	AAGGAAATGCAGCAGATTGCC
	3′ aes1	TTGGAAGTTGAATTACAAGCC
	5′ NTAPcid1	CGGGTAGTCGACTAACATTTCTTCTGCACAATTT
	3′ NTAPcid1	GCGCACTAGCGGCCGCTCACTCAGAATTGTCACCATG

APPENDIX D. Media

The following media was supplied by Cancer Research UK.

Luria-Bertani (LB) medium*

16 mg/ml bacto-tryptone (Difco)
10mg/ml bacto-yeast extract (Difco)
For agar: add 15mg/ml bacto-agar (Difco)

YE5S medium♣

5 mg/ml bacto-yeast extract (Difco)
30mg/ml glucose
225μg/ml leucine, uracil, adenine, histidine, lysine
For agar: add 20mg/ml bacto-agar (Difco)

ME4S agar

30 mg/ml bacto-malt extract (Difco)
225μg/ml leucine, uracil, adenine, histidine
20mg/ml bacto-agar (Difco)

Edinburgh Minimal Medium (EMM2)♣♠

3mg/ml potassium hydrogen phthalate
2.2mg/ml Na2HPO4
5mg/ml NH4Cl
20mg/ml glucose
20mg/ml salts (5.2mM MgCl2, 0.1mM CaCl2, 13.4mM KCl, 0.28mM Na2SO4)
1μl/ml vitamins (4.2μM pantothenic acid, 81.2μM nicotinic acid, 55.5μM inositol, 40.8μM biotin)
0.1μl/ml minerals (8.1μM boric acid, 2.37μM Mn SO4, 1.39μM ZnSO4, 0.74μM FeCl3, 0.25μM molybdic acid, 0.6μM KI, 4.76μM citric acid, 0.16μM CuSO4)
For agar: add 20mg/ml bacto-agar (Difco)

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* Ampicillin was added to a final concentration of 100μg/ml.

♣ Antibiotics and drugs were added as follows: G418 (200μg/ml), MBC (7.5 - 12μg/ml), hydroxyurea (10mM), caffeine (2.5 – 5mM), MMS (0.01%), bleomycin (5μg/ml).

♠ Amino acids were supplemented at the following concentrations: adenine, leucine, histidine (225μg/ml) and uracil (75μg/ml).